RSC Advances

PAPER

Cite this: *RSC Adv.*, 2016, **6**, 43954

Esterification of poly(γ-glutamic acid) (γ-PGA) mediated by its tetrabutylammonium salt†

M. Biagiotti,[a] G. Borghese,[a] P. Francescato,[a] C. F. Morelli,[a] A. M. Albertini,[b] T. Bavaro,[c] D. Ubiali,[cd] R. Mendichi[e] and G. Speranza*[ad]

Received 3rd April 2016
Accepted 25th April 2016

DOI: 10.1039/c6ra08567a

www.rsc.org/advances

Poly(γ-glutamic acid) is a linear anionic biopolymer synthesized by bacterial fermentation from sustainable resources. Being water soluble, biodegradable, edible and non-toxic to humans and the environment, applications of γ-PGA are of interest in a broad range of industrial sectors. However, preparation of γ-PGA derivatives is plagued by several difficulties including its scarce solubility in organic solvents. We here report a γ-PGA derivatization procedure based on the use of its tetrabutylammonium salt. The modified solubility of γ-PGA provided by counterion exchange led to the synthesis of poly(α-ethyl-γ-glutamate), poly(α-benzyl-γ-glutamate) and poly(α-*n*-butyl-γ-glutamate) under smoother conditions and an almost complete functionalization degree.

Introduction

Poly(γ-glutamic acid) (γ-PGA, **1**, Fig. 1) is a linear, anionic, water-soluble homopolyamide composed of D- and/or L-glutamic acid monomers polymerized *via* γ-amide bonds, mainly produced by Gram-positive bacteria of the genus *Bacillus*.[1] γ-PGA is biodegradable, edible, non-toxic to humans and the environment and can be obtained by bacterial fermentation from renewable resources.[1,2] The whole of these characteristics, as well as the GRAS (Generally Regarded As Safe) status of the producer bacterial strains have raised an enormous interest in γ-PGA as a novel material for many industrial and biotechnological applications.[3]

Among others, γ-PGA has been proposed as flocculant and chelating agent in waste-water treatment, cryoprotectant and texture enhancer in food industry, moisturizer in cosmetic products, biological adhesive and drug carrier in medicine, and so on.[3,4]

Even more interesting perspectives arise from the possibility to chemically modify the polymer in order to modulate its chemical–physical properties and create new materials suitable for technological as well as biomedical applications.[5]

The synthesis of esters or amides from the free α-carboxylic groups is the most commonly used approach to attain chemically modified γ-PGA derivatives.[5]

However, this is a quite challenging task, given the peculiarities of γ-PGA, *i.e.* its scarce solubility, the high viscosity of its solutions and the inherent low chemical reactivity of the α-carboxylic side groups which are highly hindered.[5] A further constraint is frequently represented by isolation and purification of the obtained products.

To date, the preparation of γ-PGA esters can be achieved by the procedure firstly reported by Kubota and coworkers.[6,7] This protocol relies on the reaction of γ-PGA with alkyl halides in the presence of sodium hydrogencarbonate in an organic solvent such as DMF, DMSO or NMP at 60 °C (Scheme 1a). Under these conditions, esterification of γ-PGA with several linear alkyl halides, up to dodecyl and including dihalogenoalkanes, was carried out.[5] The reaction outcome was found to be dependent on the size of the alkyl group, being better results obtained with short-chain alkyl halides.[7] The derivatization step must be usually reiterated in order to achieve a high degree of

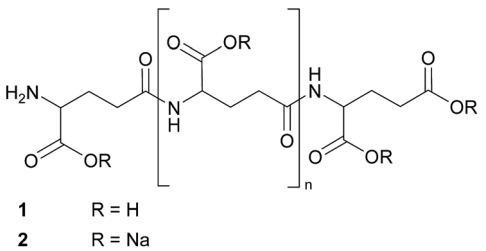


Fig. 1 Poly(γ-glutamic acid) (γ-PGA, **1**) and its sodium salt (**2**).

[a]*Dipartimento di Chimica, Università degli Studi di Milano, via C. Golgi 19, I-20133 Milano, Italy. E-mail: giovanna.speranza@unimi.it*

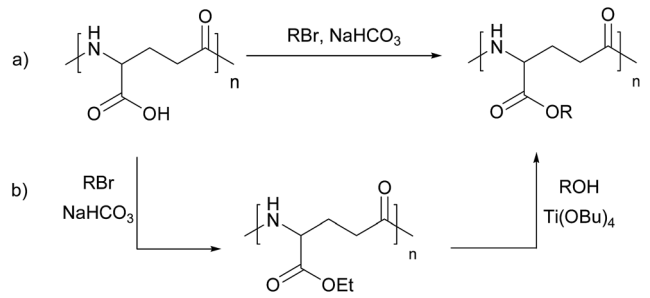
[b]*Dipartimento di Biologia e Biotecnologie "L. Spallanzani", Università degli Studi di Pavia, via A. Ferrata 9, I-27100 Pavia, Italy*

[c]*Dipartimento di Scienze del Farmaco, Università degli Studi di Pavia, viale T. Taramelli 12, I-27100 Pavia, Italy*

[d]*ISTM-CNR, via C. Golgi 19, I-20133 Milano, Italy*

[e]*ISMAC-CNR, via E. Bassini 15, I-20133 Milano, Italy*

† Electronic supplementary information (ESI) available: Molecular weight distribution (MWD) of γ-PGA (**1**) and γ-PGA derivatives (**2–6**), ^{1}H and ^{13}C NMR of γ-PGA TBA salt (**3**), ^{1}H NMR data of poly(α-ethyl γ-glutamate) (**4**), poly(α-benzyl γ-glutamate) (**5**) and poly(α-*n*-butyl γ-glutamate) (**6**). See DOI: 10.1039/c6ra08567a



Scheme 1 Procedures of esterification (a) and transesterification (b) of γ-PGA 5.

functionalization. The possibility of a reduction in molecular weight upon prolonged reaction under these conditions has been also reported.[8,9]

To overcome these limitations, a two-step esterification method was developed,[8] which was proved to be efficient also for the preparation of poly(γ-glutamate)s with medium or long alkyl chain (from 12 to 22 carbon atoms)[10] as well as with mono-, di- and tri-ethylene glycols.[11] The method consists in submitting poly(α-methyl-γ-glutamate) or poly(α-ethyl-γ-glutamate), easily obtained by the method already described, to transesterification reactions with the appropriate alcohol in the presence of $Ti(OBu)_4$ (Scheme 1b).

However, the scarce solubility of γ-PGA in organic solvents strongly narrows the broader application of these methodologies.

Following an established strategy used in polysaccharide chemistry to prepare hyaluronic acid derivatives as well as organic solvent-soluble salts of cellulose sulfate,[12,13] we here report an efficient method aimed at increasing the solubility of γ-PGA by exchanging its counterion by a quaternary ammonium salt. Such an exchange was proved to be beneficial on esterification reaction as a result of reactivity enhancement, also assisting γ-PGA manipulation.

Experimental

Materials and methods

γ-PGA Na salt (**2**) (M_w 28.3 kg mol^{-1} determined as reported below) was purchased from Natto Bioscience Co. (Japan). The acidic form (**1**) was prepared by treatment of an aqueous solution of **2** with 6 M HCl followed by precipitation with EtOH.

All other reagents and resins were purchased from Sigma-Aldrich (Milan, Italy) and/or from VWR International (Milan, Italy) and were used without further purification. All solvents were of HPLC grade and, when necessary, they were dried on molecular sieves (3 Å). ^{1}H NMR spectra were acquired at 400.13 MHz in D_2O or in DMSO-d_6 on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) interfaced with a workstation running Windows operating system and equipped with a TopSpin software package. Chemical shifts are given in ppm (δ) and are referenced to TSP [3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt, δ_{Me} 0.00 ppm] as external standard or to DMSO signal as internal standard (δ_H DMSO 2.50 ppm).

The functionalization degree was determined by ^{1}H NMR analysis, in particular measuring the ratio of intensities of the backbone α-CH signal at about 4.2 ppm and of the side-chain OCH_2 signal in the interval 4.04–5.1 ppm.

Molecular weight distribution (MWD)

The characterization of the molecular weight distribution (MWD) of the samples was performed by using a multi-angle laser light scattering (MALS) absolute detector on-line with a size exclusion chromatographic (SEC or GPC) system. The MWD, relative averages and dispersity indexes were obtained by a modular multi-detector SEC system. The SEC system consisted of an Alliance 2695 separation module from Waters (Milford, MA, USA) equipped with two on-line detectors: a MALS Dawn DSP-F photometer from Wyatt (Santa Barbara, CA, USA) and a 2414 differential refractometer (DRI) from Waters used as concentration detector. The on-line MALS detector provides absolute values of the molecular weight of the polymeric samples without external calibration, as previously reported.[16,17]

The SEC-MALS experimental conditions were selected on the basis of samples solubility. γ-PGA Na salt (**2**) and acid form (**1**): columns, 2 ultrahydrogel (2000–500 Å) from Waters; mobile phase, 0.1 M NaCl + 0.1 M phosphate buffer pH 7.0; temperature, 35 °C; flow rate, 0.8 mL min^{-1}; injection volume, 150 μL; sample concentration, about 1 or 5 mg mL^{-1}.

γ-PGA TBA salt (**3**): columns, 2 TSKGel (G4000–G3000) from Tosoh; mobile phase, 0.1 M NaCl + 0.1 M phosphate buffer pH 7.4; temperature, 35 °C; flow rate, 0.8 mL min^{-1}; injection volume, 100 μL; sample concentration, about 2 mg mL^{-1}. γ-PGA esters (4–6): columns, 2 PLgel Mixed C from Polymer Laboratories; mobile phase, DMF + 0.05 M LiBr; temperature, 50 °C; flow rate, 0.8 mL min^{-1}; injection volume, 100 μL; sample concentration, about 4 mg mL^{-1}.

Preparation of γ-PGA tetrabutylammonium salt (3, γ-PGA TBA salt)

γ-PGA sodium salt (**2**, 1.0 g, 6.6 mmol) was dissolved in 40 mL of water and then percolated through a column filled with 10 mL of wet Dowex 50WX8-200 ion-exchange resin (capacity 1.7 meq. mL^{-1} by wetted bed volume), previously activated with a 1.5 M tetrabutylammonium hydroxide solution. The resin was subsequently washed 8 times with water. The eluate was collected and lyophilized to give 1.8 g (73% yield) of γ-PGA tetrabutylammonium salt (**3**). Ammonium ion exchange degree was >99%; M_w 31.6 kg mol^{-1}.

^{1}H NMR (400 MHz, DMSO-d_6) δ 0.94 (t, J = 7.2 Hz, 12H, –$NCH_2CH_2CH_2CH_3$), 1.27–1.37 (m, 8H, –$NCH_2CH_2CH_2CH_3$), 1.55–1.62 (m, 8H, –$NCH_2CH_2CH_2CH_3$), 1.67–1.82 (broad m, 2H, –CH_2CH_2CO–), 1.86–1.97 (broad m, 1H, –CH_2CH_2CO–), 1.99–2.10 (broad m, 1H, –CH_2CH_2CO–), 3.18–3.24 (m, 8H, –NCH_2-$CH_2CH_2CH_3$), 3.55–3.63 (broad m, 1H, –*CH*COO), 6.99 (broad d, J = 4.8, 1H, –CO*NH*).

^{13}C NMR (100 MHz, DMSO-d_6) δ 13.93 N($CH_2CH_2CH_2CH_3$), 19.67 (N($CH_2CH_2CH_2CH_3$)$_4$), 23.55 (N($CH_2CH_2CH_2CH_3$)$_4$), 28.13 ($CHCH_2CH_2CO$), 32.42 ($CHCH_2CH_2CO$), 52.85 (*CH*CH_2CH_2CO), 58.07 (N($CH_2CH_2CH_2CH_3$)$_4$), 172.07 (*CO*NH); 174.33 (*C*OO).

Preparation of poly(α-ethyl γ-glutamate) (4)

Method A: alkylation of γ-PGA salts with ethyl bromide. Procedure A1: alkylation of γ-PGA sodium salt (2). To a suspension of γ-PGA sodium salt, **2** (1.0 g, 6.6 mmol) and $NaHCO_3$ (1.0 g, 11.9 mmol) in DMSO (40 mL) at 45 °C, ethyl bromide (2.2 mL, 29.6 mmol) was added and the reaction was kept at 45 °C for 3 days under vigorous stirring. The mixture was cooled to room temperature and slowly added to a diluted HCl solution (400 mL) keeping the pH at 1–2. The precipitate (**4**) was collected by filtration and shown to have a functionalization degree close to 50% by ^{1}H-NMR analysis.

A higher functionalization degree (72%) was achieved by reacting this material under the same conditions for further 2 days. 270 mg of **4** (26% yield, M_w 33.4 kg mol^{-1}) were obtained. NMR data: in agreement with those previously reported.[7]

Procedure A2: alkylation of γ-PGA TBA salt (3). γ-PGA tetrabutylammonium salt (**3**, 1.1 g, 3.0 mmol) was dissolved in 20 mL of DMSO at 45 °C and treated with $NaHCO_3$ (0.5 g, 5.9 mmol) and ethyl bromide (1.1 mL, 14.8 mmol). The reaction mixture was kept at 45 °C for 3 days under vigorous stirring and worked-up as above. Compound **4** (470 mg) was obtained in quantitative yield (99% functionalization degree, M_w 30.8 kg mol^{-1}). NMR data: in agreement with those previously reported.[7]

Method B: esterification with oxalyl chloride-catalytic DMF and alcohol. Procedure B1: esterification of γ-PGA sodium salt (2). γ-PGA (**2**, 250 mg, 1.6 mmol) was suspended in anhydrous EtOH (4 mL) under N_2 atmosphere and cooled to 0 °C. Oxalyl chloride (0.9 mL, 10.5 mmol) and a catalytic amount of anhydrous DMF (0.1 mL) were carefully added dropwise keeping the temperature under 5 °C. Once the addition was completed, the solution was stirred at r.t. for 36 hours. The reaction mixture was diluted with ethanol (14 mL) and neutralized with a saturated $NaHCO_3$ solution (20 mL). EtOH was removed under reduced pressure and the resulting residue was filtered and washed with little portions of distilled water, ethanol and diethyl ether affording **4** as a white powder (200 mg, 80% yield). Functionalization degree: 22%; M_w: 30.0 kg mol^{-1}; NMR data: in agreement with those previously reported.[7]

Procedure B2: esterification of γ-PGA TBA salt (3). γ-PGA tetrabutylammonium salt (**3**, 100 mg, 0.27 mmol) was dissolved in 3 mL of anhydrous EtOH under N_2 atmosphere and cooled to 0 °C. Oxalyl chloride (0.2 mL, 2.3 mmol) and a catalytic amount of anhydrous DMF (0.1 mL) were then carefully added dropwise keeping the temperature under 5 °C.

Once the additions were completed the solution was stirred at r.t. for 36 hours. The reaction mixture was diluted with EtOH (10 mL) and neutralized with a saturated $NaHCO_3$ solution (15 mL). After removal of EtOH under reduced pressure, the residue was worked-up as above to give 30 mg of **4** as a white powder (70% yield, functionalization degree: 100%, M_w 21.8 kg mol^{-1}). NMR data: in agreement with those previously reported.[7]

Preparation of poly(α-benzyl γ-glutamate) (5) (procedure B2)

γ-PGA tetrabutylammonium salt (**3**, 100 mg, 0.27 mmol) was dissolved in 4 mL of anhydrous benzyl alcohol and the reaction was carried out as described above. The product (**5**, 38 mg) was obtained as a white powder. Yield: 63%; functionalization degree: 100%; M_w 39.5 kg mol^{-1}; NMR data: in agreement with those previously reported.[6]

Preparation of poly(α-*n*-butyl γ-glutamate) (6) (procedure B2)

γ-PGA tetrabutylammonium salt (**3**, 100 mg, 0.27 mmol) was dissolved in 12 mL of anhydrous *n*-butanol and the reaction was carried out as described above. The product (**6**, 23 mg) was obtained as a white powder. Yield: 45%; functionalization degree: 99%; M_w 20.8 kg mol^{-1}; NMR data: in agreement with those previously reported.[7]

Solubility assays

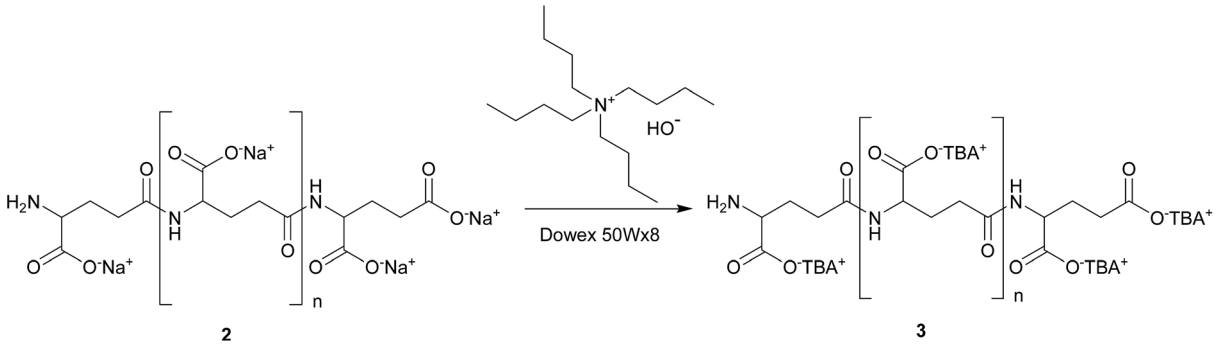
Each test was performed by weighting an amount of about 15 μmol of substance (corresponding to 2.0 mg, 2.3 mg and 5 mg of **1**, **2** and **3**, respectively) and adding stepwise 50 μL of the selected solvent till complete dissolution or up to a final volume of 2 mL. Assays were performed under magnetic stirring at room temperature for 8 hours.

Results and discussion

It is well known that the nature of the counterion has an important influence on the physicochemical properties of ionic materials, including solubility. Specifically the solubility in organic solvents increases markedly when a large "onium" cation is substituted for the usual alkali metal cation.[14] We reasoned therefore that the substitution of a metal counterion with a tetrabutylammonium ion would have been beneficial for the solubility profile of γ-PGA. In order to test our hypothesis, commercially available sodium salt of γ-PGA (**2**) was solubilized in water and the resulting solution was percolated through a column filled with the sulphonic resin Dowex 50Wx8 in the form of tetrabutylammonium salt (TBA). The eluate was collected, freeze-dried to give γ-PGA TBA salt (**3**) in 73% yield (Scheme 2).[12] Analysis of NMR spectra indicated that under these conditions, almost a complete substitution of sodium with TBA ion occurred.

Solubility tests carried out on **3** as well as on γ-PGA (**1**) and γ-PGA sodium salt (**2**) (see Table 1) indicated that the presence of the TBA counterion improves the solubility of the biopolymer in organic solvent, particularly in the case of aprotic polar solvents such as DMF and DMSO, and short-chain alcohols (MeOH, EtOH, *n*-PrOH). However, γ-PGA TBA salt (**3**) still remains insoluble in ethers and branched alcohols (iso-PrOH and *t*-BuOH).

To evaluate the effect of the solubility enhancement on reactivity, we investigated the esterification of the biopolymer and, specifically, the preparation of poly(α-ethyl γ-glutamate) (**4**) as a reference reaction. Poly(α-alkyl-γ-glutamate)s are among the most promising derivatives of PGA *e.g.* alkyl esters of PGA display improved thermal and mechanical properties with respect to the polyacid and have been investigated for their capability to form biodegradable fibers and films that can replace currently used non-biodegradable polymers.[2,5]



Scheme 2 Synthesis of γ-PGA TBA salt (**3**).

Table 1 Solubilities of γ-PGA (**1**), γ-PGA sodium salt (**2**), and γ-PGA TBA salt (**3**), respectively, in different organic solvents[a]

Solvent	γ-PGA (**1**)	γ-PGA Na (**2**)	γ-PGA TBA (**3**)
MeOH	−	−	+
EtOH	−	−	+
n-PrOH	−	−	+
i-PrOH	−	−	−
n-BuOH	−	−	±
t-BuOH	−	−	−
Benzyl alcohol	−	−	+
2,2,2-Trifluoroethanol	−	−	+
Diethyl ether	−	−	−
Dioxane	−	−	−
Acetonitrile	−	−	−
DMF	−	±	++
DMSO	±	±	++

[a] Each test was performed by weighting an amount of about 15 μmol of substance (corresponding to 2.0 mg, 2.3 mg and 5 mg of **1**, **2** and **3**, respectively) and adding stepwise 50 μL of the selected solvent till complete dissolution or up to a final volume of 2 mL, under magnetic stirring at room temperature for 8 hours. Legend: −, insoluble; +, soluble; ++, very soluble; ±, sparingly soluble.

Two methodologies were used. The former method (namely, A) is the one already described above, firstly reported by Kubota[5–7] and based on carboxylate chemistry, which was found to be effective when γ-PGA sodium salt (**2**) is used as the starting material (see Scheme 3, Method A).

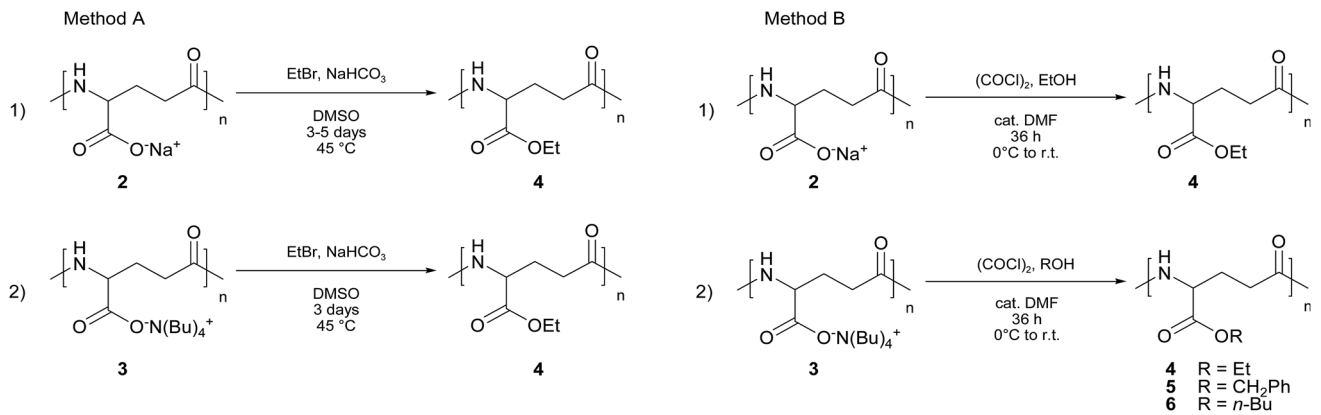
The latter route (namely, B) was set up in our laboratories and consists in the *in situ* formation of the acyl chloride of poly(γ-glutamic acid) by treatment of both **2** and **3** with oxalyl chloride in the presence of a catalytic amount of dimethylformamide (DMF),[15] followed by direct esterification with the proper alcohol (see Scheme 3, Method B).

Using γ-PGA sodium salt (**2**) as the starting material our strategy appears advantageous with respect to the Kubota's methodology in terms of reaction time (120 hours *vs.* 36 hours) and final yield (26% *vs.* 80%). Nevertheless, the functionalization degree is far to be excellent (72% *vs.* 22%, see Table 2, entries A1 and B1).

Contrariwise, when γ-PGA TBA salt (**3**) was used as the starting material in both procedures, an overall improvement of the reaction outcome was observed in terms of reaction time as well as yield and functionalization degree. In the case of Method A, the reaction time was reduced from 120 to 72 hours and, mostly important, both the functionalization degree and the yield were almost complete (*cf.* entries A1 and A2 in Table 2).

On the other hand, the use of γ-PGA TBA salt (**3**) in the procedure B, under the same experimental conditions (r.t., 36 hours), afforded an almost complete functionalized product (**4**), although with a slightly lower yield (*cf.* Table 2, entries B1 and B2). It is also worth noting that Method B does not require any heating.

Since the acyl chloride-based approach gave promising results for the preparation of poly(α-ethyl γ-glutamate) (**4**), we



Scheme 3 Alkylation of γ-PGA salts with ethyl bromide (Method A) and esterification with oxalyl chloride-catalytic DMF and alcohol (Method B).

Table 2 Comparison of different γ-PGA esterification methods

Method[a]	Product[a]	T (°C)	Time (h)	Yield (%)	Functionalization degree[b] (%)
A1	4	45	120	26	72
A2	4	45	72	99	100
B1	4	r.t	36	80	22
B2	4	r.t.	36	70	100
	5	r.t.	36	63[c]	100
	6	r.t.	36	45	99

[a] See Scheme 3. [b] The functionalization degree was determined by ^{1}H NMR analysis, in particular measuring the ratio of intensities of the backbone α-CH signal at about 4.2 ppm and of the side-chain OCH_2 signal in the interval 4.04–5.1 ppm. [c] Recovery due to the presence of solvent residues.

extended our methodology to the synthesis of other poly(α-alkyl γ-glutamate)s, in particular benzyl and *n*-butyl derivatives (**5** and **6**, respectively). These esters were chosen taking into account the different solubility of the γ-PGA TBA (**3**) in benzyl and *n*-butyl alcohols (Table 1). For both **5** and **6** a very high functionalization degree was achieved even if accompanied by a moderate yield in the case of **6**. The effect of the lower solubility of **3** in *n*-butyl alcohol cannot be ruled out.

Conclusions

In conclusion, γ-PGA TBA (**3**) was shown to be a valid starting material for a straightforward chemical modification of the biopolymer. The routinely used esterification procedure based on the reaction with alkyl bromides and the newly developed method based on the activation of the carboxylic groups with oxalyl chloride were successfully applied for the preparation of three esters (**4–6**). Taking into account the interest in γ-PGA esters as materials endowed with improved thermal and mechanical properties, the preparation of γ-PGA TBA and the set-up of a new method of esterification may assist the practical applications of this unexploited biopolymer.

Acknowledgements

This work was financially supported by Alma Mater Ticinensis Foundation (Pavia, Italy) to A. M. A., Call 2010–2011 "Promuovere la ricerca d'eccellenza". Project: "Poly(γ-glutamate) (γ-PGA): a biocompatible and biodegradable material for the immobilization of biologically active molecules".

Notes and references

1 A. Ogunleye, A. Bhat, V. U. Irorere, D. Hill, C. Williams and I. Radecka, *Microbiology*, 2015, **161**, 1–17.
2 I.-L. Shih and J.-Y. Wu, in *Microbial Production of Biopolymers and Polymer Precursors: Applications and Perspectives*, ed. B. H. A. Rehm, Caister Academic Press, Norfolk, UK, 2009.
3 H. Poo, C. Park, M.-S. Kwak, D.-Y. Choi, S.-P. Hong, I.-H. Lee, Y. T. Lim, Y. K. Choi, S.-R. Bae, H. Uyama, C.-J. Kim and M.-H. Sung, *Chem. Biodiversity*, 2010, **7**, 1555–1562.
4 I. Bajaj and R. Singhal, *Bioresour. Technol.*, 2011, **102**, 5551–5560.
5 S. Muñoz-Guerra, M. García-Alvarez and J.-A. Portilla-Arias, *J. Renewable Mater.*, 2013, **1**, 42–60.
6 H. Kubota, Y. Nambu and T. Endo, *J. Polym. Sci., Part A: Polym. Chem.*, 1993, **31**, 2877–2878.
7 H. Kubota, Y. Nambu and T. Endo, *J. Polym. Sci., Part A: Polym. Chem.*, 1995, **33**, 85–88.
8 J. Melis, M. Morillo, A. Martínez de Ilarduya and S. Muñoz-Guerra, *Polymer*, 2001, **42**, 9319–9327.
9 A. Martínez de Ilarduya, N. Ittobane, M. Bermúdez, A. Alla, M. El Idrissi and S. Muñoz-Guerra, *Biomacromolecules*, 2002, **3**, 1078–1086.
10 M. Morillo, A. Martínez de Ilarduya and S. Muñoz-Guerra, *Macromolecules*, 2001, **34**, 7868–7875.
11 G. Pérez-Camero, B. Vázquez and S. Muñoz-Guerra, *J. Appl. Polym. Sci.*, 2001, **82**, 2027–2036.
12 D. Bellini and A. Topai, WO2000001733 A1, 2011.
13 R. G. Schweiger, US 3,637,520, 1972.
14 E. V. Dehmlow and S. S. Dehmlow, *Phase Transfer Catalysis*, VCH, Weinheim, 3rd edn, 1993.
15 A. Wissner and C. V. Grudzinskas, *J. Org. Chem.*, 1978, **43**, 3972–3974.
16 R. Mendichi and A. Giacometti Schieroni, *Current Trends in Polymer Science*, ed. S. G. Pandalai, Trans-World Research Network, Trivandrum, India, 2001, vol. 6, pp. 17–32.
17 P. J. Wyatt, *Anal. Chim. Acta*, 1993, **272**, 1–40.

