



## RESEARCH ARTICLE

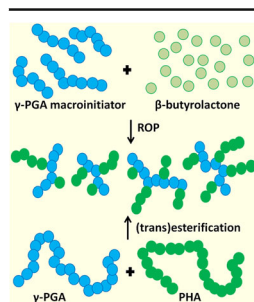
# The Synthesis and Structural Characterization of Graft Copolymers Composed of $\gamma$ -PGA Backbone and Oligoesters Pendant Chains

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**Abstract.** The novel copolymers composed of poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) and oligoesters have been developed. The structures of the obtained copolymers including variety of end groups were determined at the molecular level with the aid of electrospray ionization multistage mass spectrometry (ESI-MS<sup>n</sup>). The fragmentation experiment performed for the selected sodium adducts of the copolymers confirmed that the developed methods lead to the formation of graft copolymers composed of poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) backbone and oligoesters pendant chains. Moreover, it was established that fragmentation of selected sodium adducts of graft copolymers proceeded via random breakage of amide bonds along the backbone and ester bonds of the

oligoesters pendant chains. Considering potential applications of the synthesized copolymers in the area of biomaterials, the hydrolytic degradation under laboratory conditions and *in vitro* cytotoxicity tests were performed. The ESI-MS<sup>n</sup> technique applied in this study has been proven to be a useful tool in structural studies of novel graft copolymers as well as their degradation products.

**Keywords:** Biopolymers, Graft copolymers, Polyamides, Polyesters, Mass spectrometry

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## Introduction

Poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA), a commercially available biopolymer made of D- or L-glutamic acid units connected by amide linkages, is produced during fermentation by various bacteria. Production of this polymer by microbial fermentation has been widely investigated and it was found that selection of bacterial strain as well as nutrient type, ionic strength, and fermentation conditions are factors that affect the enantiomeric composition and molecular mass of obtained  $\gamma$ -PGA [1–3]. The  $\gamma$ -PGA is biodegradable, nontoxic for humans, and edible;

therefore it has been used in the synthesis of various materials, which were applied in wide range of fields [4, 5]. For example, the  $\gamma$ -PGA/chitosan composite [6] or the poly( $\gamma$ -glutamic acid)-*graft*-chondroitin sulfate/polycaprolactone composite [7] were used as scaffolds in tissue engineering. The hydrogels prepared by cross-linking of  $\gamma$ -PGA with dihalogenoalkanes [8, 9], alkanediamine [10], or various saccharides [11] have potential application as controlled release systems. Moreover, the delivery systems based on  $\gamma$ -PGA have been developed. The  $\gamma$ -PGA-based delivery systems have been designed for various active substances, such as anticancer drug pixantrone dimaleate [12], cisplatin [13], insulin [14], protein [15], or fibroblast growth factor and heparin [16]. Effectiveness of these systems have been proven during *in vitro* and *in vivo* tests. Owing to the edibility of  $\gamma$ -PGA, this biopolymer could find applications in food industry, for example as a bitterness relieving agent [17], texture modifier for baked foods like wheat bread [18], or

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cryoprotectant for probiotic bacteria [19]. On the other hand, the  $\gamma$ -PGA has been successfully applied for the removal of heavy metals from wastewaters [20, 21].

Polyhydroxyalkanoates, a family of biodegradable polyesters, are produced from renewable resources (e.g., glucose) by numerous microorganisms. Use of waste and non-food competing sources as substrates is the focus of interest of recent research studies [22–24]. Synthetic polyhydroxyalkanoates can be obtained via anionic ring-opening polymerization (ROP) of  $\beta$ -substituted  $\beta$ -lactones [25–27]. Bacterial PHA, as well as synthetic PHA, have been exploited widely for various applications, including the medical field, for example as drug delivery systems [28, 29], scaffolds [30, 31], vascular systems [32, 33], or sutures [34, 35].

Taking into account the already known materials based on poly- $\gamma$ -glutamic acid and their properties, the  $\gamma$ -PGA seems to be a promising starting compound for further modifications to obtain biomaterials with many potential applications. However, there are some difficulties associated to the derivatization of  $\gamma$ -PGA, mostly related to the poor solubility of this biopolymer in solvents commonly used in organic synthesis [36]. We report synthetic approaches that overcome the problem of poor solubility of  $\gamma$ -PGA and enable to obtain derivatives of this biopolymer. However, it is noteworthy that in case of polymers with potential applications as biomaterials, it is necessary to confirm the molecular structure of obtained products. Therefore, to verify the structures of the obtained graft copolymers composed of  $\gamma$ -PGA backbone and oligoesters pendant chains, electrospray ionization multistage mass spectrometry (ESI-MS<sup>n</sup>) has been used. The ESI-MS<sup>n</sup> technique has been successfully used in various polyester studies as well as in polyamides studies. The ESI-MS<sup>n</sup> technique has been applied to get detailed structural information of numerous (co)polyesters, such as poly(butylene adipate-co-butylene terephthalate) [37], poly(2-methyl-3-hydroxyoctanoate) [26], poly(3-hydroxybutyrate-co-3-hydroxy-4-ethoxybutyrate) [27], or poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [38]. In studies related to polyamides, the ESI-MS<sup>n</sup> technique was used, for example, for identification of polyamide cyclic oligomers [39], structural studies of polyamide dendrimer [40], or to probe the binding selectivity of a flexible cyclic polyamide [41]. Taking into consideration effectiveness of ESI-MS in structural studies of polyesters and polyamides, we assume that the use of this technique will provide detailed information about the structure of the graft copolymers studied as well as their degradation products.

## Experimental

### Materials

The poly(3-hydroxybutyrate-co-4-hydroxybutyrate) ( $M_n = 250,000$  g/mol; dispersity index  $M_w/M_n = 2.5$ ; the 4HB unit content 8.8 mol %) was purchased from Tianjin Green Bio-Science (Tianjin, China); the high molecular weight poly- $\gamma$ -glutamic acid ( $M_n = 150,000$  g/mol; dispersity index  $M_w/M_n =$

2.05) and ultra-low molecular weight poly- $\gamma$ -glutamic acid ( $M_n = 2000$  g/mol; dispersity index  $M_w/M_n = 1.43$ ) were purchased from Shandong Freda Biotechnology Co., Ltd. (Shandong, China). Tetradecyltrimethylammonium bromide, 4-toluenesulfonic acid monohydrate, and  $[R,S]$ - $\beta$ -butyrolactone were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Dimethyl sulfoxide (DMSO) and  $N,N$ -dimethylformamide (DMF) were purchased from POCH SA (Gliwice, Poland). Dialysis membrane Spectra/Por (MWCO 1000) was purchased from Carl Roth (Karlsruhe, Germany).

### Methods

Proton nuclear magnetic resonance (<sup>1</sup>H NMR) analyses were performed in CDCl<sub>3</sub> on an Avance II 600 MHz Ultrashield Plus spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). FTIR spectroscopy analysis was performed on Jasco FT-IR-6700 spectrometer (Jasco Corporation, Tokyo, Japan) using MultiLoop-MIR fiber probe (Harrick Scientific Products Inc., Pleasantville, NY, USA) connected to a FiberMate2 fiber optic coupler (Harrick Scientific Products Inc.). Electrospray mass spectrometry (ESI-MS<sup>n</sup>) analyses were performed in positive-ion mode on a Thermo LCQ Fleet ion-trap mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA). Solutions of samples were introduced into the ESI source by continuous infusion by means of the instrument syringe pump with 10  $\mu$ L/min flow rate. Spray voltage was set at 4.8 kV; capillary temperature was set at 200 °C; nitrogen was used as sheath gas; helium was used as the auxiliary gas. In ESI-MS/MS experiments, the precursor ions were isolated in the ion trap and activated by the collision.

### Synthesis of Graft Copolymers Via “Grafting From” Method

The macroinitiators for “grafting from” method was obtained from ultra-low molecular weight poly- $\gamma$ -glutamic acid and tetradecyltrimethylammonium bromide. Macroinitiator was subjected to benzylation by treatment with benzyl bromide in DMSO in order to estimate carboxylate active centers, similar to a procedure known from the literature [42]. Details of “grafting from” method are in the [Supplementary Material](#).

### Synthesis of Graft Copolymers Via (Trans)Esterification Reaction

The graft copolymers were obtained via (trans)esterification reaction from high molecular weight poly- $\gamma$ -glutamic acid and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in the presence of 4-toluenesulfonic acid monohydrate. Details of (trans)esterification method are in [Supplementary Material](#).

### Hydrolytic Degradation of Graft Copolymers

Studies of hydrolytic degradation of graft copolymers were performed under laboratory conditions. Glass vials containing samples of graft copolymers (10 mg) and deionized water (5 cm<sup>3</sup>) were placed

in a thermostatically controlled incubator set at 25 °C. Vials with samples were withdrawn in triplicate from the incubator after 1, 5, 10, and 20 wk; samples were analyzed using ESI-MS technique.

*Assessment of Cytocompatibility of  $\gamma$ -PGA-Graft-(3HB-co-4HB) Copolymer* In vitro cytotoxicity was analyzed after an indirect contact of CCD-11Lu fibroblasts with extracts of the  $\gamma$ -PGA-graft-(3HB-co-4HB) copolymer by means of sulforhodamine B-based assay (In Vitro Toxicology Assay

Kit, Sulforhodamine B-based; Sigma-Aldrich). Details are in [Supplementary Material](#).

## Results and Discussion

### Grafting From Method

The first synthetic strategy for obtaining graft copolymers composed of  $\gamma$ -PGA backbone and oligoesters pendant chains was the anionic grafting of racemic  $\beta$ -butyrolactone on  $\gamma$ -PGA

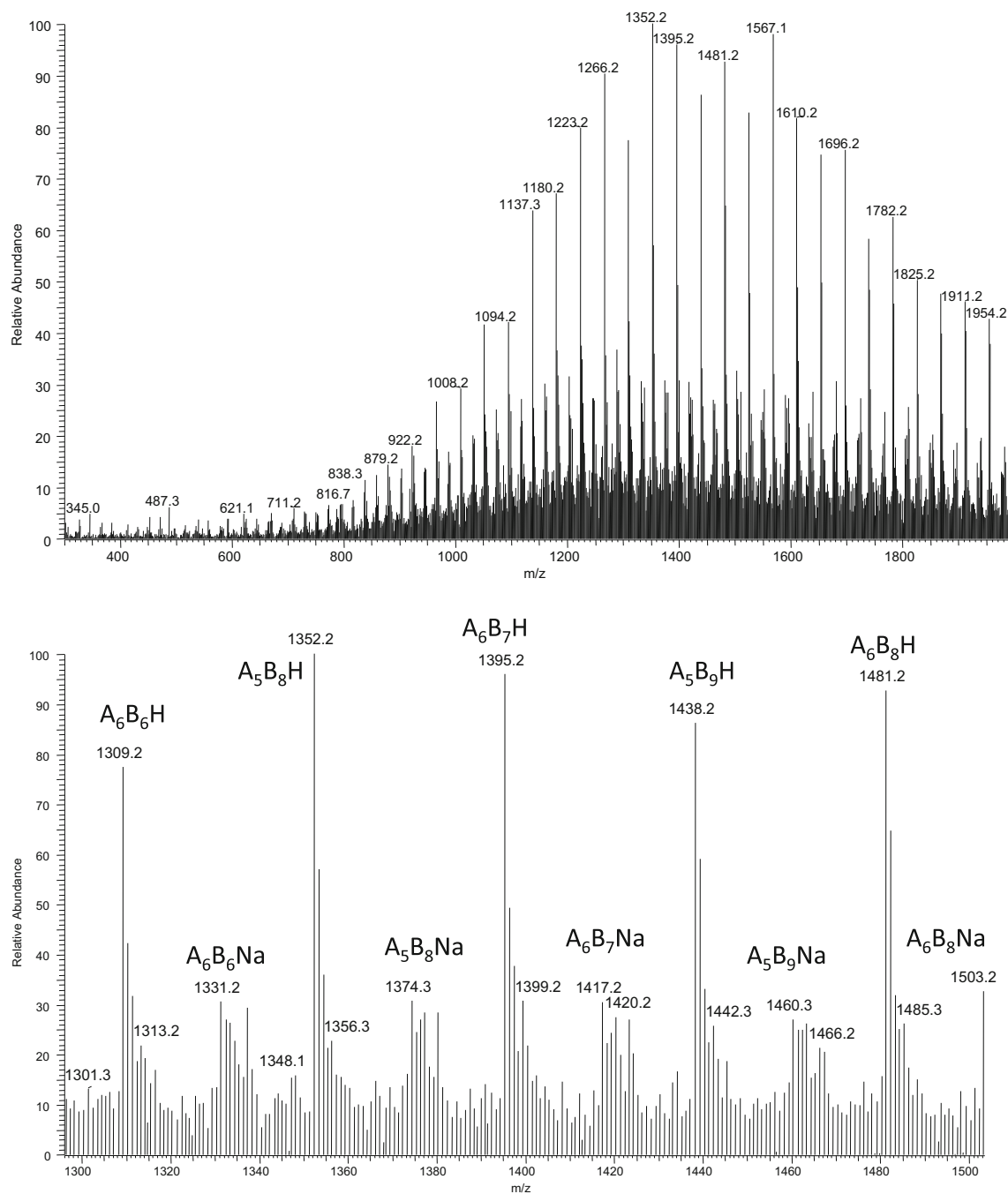
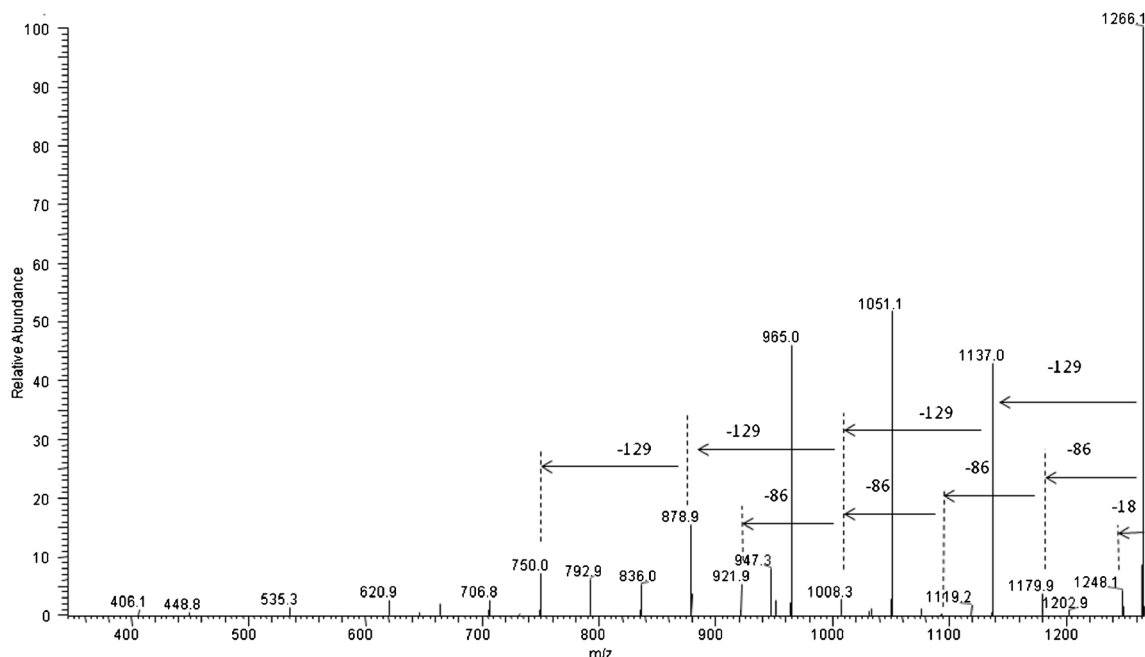


Figure 1. ESI-MS spectrum of  $\gamma$ -PGA-graft-3HB cooligomers and spectral expansion in the range  $m/z$  1290–1505

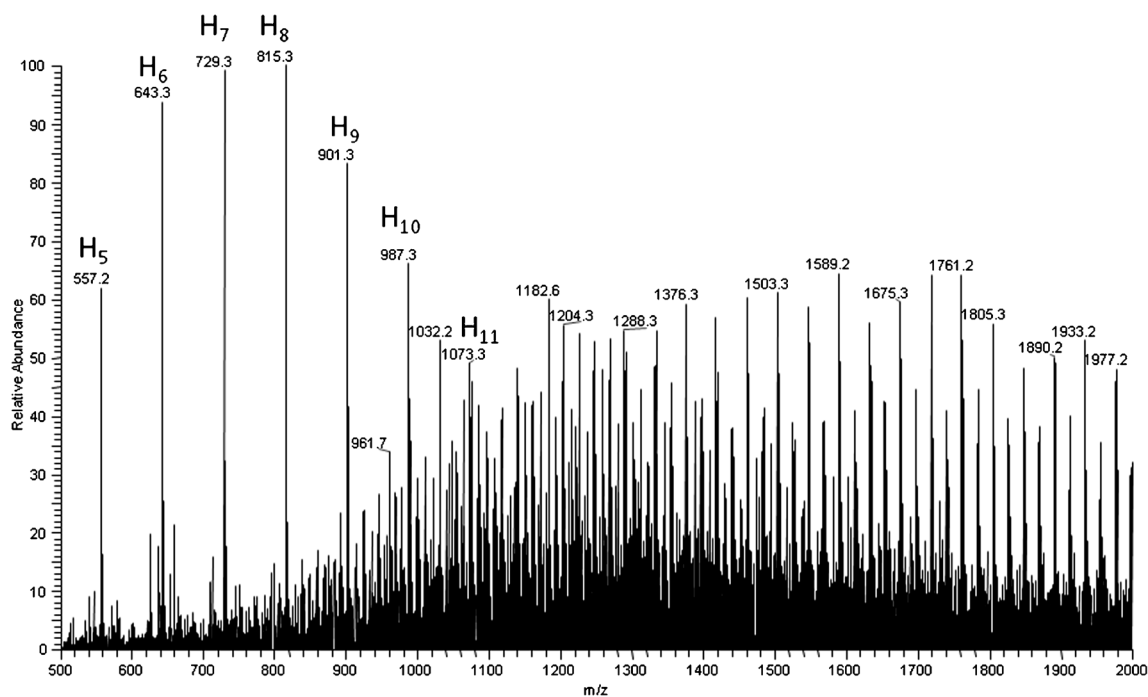




**Figure 3.** ESI-MS<sup>3</sup> spectrum (in positive-ion mode) of the sodium adduct of  $\gamma$ -PGA-*graft*-3HB co-oligomers at  $m/z$  1266 selected from the ESI-MS<sup>2</sup> spectrum of  $\gamma$ -PGA-*graft*-3HB cooligomers at  $m/z$  1395

Signals of protonated or sodiated cooligomer macromolecules in expanded mass spectrum (Figure 1) were labeled in formula  $A_xB_yH$  or  $A_xB_yNa$ , where “x” equals the number of  $\gamma$ -glutamate repeating units with molecular mass 129 Da, whereas “y” equals the number of 3-hydroxybutyric units (86 Da). The molecular mass of N-terminal end group and C-terminal end group in  $\gamma$ -PGA are 130 Da and 146 Da, respectively. Therefore, the  $m/z$  value for protonated cooligomer

macromolecules was calculated with formula  $A_xB_yH$ :  $130+(x-2)\cdot 129+146+y\cdot 86+1$ , whereas the  $m/z$  value for  $A_xB_yNa$  formula was calculated:  $130+(x-2)\cdot 129+146+y\cdot 86+23$ . Moreover, molecular weights of three 3-hydroxybutyric units (3·86 Da) and two  $\gamma$ -glutamate repeating units (2·129 Da) have the same value (258 Da). Therefore, signal assignment presented in expanded mass spectrum shows only one of many possibilities.



**Figure 4.** ESI-MS spectrum of  $\gamma$ -PGA-*graft*-3HB cooligomers after 20 wk of incubation in water at 25 °C



Apart from  $\gamma$ -PGA-*graft*-3HB cooligomers, the 3-hydroxybutyric acid oligomers with carboxyl and crotonate end groups have been detected in lower mass range. The formation of crotonate end groups in the  $\beta$ -butyrolactone polymerization might be caused by a chain-transfer reaction to the monomer and/or by intermolecular carboxylate-induced  $\alpha$ -deprotonation, which has been already reported [50, 51].

To verify the structure of obtained grafted copolymers, the ESI-MS<sup>n</sup> experiments were performed for selected ions visible in ESI-MS spectrum (Figure 1). Figure 2 shows the results of an ESI-MS<sup>2</sup> experiment performed for the sodium adducts of co-oligomers at  $m/z$  1395 (A<sub>6</sub>B<sub>7</sub>H) selected from ESI-MS spectrum of  $\gamma$ -PGA-*graft*-3HB cooligomers (Figure 1). All of the active carboxylic groups present in the  $\gamma$ -PGA macroinitiator should initiate anionic oligomerization of  $\beta$ -butyrolactone; however, oligo3-hydroxybutyrate pendant chain bonded to the  $\gamma$ -PGA backbone might have a different length. One of the probable structures, which consisted of six  $\gamma$ -glutamate repeating units and seven 3-hydroxybutyric repeating units, and the theoretical fragmentation pathway of this structure, are shown in Figure 2. The product ions at  $m/z$  1309; 1223; 1137; 1051 etc. correspond to the cooligomers formed by the loss of the 3-hydroxybutyric repeating units from oligoesters pendant chains, one (86 Da), two (172 Da), three (258 Da), four (344 Da) etc. respectively. In addition, the  $\gamma$ -PGA backbone underwent fragmentation; for example the product ion at  $m/z$  1094 corresponds to the oligomer formed by loss of the  $\gamma$ -glutamate repeating unit bonded with two 3-hydroxybutyric repeating units (301 Da), the product ion at  $m/z$  1248 corresponds to the oligomer formed by the loss of the  $\gamma$ -glutamic acid (147 Da), the product ion at  $m/z$  1266 corresponds to the co-oligomer formed by the loss of the pyroglutamic acid (129 Da). The product ion at  $m/z$  1377 corresponds to the cooligomer formed by the loss of the water molecule (18 Da).

In order to confirm the structure of the individual cooligomer, further fragmentation experiments were performed.

Figure 3 shows ESI-MS<sup>3</sup> spectrum of the sodium adduct of  $\gamma$ -PGA-*graft*-3HB cooligomers at  $m/z$  1266 selected from the ESI-MS<sup>2</sup> spectrum of  $\gamma$ -PGA-*graft*-3HB cooligomers at  $m/z$  1395. The product ions at  $m/z$  1180; 1094; 1008; 922 etc. correspond to the cooligomers formed by the loss of the repeating units from oligoesters pendant chains: one (86 Da), two (172 Da), three (258 Da), four (344 Da) etc., respectively. The product ions at  $m/z$  1137; 1008; 879 etc. correspond to the cooligomers formed by the loss of one, two, three etc. repeating units from  $\gamma$ -PGA backbone.

### Hydrolytic Degradation of $\gamma$ -PGA-Graft-3HB Cooligomers

Considering the prospective application of the obtained graft copolymers as biomaterials, preliminary hydrolytic degradation studies were performed. The products of hydrolytic degradation of graft copolymers under laboratory condition were analyzed with the aid of mass spectrometry. A significant change in the distribution of the ion patterns has been observed when comparing ESI-MS spectrum of the sample after 10 wk of hydrolytic degradation (Figure 4) with ESI-MS spectrum of starting sample (Figure 1). Moreover, in Figure 4, the signals corresponding to sodium adduct of oligo(3-hydroxybutyrate) oligomers hydroxyl and carboxyl end groups appeared in mass range  $m/z$  500–1100. These oligomers were formed as a product of the hydrolytic degradation of oligoesters pendant chains. Signals corresponding to sodium adduct of 3-hydroxybutyrate oligomers with hydroxyl and carboxyl end groups (Figure 4) were labeled as H<sub>y</sub>, where “y” equals to the number of 3-hydroxybutyric units (86 Da). The molecular mass of 3-hydroxybutyrate end group is 104 Da; therefore, the  $m/z$  value for H<sub>y</sub> was calculated according to the formula  $104+y\cdot 86+23$ .

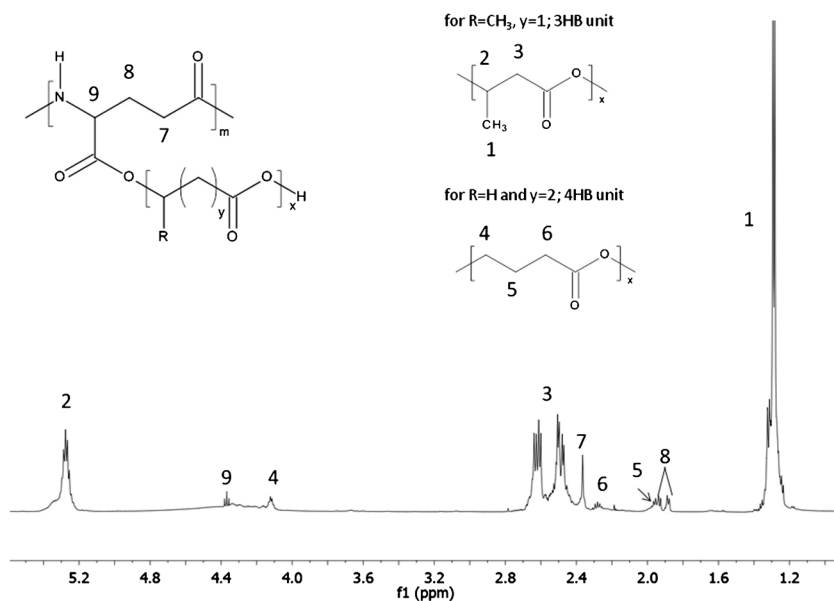


Figure 5. The <sup>1</sup>H NMR spectrum of  $\gamma$ -PGA-*graft*-(3HB-co-4HB) cooligomers (for 3HB units R = CH<sub>3</sub>, y = 1, for 4HB units R = H and y = 2)

During hydrolytic degradation studies, products of hydrolysis of  $\gamma$ -PGA backbone have not been detected. The slower hydrolytic degradation rate of oligoamide chain might have been expected because amide bonds undergo hydrolysis at considerably more vigorous conditions than ester bonds [52].

### (Trans)esterification

In further research, the second approach for obtaining the copolymers composed of  $\gamma$ -PGA backbone and oligoesters

pendant chains based on (trans)esterification reaction has been developed. Previously, we reported (trans)esterification reaction as the method for synthesis of conjugates of model bioactive compounds with oligomer from selected biopolymers [53, 54]. In the next step of our research, it was found that the “one-pot” solvent-free synthesis enabled obtaining  $\gamma$ -PGA-graft-(3HB-co-4HB) cooligomers. Such graft copolymers were obtained via (trans)esterification reaction of the poly- $\gamma$ -glutamic acid with poly(3-hydroxybutyrate-co-4-hydroxybutyrate) mediated by 4-toluenesulfonic acid monohydrate (TSA · H<sub>2</sub>O) carried out in the melt (Scheme S2, Supplementary Material).

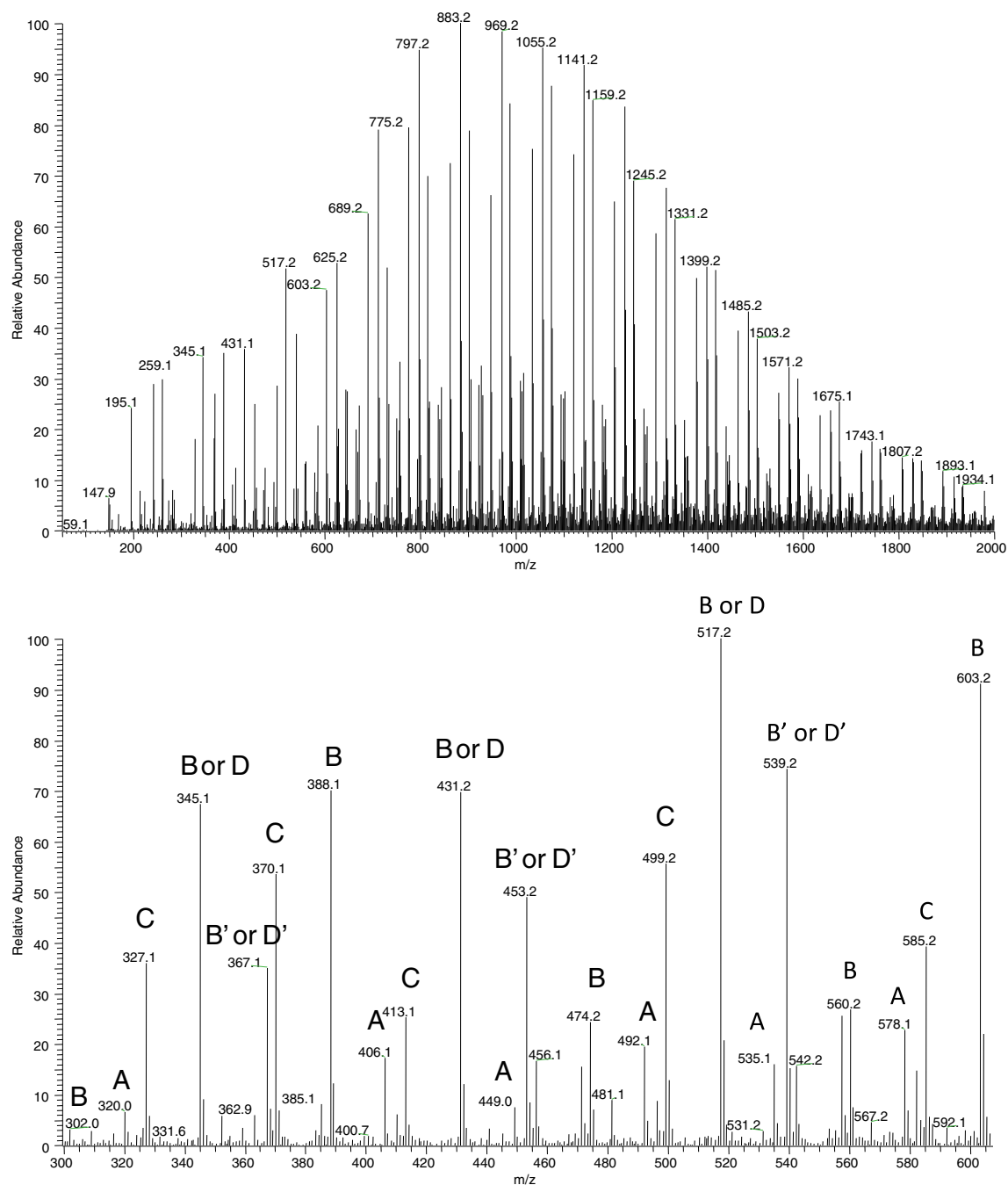
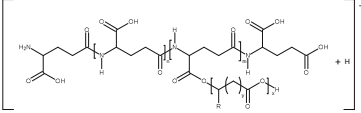
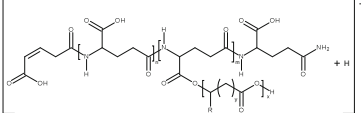
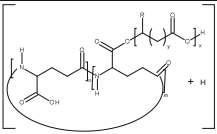
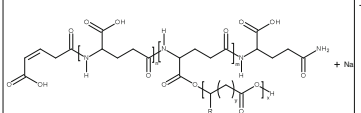
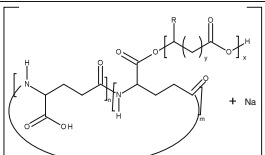
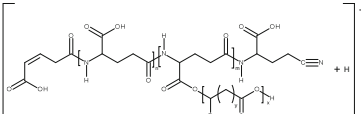
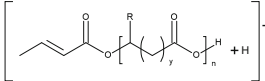
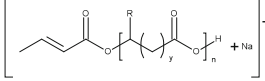


Figure 6. The ESI-MS of  $\gamma$ -PGA-graft-(3HB-co-4HB) cooligomers and spectral expansion in the range  $m/z$  300–610

**Table 1.** Structural Assignments of the Ions Appearing in the Expanded Regions at  $m/z$  300–610 of the ESI-MS Spectrum presented in Figure 6

	Structure	Ions [ $m/z$ ]
A		320; 406; 492; 535
B	 	302; 345; 388; 431; 474; 517; 560; 603
B'	 	367; 453; 539
C		327; 370; 413; 499; 585
D		345; 431; 517
D'		367; 453; 539



The  $^1\text{H}$  NMR spectrum of the products obtained through the (trans)esterification reaction poly- $\gamma$ -glutamic acid with poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) in the presence of 4-toluenesulfonic acid monohydrate is presented in Figure 5. In this spectrum, signals corresponding to the protons of  $\gamma$ -PGA backbone (labeled 7–9) as well as signals corresponding to protons of oligo(3-hydroxybutyrate-*co*-4-hydroxybutyrate) pendant chains (labeled 1–6) were observed.

Under the (trans)esterification reaction conditions both biopolymers underwent partial hydrolysis because of the presence of water (introduced with 4-toluenesulfonic acid monohydrate). The partial thermal degradation of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) via a random chain scission mechanism involving the  $\beta$ -CH hydrogen transfer at the 3-hydroxybutyrate repeating units also occurred [55]. Moreover,  $\gamma$ -PGA underwent partial thermal degradation via typical for polyamides mechanisms, which leads to the formation of cyclic amides [56, 57] or oligomers with unsaturated alkyl and amide end groups further transformed into nitriles [58, 59]. Owing to a variety of end groups formed via different known mechanisms, as well as the diversity of probable structure, further structural investigation was needed. For this purpose, the ESI-MS<sup>n</sup> technique has been successfully applied.

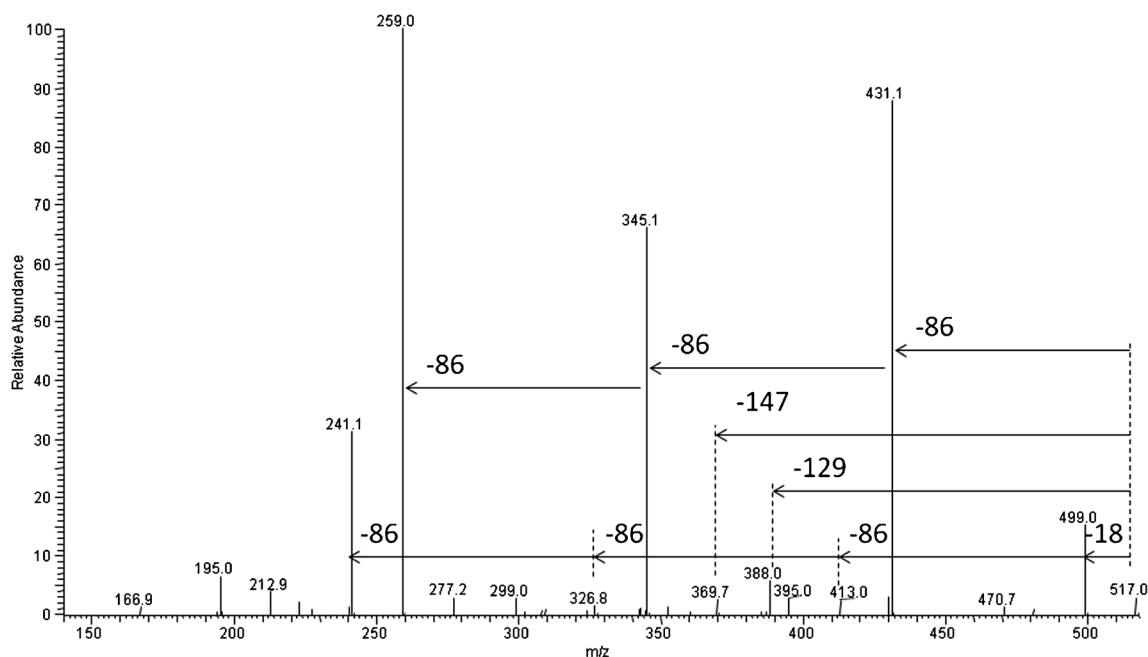
The structures of the ions visible in the ESI-MS spectrum in Figure 6 were assigned to the structures placed in Table 1 based on different mechanisms of thermal decomposition of polyamides discussed in literature [56–59]. Signals in ESI-MS spectrum correspond to sodium or proton adducts of  $\gamma$ -PGA-*graft*-(3HB-*co*-4HB) cooligomers obtained in (trans)esterification of  $\gamma$ -PGA with poly(3HB-*co*-4HB) mediated by 4-toluenesulfonic acid monohydrate. Among proposed structure

of  $\gamma$ -PGA-*graft*-(3HB-*co*-4HB) cooligomers, both linear and cyclic  $\gamma$ -PGA backbones were taken into consideration.

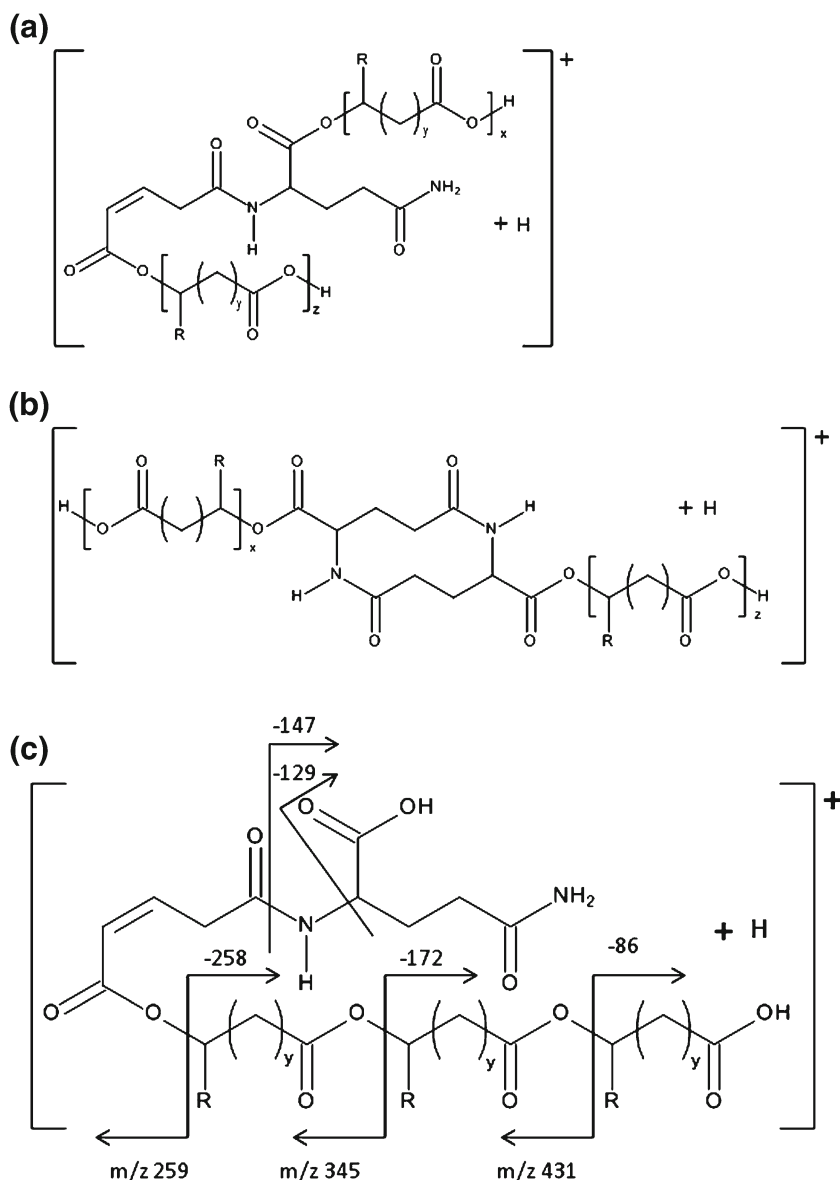
As a part of structural analysis, the ESI-MS<sup>2</sup> experiments for selected ions were carried out. Figure 7 shows the results of the ESI-MS<sup>2</sup> experiment performed for the ion at  $m/z$  517 selected from ESI-MS spectrum (labeled B, see structure in Table 1). This ion corresponds to sodium adduct of  $\gamma$ -PGA-*graft*-(3HB-*co*-4HB) cooligomers containing three repeating units derivative from poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and two repeating units derivative from  $\gamma$ -PGA. The oligoamide part of such ion could be cyclic or might contain unsaturated alkyl and amide end groups (see Scheme 1). The product ion at  $m/z$  370 corresponds to the cooligomer formed by the loss of the  $\gamma$ -glutamic acid (147 Da). The product ion at  $m/z$  388 corresponds to the cooligomer formed by the loss of the pyroglutamic acid (129 Da). The product ion at  $m/z$  499 corresponds to the cooligomer formed by the loss of the water molecule (18 Da). The product ions at  $m/z$  431; 345; 259 correspond to the cooligomer formed by the loss of the one (86 Da), two (172 Da), and three (258) 3-hydroxybutyric or 4-hydroxybutyric repeating units.

## Results of Cytocompatibility Studies of $\gamma$ -PGA-Graft-(3HB-*co*-4HB) Copolymer

The cell viability was not affected by  $\gamma$ -PGA-*graft*-(3HB-*co*-4HB) copolymer over the entire range of concentrations (Figure S1, Supplementary Material). These results indicate that the novel materials obtained via (trans)esterification reaction of the



**Figure 7.** ESI-MS<sup>2</sup> product ion spectrum of the sodiated  $\gamma$ -PGA-*graft*-(3HB-*co*-4HB) cooligomers at  $m/z$  517 and theoretical fragmentation pathway of this ion



**Scheme 1.** Probable structures of sodium adduct of  $\gamma$ -PGA-graft-(3HB-co-4HB) cooligomers at  $m/z$  517 ( $x + z = 3$ ;  $R = CH_3$ ,  $y = 1$  for 3HB units;  $R = H$ , and  $y = 2$  for 4HB units): linear (a), cyclic (b), and fragmentation pathway of one of possible structure of this ion (c)

poly- $\gamma$ -glutamic acid with poly(3-hydroxybutyrate-co-4-hydroxybutyrate) mediated by 4-toluenesulfonic acid monohydrate carried out in melt did not affect negatively the viability of the treated cells.

## Conclusions

Two methods of obtaining graft copolymers composed of poly- $\gamma$ -glutamic acid backbone and oligoesters pendant chains were developed. The first elaborated synthetic strategy is based on the anionic grafting of racemic  $\beta$ -butyrolactone on  $\gamma$ -PGA backbone. The second developed method is based on the (trans)esterification reaction of  $\gamma$ -PGA with poly(3-

hydroxybutyrate-co-4-hydroxybutyrate) mediated by 4-toluenesulfonic acid monohydrate.

The structural studies with the aid of electrospray ionization multistage mass spectrometry technique confirmed the structure of graft copolymers obtained via both methods. Moreover, it was established that fragmentation of selected sodium adducts of graft copolymers proceeded via random breakage of amide bonds along the backbone and ester bonds of the oligoesters pendant chains. The ESI-MS allowed determining a variety of end groups in final products in case of applying the second method. The presence of various end groups was attributable to hydrolysis and thermal degradation, both of which occurred under reaction conditions. However, obtained copolymers, despite having a variety of end groups, did not affect

negatively the viability of the treated cells during in vitro cytotoxicity tests.

In addition, the ESI-MS technique has allowed us to monitor the progress of hydrolytic degradation process of obtained copolymers and to determine the degradation products. The performed tests confirmed that hydrolytic degradation of oligoesters pendant chains proceeds faster than hydrolytic degradation of  $\gamma$ -PGA backbone.

This first method of synthesis of graft copolymers will be further developed in order to obtain copolymers with higher molecular weight, which should have thermo-mechanical properties required to find application in the field of biomaterials.

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## References

- Bajaj, I., Singhal, R.: Poly (glutamic acid) – an emerging biopolymer of commercial interest. *Bioresour. Technol.* **102**, 5551–5561 (2011)
- Ogunleye, A., Bhat, A., Irorere, V.U., Hill, D., Williams, C., Radecka, I.: Poly- $\gamma$ -glutamic acid: production, properties, and applications. *Microbiology* **161**, 1–17 (2015)
- Luo, Z., Guo, Y., Liu, J., Qiu, H., Zhao, M., Zou, W., Li, S.: Microbial synthesis of poly- $\gamma$ -glutamic acid: current progress, challenges, and future perspectives. *Biotechnol. Biofuels* **9**, 134–145 (2016)
- Sung, M.H., Park, C., Kim, C.J., Poo, H., Soda, K., Ashiuchi, M.: Natural and edible biopolymer poly- $\gamma$ -glutamic acid: synthesis, production, and application. *Chem. Rec.* **5**, 352–366 (2005)
- Ashiuchi, M.: Microbial production and chemical transformation of poly- $\gamma$ -glutamate. *Microbiol. Biotechnol.* **6**, 664–674 (2013)
- Hsieh, C.-Y., Tsai, S.-P., Wang, D.-M., Chang, Y.-N., Hsieh, H.-J.: Preparation of  $\gamma$ -PGA/chitosan composite tissue engineering matrices. *Biomaterials* **26**, 5617–5623 (2005)
- Chang, K.-Y., Cheng, L.-W., Ho, G.-H., Huang, Y.-P., Lee, Y.-D.: Fabrication and characterization of poly( $\gamma$ -glutamic acid)-graft-chondroitin sulfate/polycaprolactone porous scaffolds for cartilage tissue engineering. *Acta Biomater.* **5**, 1937–1947 (2009)
- Gonzales, D., Fan, K., Sevoian, M.: Synthesis and swelling characterizations of a poly( $\gamma$ -glutamic acid) hydrogel. *J. Polym. Sci. A Polym. Chem.* **34**, 2019–2027 (1996)
- Fan, K., Gonzales, D., Sevoian, M.: Hydrolytic and enzymatic degradation of poly( $\gamma$ -glutamic acid) hydrogels and their application in slow-release systems for proteins. *J. Environ. Polym. Degrad.* **4**, 253–260 (1996)
- Kunioka, M., Furusawa, K.: Poly( $\gamma$ -glutamic acid) hydrogel prepared from microbial poly( $\gamma$ -glutamic acid) and alkanediamine with water-soluble carbodiimide. *J. Appl. Polym. Sci.* **65**, 1889–1896 (1997)
- Murakami, S., Aoki, N.: Bio-based hydrogels prepared by cross-linking of microbial poly( $\gamma$ -glutamic acid) with various saccharides. *Biomacromolecules* **7**, 2122–2127 (2006)
- Meng, L., Ji, B., Huang, W., Wang, D., Tong, G., Su, Y., Zhu, X., Yan, D.: Preparation of pixantone/poly( $\gamma$ -glutamic acid) nanoparticles through complex self-assembly for oral chemotherapy. *Macromol. Biosci.* **12**, 1524–1533 (2012)
- Ye, H., Jin, L., Hu, R., Yi, Z., Li, J., Wu, Y., Xi, X., Wu, Z.: Poly(L-glutamic acid)-cisplatin conjugate effectively inhibits human breast tumor xenografted in nude mice. *Biomaterials* **27**, 5958–5965 (2006)
- Sonaje, K., Chen, Y.J., Chen, H.L., Wey, S.P., Juang, J.H., Nguyen, H.N., Hsu, C.W., Lin, K.J., Sung, H.W.: Enteric-coated capsules filled with freeze-dried chitosan/poly(-glutamic acid) nanoparticles for oral insulin delivery. *Biomaterials* **31**, 3384–3394 (2010)
- Zhu, Y., Akagi, T., Akashi, M.: Self-assembling stereocomplex nanoparticles by enantiomeric poly( $\gamma$ -glutamic acid)-poly(lactide) graft copolymers as a protein delivery carrier. *Macromol. Biosci.* **14**, 576–587 (2014)
- Tang, D.W., Yu, S.H., Ho, Y.C., Mi, F.L., Kuo, P.L., Sung, H.W.: Heparinized chitosan/poly(-glutamic acid) nanoparticles for multi-functional delivery of fibroblast growth factor and heparin. *Biomaterials* **31**, 9320–9332 (2010)
- Sakai, K., Sonoda, C., Murase, K.: Bitterness relieving agent. WO0021390, Japan (2000)
- Shyu, Y.-S., Hwang, J.-Y., Hsu, C.-K.: Improving the rheological and thermal properties of wheat dough by the addition of  $\gamma$ -polyglutamic acid. *LWT-Food Sci. Technol.* **41**, 982–987 (2008)
- Bhat, A.R., Irorere, V.U., Bartlett, T., Hill, D., Kedia, G., Morris, M.R., Charalampopoulos, D., Radecka, I.: *Bacillus subtilis* natto: a nontoxic source of poly- $\gamma$ -glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *AMB Express* **3**, 36–44 (2013)
- Bhattacharyya, D., Hestekin, J.A., Brushaber, P., Cullen, L., Bachas, L.G., Sikdar, S.K.: Novel poly-glutamic acid functionalized microfiltration membranes for sorption of heavy metals at high capacity. *J. Membr. Sci.* **141**, 121–135 (1998)
- Inbaraj, B.S., Wang, J.S., Lu, J.F., Siao, F.Y., Chen, B.H.: Adsorption of toxic mercury(II) by an extracellular biopolymer poly( $\gamma$ -glutamic acid). *Bioresour. Technol.* **100**, 200–207 (2009)
- Koller, M., Salerno, A., Dias, M., Reiterer, A., Braunegg, G.: Modern biotechnological polymer synthesis: a review. *Food Technol. Biotechnol.* **48**, 255–269 (2010)
- Kushwah, B.S., Kushwah, A.V.S., Singh, V.: Towards understanding polyhydroxyalkanoates and their use. *J. Polym. Res.* **23**, 153–166 (2016)
- Radecka, I., Irorere, V., Jiang, G., Hill, D., Williams, C., Adamus, G., Kwiecień, M., Marek, A., Zawadiak, J., Johnston, B., Kowalczyk, M.: Oxidized polyethylene wax as a potential carbon source for PHA production. *Materials* **9**, 367–383 (2016)
- Jedliński, Z., Kowalczyk, M., Główkowski, W., Grobelny, J., Szwarc, M.: Novel polymerization of  $\beta$ -butyrolactone initiated by potassium naphthalenide in the presence of a crown ether or a cryptand. *Macromolecules* **24**, 349–352 (1991)
- Arkin, A.H., Hazer, B., Adamus, G., Kowalczyk, M., Jedliński, Z., Lenz, R.W.: Synthesis of poly(2-methyl-3-hydroxyoctanoate) via anionic polymerization of  $\alpha$ -methyl- $\beta$ -pentyl- $\beta$ -propiolactone. *Biomacromolecules* **2**, 623–627 (2001)
- Adamus, G.: Molecular level structure of (R, S)-3-hydroxybutyrate/(R, S)-3-hydroxy-4-ethoxybutyrate copolyesters with dissimilar architecture. *Macromolecules* **42**, 4547–4557 (2009)
- Shishatskaya, E.I., Goreva, A.V., Voinova, O.N., Inzhevatin, E.V., Khlebopros, R.G., Volova, T.G.: Evaluation of antitumor activity of rubomycin deposited in absorbable polymeric microparticles. *Bull. Exp. Biol. Med.* **145**, 358–361 (2008)
- Michalak, M., Marek, A.A., Zawadiak, J., Kawalec, M., Kurcok, P.: Synthesis of PHB-based carrier for drug delivery systems with pH-controlled release. *Eur. Polym. J.* **49**, 4149 (2013)
- Xu, X.Y., Li, X.T., Peng, S.W., Xiao, J.F., Liu, C., Fang, G., Chen, K.C., Chen, G.Q.: The behavior of neural stem cells on polyhydroxyalkanoate nanofiber scaffolds. *Biomaterials* **31**, 3967–3975 (2010)
- Kawalec, M., Sitkowska, A., Sobota, M., Sieroń, A.L., Komar, P., Kurcok, P.: Human procollagen type I surface-modified PHB-based nonwoven textile scaffolds for cell growth: preparation and short-term biological tests. *Biomed. Mater.* **9**, 065005 (2014)
- Unverdorben, M., Spielberger, A., Schywalsky, M., Labahn, D., Hartwig, S., Schneider, M., Looz, D., Behrend, D., Schmitz, K., Degenhardt, R., Schaldach, M., Vallbracht, C.: A polyhydroxybutyrate biodegradable stent: preliminary experience in the rabbit. *Cardiovasc. Intervent. Radiol.* **25**, 127–132 (2002)
- Adamus, G., Sikorska, W., Janeczek, H., Kwiecień, M., Sobota, M., Kowalczyk, M.: Novel block copolymers of atactic PHB with natural

- PHA for cardiovascular engineering: synthesis and characterization. *Eur. Polym. J.* **48**, 621–631 (2012)
34. Volova, T., Shishatskaya, E., Sevastianov, V.: Results of biomedical investigations of PHB and PHB/PHV fibers. *Biochem. Eng. J.* **16**, 125–133 (2003)
35. He, Y., Hu, Z., Ren, M., Ding, C., Chen, P., Gu, Q., Wu, Q.: Evaluation of PHBHHx and PHBV/PLA fibers used as medical sutures. *J. Mater. Sci. Mater. Med.* **25**, 561–571 (2014)
36. Pacini, A., Caricato, M., Ferrari, S., Capsoni, D., Ilarduya, A.M., Munoz-Guerra, S., Pasini, D.: Poly( $\gamma$ -glutamic acid) esters with reactive functional groups suitable for orthogonal conjugation strategies. *J. Polym. Sci. A Polym. Chem.* **50**, 4790–4799 (2012)
37. Song, J., Šišková, A., Simons, M.G., Kowalski, W.J., Kowalczyk, M.M., van den Brink, O.F.: LC-multistage mass spectrometry for the characterization of poly(butylene adipate-co-butylene terephthalate) copolyester. *J. Am. Soc. Mass Spectrom.* **22**, 641–648 (2011)
38. Adamus, G., Sikorska, W., Kowalczyk, M., Noda, I., Satkowski, M.M.: Electrospray ion-trap multistage mass spectrometry for characterization of co-monomer compositional distribution of bacterial poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) at the molecular level. *Rapid Commun. Mass Spectrom.* **17**, 2260–2266 (2003)
39. Tran, J.C., Doucette, A.A.: Cyclic polyamide oligomers extracted from nylon 66 membrane filter disks as a source of contamination in liquid chromatography/mass spectrometry. *J. Am. Soc. Mass Spectrom.* **17**, 652–656 (2006)
40. Li, Y., Chao, M., Xiuqiang, Z., Afang, Z.: Synthesis of dendritic polyamide with protected amino functional groups in the periphery. *Front. Chem. Chin.* **4**, 469–473 (2006)
41. Cui, X., Zhang, Q., Chen, H., Zhou, J., Yuan, G.: ESI mass spectrometric exploration of selective recognition of G-quadruplex in c-myc oncogene promoter using a novel flexible cyclic polyamide. *J. Am. Soc. Mass Spectrom.* **25**, 684–691 (2014)
42. Krecz, A., Pocsí, I., Borbely, J.: Preparation and chemical modification of poly- $\gamma$ -L-glutamic acid. *Folia Microbiol.* **46**, 183–186 (2001)
43. Ponomarenko, E.A., Waddon, A.J., Bakeev, K.N., Tirrell, D.A., MacKnight, W.J.: Self-assembled complexes of synthetic polypeptides and oppositely charged low molecular weight surfactants. Solid-state properties. *Macromolecules* **29**, 4340–4345 (1996)
44. Pérez-Camero, G., García-Alvarez, M., de Ilarduya, A.M., Fernández, C., Campos, L., Muñoz-Guerra, S.: Comb-like complexes of bacterial poly( $\gamma$ -D-glutamic acid) and cationic surfactants. *Biomacromolecules* **5**, 144–152 (2004)
45. Biagiotti, M., Borghese, G., Francescato, P., Morelli, C.F., Albertini, A.M., Bavaro, T., Ubiali, D., Mendichi, R., Speranza, G.: Esterification of poly( $\gamma$ -glutamic acid) ( $\gamma$ -PGA) mediated by its tetrabutylammonium salt. *RSC Adv.* **6**, 43954–43958 (2016)
46. Adamus, G., Kwiecień, I., Maksymiak, M., Bałakier, T., Jurczak, J., Kowalczyk, M.: Molecular level structure of novel synthetic analogues of aliphatic biopolyesters as revealed by multistage mass spectrometry. *Anal. Chim. Acta* **808**, 104–114 (2014)
47. Kwiecień, I., Bałakier, T., Jurczak, J., Kowalczyk, M., Adamus, G.: Molecular architecture of novel potentially bioactive (co)oligoesters containing pesticide moieties established by electrospray ionization multistage mass spectrometry. *Rapid Commun. Mass Spectrom.* **29**, 533–544 (2015)
48. Michalak, M., Kwiecień, M., Kawalec, M., Kurcok, P.: Oxidative degradation of poly(3-hydroxybutyrate). A new method of synthesis for the malic acid copolymers. *RSC Adv.* **6**, 12809–12818 (2016)
49. Maksymiak, M., Bałakier, T., Jurczak, J., Kowalczyk, M., Adamus, G.: Bioactive (co)oligoesters with antioxidant properties - synthesis and structural characterization at the molecular level. *RSC Adv.* **6**, 57751–57761 (2016)
50. Kurcok, P., Śmiga, M., Jedliński, Z.:  $\beta$ -Butyrolactone polymerization initiated with tetrabutylammonium carboxylates: a novel approach to biomimetic polyester synthesis. *J. Polym. Sci. A Polym. Chem.* **40**, 2184–2189 (2002)
51. Kawalec, M., Adamus, G., Kurcok, P., Kowalczyk, M., Foltran, I., Focarete, M.L., Scandola, M.: Carboxylate-induced degradation of poly(3-hydroxybutyrate)s. *Biomacromolecules* **8**, 1053–1058 (2007)
52. Carey, F.A., Sundberg, R.J.: Reactions of carbonyl compounds. In: *Advanced organic chemistry: Part A: Structure and mechanisms*, 4th ed., p. 481. Kluwer Academic Publishers: New York (2000)
53. Kwiecień, I., Radecka, I., Kowalczyk, M., Adamus, G.: Transesterification of PHA to oligomers covalently bonded with (bio)active compounds containing either carboxyl or hydroxyl functionalities. *PLoS ONE* **10**, e0120149 (2015)
54. Kwiecień, I., Radecka, I., Kwiecień, M., Adamus, G.: Synthesis and structural characterization of bioactive PHA and  $\gamma$ -PGA oligomers for potential applications as a delivery system. *Materials* **9**, 307–319 (2016)
55. Abate, R., Ballistreri, A., Montaudo, G., Giuffrida, M., Impallomeni, G.: Separation and structural characterization of cyclic and open chain oligomers produced in the partial pyrolysis of microbial poly(hydroxybutyrate)s. *Macromolecules* **28**, 7911–7916 (1995)
56. Ballistreri, A., Garozzo, D., Giuffrida, M., Impallomeni, G., Montaudo, G.: Primary thermal decomposition processes in aliphatic polyamides. *Polym. Degrad. Stab.* **23**, 25–41 (1989)
57. Levchik, S.V., Weil, E.D., Lewin, M.: Thermal decomposition of aliphatic nylons. *Polym. Int.* **48**, 532–557 (1999)
58. Bahr, U., Lüderwald, I., Müller, R., Schulten, H.R.: Pyrolysis field desorption mass spectrometry of polymers. III. Aliphatic polyamides. *Angew. Makromol. Chem.* **120**, 163–175 (1984)
59. Mailhos-Lefevre, V., Sallet, D., Martel, B.: Thermal degradation of pure and flame-retarded polyamides 11 and 12. *Polym. Degrad. Stab.* **23**, 327–336 (1989)