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TESI DI DOTTORATO DI RICERCA

STUDIES ON NATURAL AND EDIBLE BIOPOLYMERS. ISOLATION, CHARACTERIZATION AND CHEMICAL MODIFICATIONS OF POLY(Γ-GLUTAMIC ACID) (γ-PGA) FROM *BACILLUS SUBTILIS*

CHIM/06

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INTRODUCTION

Biopolymers

Recently, naturally occurring polymers have been attracting the interest of material scientists as well as of biologists, in a quest for the production of environmentally friendly novel materials, possibly offering an alternative to petroleum-based, non biodegradable polymers, whose supply is limited in time.

Biopolymers, in fact, offer several advantages in this regard: their production, based on renewable sources, is sustainable in environmental terms. First of all, they are obviously biodegradable, being fully decomposed and mineralized to CO_2 and water when exposed to microbial flora present in soil and water. Moreover, they are often also biocompatible (*i.e.* they are non- toxic and therefore they don't cause an immune response), therefore they are suitable for several medical applications, in which they are used as scaffold, or matrices, for tissue engineering, wound healing and drug delivery.

Bacteria can produce four major classes of polymers: polysaccharides, polyesters, inorganic polyanhydrides (such as polyphosphates) and polyamides (Figure 1), with different chemical and material properties, from various carbon sources (Rehm, 2010). A few of them is intracellular, while a vast amount of extracellular ones is known. These macromolecules cover a broad range of biological functions, such as reserve material, or constituent of a protective structure, and help bacteria survive under stressing environmental conditions. Their biosynthesis, as well as their properties, is controlled by quite complex regulatory paths, acting in response of external stimuli.

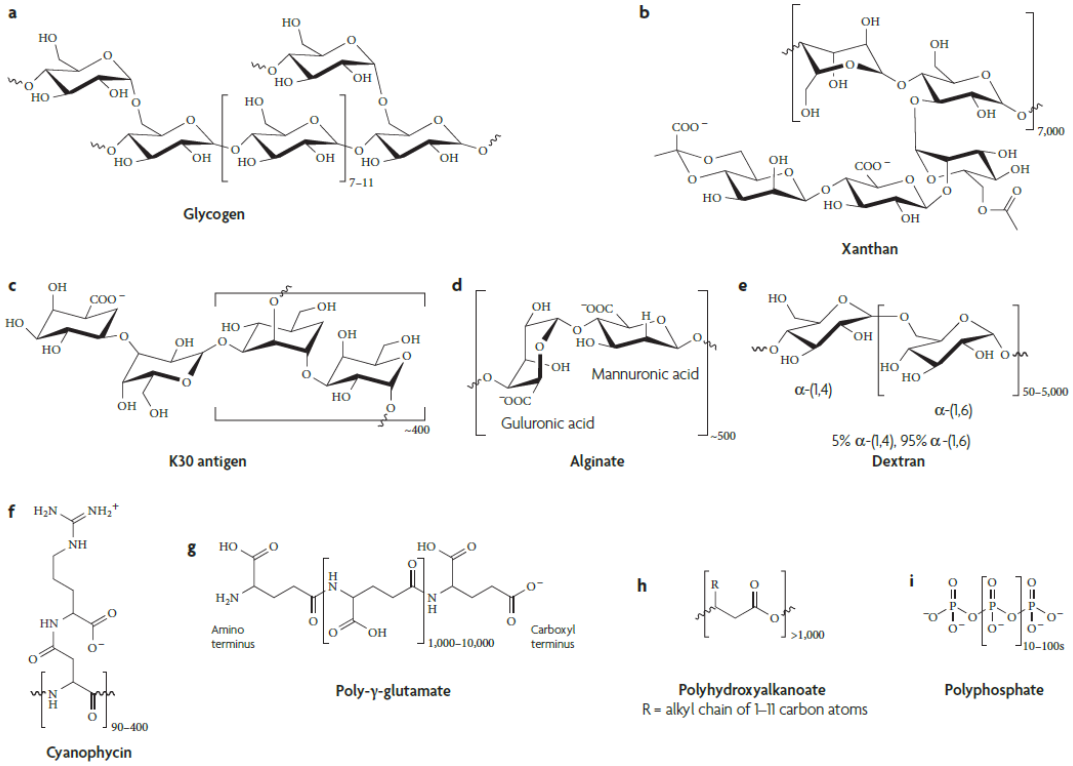


Figure 1: some examples of polymers of bacterial origin. Intracellular polymers are represented by Glycogen (a), a polysaccharide, Cyanophycin (f), a polyamide, polyhydroxyalkanoate (h) and Polyphosphate, a polyanhydride (i); among extracellular ones we find the polysaccharides Xanthan (b), Alginate (d), which is secreted and/or is part of cyst cell wall, Dextran (e) and the secreted polyamide poly-γ-glutamate (g). A capsular polymer is the K30 antigen (c), a polysaccharide. The most common polymerization degree found for each polymer is indicated by numbers below the chemical structure brackets. Source: B.H.A. Rehm, *Nature Reviews Microbiology*, 2010.

In the last years, the biosynthesis of bacterial polymer has been extensively investigated, particularly dealing with its molecular mechanism and regulatory aspects; discoveries in the field represent a strong advance for biotechnology, and are the first, necessary step to the production of tailor-made biopolymers, suitable for specific, high-value industrial as well as medical application, by means of rational engineering of bacteria. Table 1 summarizes current knowledge about bacterial polymers and their possible field of application.

Table 1: main characteristics and applications of bacterial polymers. Source: B.H.A. Rehm, *Nature Reviews Microbiology*, 2010.

Polymer class	Polymer localization	Primary structure	Main components	Precursors	Polymerizing enzyme*	Producer	Industrial applications‡
Polysaccharides							
Glycogen	Intracellular	α-(1,6)-branched α-(1,4)-linked homopolymer	Glucose	ADP–glucose	Glycogen synthase (GlgA)	Bacteria and archaea	NA
Alginate	Extracellular	β-(1,4)-linked non-repeating heteropolymer	Mannuronic acid and guluronic acid	GDP–mannuronic acid	Glycosyl transferase (Alg8)	Pseudomonas spp. and Azotobacter spp.	Biomaterial (for example, as a tissue scaffold or for drug delivery)
Xanthan§	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of pentasaccharide units	Glucose, mannose and glucuronate	UDP–glucose, GDP–mannose and UDP–glucuronate	Xanthan polymerase (GumE)	Xanthomonas spp.	Food additive (for example, as a thickener or an emulsifier)
Dextran§	Extracellular	α-(1,2)/α-(1,3)/α-(1,4)-branched α-(1,6)-linked homopolymer	Glucose	Saccharose	Dextransucrase (DsrS)	Leuconostoc spp. and Streptococcus spp.	Blood plasma extender and chromatograp-hy media
Curdlan§	Extracellular	β-(1,3)-linked homopolymer	Glucose	UDP–glucose	Curdlan synthase (CrdS)	Agrobacterium spp., Rhizobium spp. and Cellulomonas spp.	Food additive (for example, as a thickener or a gelling agent)
Gellan§	Extracellular	β-(1,3)-linked repeating heteropolymer consisting of tetrasaccharide units	Glucose, rhamnose and glucuronate	UDP–glucose, dTDP–rhamnose and UDP–glucuronate	Gellan synthase (GelG)	Sphingomonas spp.	Culture media additive, food additive (for example, as a gelling agent) or for encapsulation
Colanic acid	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of hexasaccharide units	Fucose, glucose, glucuronate and galactose	GDP–L-fucose, UDP–D-glucose, UDP–D-galactose and UDP–D-glucuronate	Colanic acid polymerase (WcaD)	Escherichia coli, Shigella spp., Salmonella spp. and Enterobacter spp.	NA
K30 antigen	Capsular	β-(1,2)-linked repeating heteropolymer consisting of tetrasaccharide units	Mannose, galactose and glucuronate	UDP–D-glucose, UDP–D-galactose and UDP–D-glucuronate	Polysaccharide polymerase (Wzy)	Escherichia coli	NA
Cellulose§	Extracellular	β-(1,4)-linked homopolymer	D-glucose	UDP–D-glucose	Cellulose synthase (BcsA)	Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Gram-positive bacteria	Food, diaphragms of acoustic transducers and wound dressing
Hyaluronic acid§	Extracellular	β-(1,4)-linked repeating heteropolymer consisting of disaccharide units	Glucuronate and N-acetyl glucosamine	UDP–D-glucuronate and UDP–N-acetyl glucosamine	Hyaluronan synthase (HasA)	Streptococcus spp. and Pasteurella multocida	Cosmetics, visco-supplementation, tissue repair and drug delivery
Polyamides							
Cyanophycin granule peptide	Intracellular	Repeating heteropolymer consisting of dipeptide units	Aspartate and arginine	(β-spartate-arginine)$_3$-phosphate, ATP, L-arginine and L-aspartate	Cyanophycin synthetase (CphA)	Cyanobacteria, Acinetobacter spp. and Desulfitobacterium spp.	Dispersant and water softener (after removal of arginyl residues)
Poly-γ-glutamate	Extracellular or capsular	Homopolymer	D-glutamate and/or L-glutamate	(Glutamate)$_n$-phosphate, ATP and glutamate	Poly-γ-glutamate synthetase (PgsBC; also known as	Bacillus spp. and a few Gram-positive bacteria, the Gram-negative bacterium Fusobacterium	Replacement of polyacrylate, thickener, humectant, drug delivery and

Polymer class	Polymer localization	Primary structure	Main components	Precursors	Polymerizing enzyme*	Producer	Industrial applications‡
					CapBC)	nucleatum and the Archaea Natronococcus occultus and Natrialba aegyptiaca	cosmetics
ε-poly-L-lysine	Extracellular	Homopolymer	L-lysine	L-lysine, ATP and L-lysine–AMP	ε-poly-L-lysine synthetase (Pls)	Streptomyces albulus subsp. lysinopolymerus	Feed preservative and, when cross-linked, adsorbent (in medicine)
Polyester							
Polyhydroxyalkanoates§	Intracellular	Heteropolymer	(R)-3-hydroxy fatty acids	(R)-3-hydroxyacyl CoA	Polyhydroxyalkanoate synthase (PhaC)	Bacteria and archaea	Bioplastic, biomaterial and matrices for displaying or binding proteins
Polyanhydrides							
Polyphosphate	Intracellular	Homopolymer	Phosphate	ATP	Polyphosphate kinase (PPK)	Bacteria and archaea	Replacement of ATP in enzymatic synthesis and flavour enhancer

dTDP, deoxythymidine diphosphate; NA, not applicable.
*All polymerizing enzymes involved in the synthesis of intracellular polymers localize to the cytosol. All polymerizing enzymes involved in the synthesis of extracellular or capsular polymers localize to the cytosolic membrane, except for dextran sucrase, which is secreted and anchored to the cell wall.
‡For those polymers that are not commercially produced, potential applications are suggested.
§Commerically produced polymers.

Polysaccharides

Polysaccharides represent the most numerous class of bacterial polymers. They can be categorised into exopolysaccharides, (such as dextran, xanthan, hyaluronic acid, and alginate) which are excreted or formed in the environment surrounding the cell, intracellular ones (such as glycogen) and capsular ones (such as the K30 antigen). They can alternatively be classified, according to their structure, into repeat unit polymers (*e.g.* xanthan and alginate), repeating polymers (*e.g.* cellulose) and non-repeating polymers (alginate). Moreover, many polysaccharides with structural functions contribute to form cell walls, but they will not be discussed here.

Obviously, given the huge variety of polysaccharides structures, their synthesis involves the action and coordination of several enzymes and proteins.

A wide number of Bacteria and Archea is known to produce exopolysaccharides, many of which are of interest for their material properties.

Dextrans are synthesized thanks to a key enzyme called dextransucrase, with an average molecular weight around 160 kDa, which is anchored to the cell wall; it belongs to the family of glycoside hydrolase. The glycosidic bond in sucrose is hydrolyzed by dextransucrase, and then glucose is transferred to the reducing end of the growing glucan chain which is covalently bond. This reaction happens *via* an insertion mechanism granted by two separate catalytic sites present in the same enzymatic active site. Driving force of the process is the hydrolysis of the glycosidic bond (Robyt, 2008). The resulting polymer is quite polydisperse (molecular mass is between 10^6 and 10^9 Da) and soluble in water, where it has a Newtonian behavior, with a viscosity that depends on concentration, molecular mass and temperature (Carrasco, 1989). Moreover, on the biological point of view, it has a very low immunogenicity, and thus it has been used for many pharmaceutical applications (Leathers, 2005).

Alginate is produced by a multiprotein complex extending between the periplasm, the cytoplasmic membrane and the outer membrane (Kim, 1994) that, although extensively studied, has still not disclosed all the aspects of the polymerization molecular mechanism. Still, it is possible to genetically engineer bacteria constitutively able to produce alginate (such as *Pseudomonas fluorescens* and *Azobacter vinelandii)* to get polymers with controlled characteristics and material properties, which depend on factors such as the molecular mass, degree of acetylation, sequence and molar ratio of glucoronic and mannuronic acid constituents (Remminghorst, 2006; Steigedal, 2008). The interest of material scientists for alginates is justified by the ability as viscosifying, stabilizing, and water retaining agents.

Xanthan is another polymer of bacterial origin whose technological properties have attracted much interest; it is an exopolysaccharide, and its structure is that of a repeating unit heteropolymer, formed by pentasaccharides. Its biosynthesis has been elucidated

taking as a paradigm capsular polysaccharides (such as the K30 antigen); that don't have commercial interest themselves, since they often have the functions of virulence factors. However, the study of their biosynthesis for medical and pharmaceutical reasons provided a good model for the class of repeating unit polymers, which xanthane belongs to. The process is initiated by glycosyl transferase, WbaP, a polyisoprenyl sugar phosphate transferase anchored to the cell wall, that transfers the sugar phosphate from the corresponding nucleotide sugar to undecaprenyl phosphate, and another monofunctional glycosyl transferase enzyme called GumK. Polymerization reaction happens at the cytoplasmic membrane, on the periplasmic side, thanks to a transport protein called Wzx, specific for polysaccharides, which transfers the undecaprenyl phosphate bond repeat unit across the membrane. It is believed that this enzyme has also a role in controlling the polymer's length (Wang, 1994; Wang, 1996; Drummelsmith, 1999; Tocilj, 2008).

Polyesters

When growth is limited by lack of nutrients like nitrogen or phosphorus, but at the same time a rich carbon source is available, bacteria can accumulate a carbon reserve in the form of polyhydroxyalkanoate (PHA) (Anderson, 1990; Kessler, 2001). The polymer is kept inside the cell as an inclusion in which a hydrophobic core of PHA is surrounded by the proteins which regulate its metabolism (Grage, 2009; Jendrossek, 2009). PHAs are usually classified according to their chain length, into medium- chain- length (C_6-C_{14}) and short-chain-length (C_3-C_5) ones; the former are produced by *Pseudomonads*, the latter by numerous *Bacteria* and *Archea*. The biosynthesis of polyhydroxyalkanoate is made possible by the PHA synthase enzyme (PhaC); almost any organic molecule endowed with a hydroxyl and a carbonyl group can theoretically be polymerized to form PHA, *via* the transformation in the corresponding CoA thioesther. This is possible

thanks to the broad substrate specificity of PhaC (Rehm, 1999). Its composition is extremely variable, since more than 150 constituents have been identified so far, therefore also its physical-chemical properties can dramatically differ: for instance, melting temperatures range from 50 to 180°C, and crystallinity between 30 and 70%. This fact is of great interest with regard to the possible technological use of PHA as biocompatible and biodegradable plastic, replacing oil-based products, particularly in the field of thermoplastic resins, considering that some two-thirds of the current oil-deriving commodities belong to this category (Andrade, 2003).

Polyanhydrides

Polyphosphate is the only polyanhydride present in living cells. Bacteria use it to perform a variety of tasks, such as creating storage particles in the cell, or helping the uptake of metal ions and DNA thanks to the formation of a membrane complex with polyhydroxybutyrate (Reusch, 1988). Polyphosphate is also involved in bacterial motility, response to stress, pathogenicity, and many other physiological activities (Rao, 2009). Its biosynthesis is performed by the key enzyme polyphosphate kinase (PPK), which can produce it from ATP. Since it is also able to perform the inverse reaction, namely the phosphorilation of ADP to give ATP, this enzyme is also used in enzymatic synthetic processes that are ATP dependent to recycle expensive ATP using cheap polyphosphate (Kameda, 2001). The polymer itself has many applications, from flavor enhancer in food industry to flame retardant, with a performance comparable to that of asbestos. Its production by bacteria is not economically affordable since it is easily obtained by inorganic resources, but it can be exploited as a means to remove phosphate from industrial waste water, thus contributing to the removal of pollutants.

Polyamides

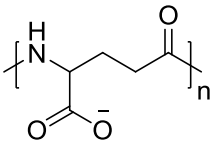
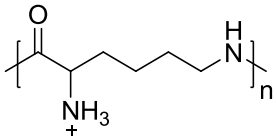
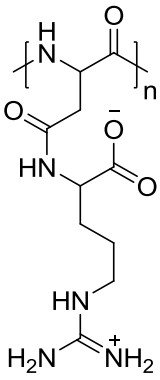
Among other biopolymers which bacteria are able to synthesize, three different polyamides are known, namely poly-γ-glutamic acid (PGA), poly-ε-lysine, and multi-L-arginyl-poly (L-aspartic acid), also known as cyanophycin. Their main producers belong to the genus *Bacillus,* as well as to *Cnidaria* and cyanobacteria. Their possible use in material science makes them a remarkable subject of research.

Polyamides are defined as polymeric compounds whose constituents are linked by amide bonds. They can be further divided in homopolyamides, which are composed by just one type of amino acid monomers, and copolyamides, consisting of different amino acids. Obviously, proteins represent the most numerous category of known copolyamides; a small number of compounds are indeed known as poly (amino acids), since their biosynthesis is significantly different from proteins' one. In particular, four capital features distinguish proteins from poly(amino acids): in poly(amino acids), only one amino acid monomer is present, at least in its backbone, while proteins incorporate usually up to 21 amino acids; the biosynthesis of poly(amino acid) does not involve ribosomial transcription, but is effected by enzymes, whose characteristics and coordination is relatively simple; thus, poly(amino acids), differently from proteins, are highly polydisperse and show a great size distribution, while proteins present monodispersity and a well defined and precise length. Finally, in poly (amino acids) the amide bonds which keep the structure together involve side chain functions and not just α- amino and α- carboxylic groups as it happens in proteins.

In nature, three poly(amino acids) have been discovered to date, i.e. cyanophicycin, composed by α-aspartic acid monomers bond to arginine residues at the β-carboxylic group position, poly(lysine), formed by lysine monomers connected by linkages between the α-carboxylic group and the ε- amino group, and poly-γ-glutamic acid

(PGA), in which the polymer backbone is formed by glutamic acid residues whose α-amino group is bond to the γ-carboxylic one (Table 2).

Table 2: poly (amino acid)s in Nature and their main features. Adapted from Oppermann- Sanio, 2002.

Poly(amino acid)	Distribution in organisms	Applications
 Γ-poly (glutamic acid)	*Bacillus anthracis* *B. licheniformis* *B. megaterium* *B. subtilis* *Sporosarcina halophila* *Planococcus halophila* *Natrialba aegyptiaca* Nematocysts of *Cnidaria*	Dispersant Water softener Waste water treatment Thickener in food and cosmetics Superabsorber Drug delivery devices Humectant in cosmetics Treatment of leather
 Poly (lysine)	*Streptomyces albulus* *lysinopolymerus*	Superabsorber Drug delivery devices Additive to animal feeding stuff
 Cyanophycin	Most cyanobacterial species	Dispersant Water softener (after removal of arginyl residues)

Although evidence so far indicates the existence of these very three poly (amino acids), it is indeed possible that many others are still waiting to be discovered and investigated.

Cyanophycin

Cyanophycin granule polypeptide (CGP), also called cyanophycin, is a compound occurring exclusively (Simon 1987) in all cyanobacterial groups, of all kinds, nitrogen fixing and non-nitrogen fixing, unicellular and filamentous (Lawry, 1982; Allen, 1988; Golecki, 1991). The discovery of it by Borzi, who detected the material microscopically as cell inclusions with highly refractive features, dates back to 1887. The physiological role of cyanophycin is that of intracellular temporary nitrogen storage. In fact, every polymer's building block contains five nitrogen atoms, and the polymer itself, at cell internal pH and ionic strength, is insoluble. Cyanophycin is accumulated during stationary growth phase of the cell, while it is present at very low levels during exponential and balanced growth phase. Moreover, cyanobacteria are able to detect the nitrogen level in the medium surrounding them, and therefore to trigger the production of cyanophycin to protect themselves from nitrogen depletion (Liotenber, 1996). The polymerization of cyanophycin is catalysed by an enzyme called CGP synthetase or CphA; polymer synthesis requires the presence of ATP, ions such as K^+ and Mg^{2+}, a thiol reagent and a CPG primer (Aboumalgd,. 2001; Simon, 1976; Ziegler, 1998) ; and probably resembles an amide-ligase dependent process (Berg, 2000). Experimental data suggests that CphA synthetase should form a homodimer endowed with two binding sites for ATP and substrate, respectively in order to include both arginine and aspartate in the polymer (Krehenbrink, 2004). Degradation of the polymer to allow bacteria to exploit the nitrogen reserve is granted by the enzyme cyanophycinase, or CphB.

Isolation of cyanophycin is possible by repeated centrifugation of disrupted cells from culture broth (Simon 1971); polymer purification is achieved, taking advantage of its solubility properties, by repeated dissolution in acidic solution, followed by centrifugation and precipitation in neutral conditions. In fact, cyanphycin is soluble in

acidic conditions, under pH 2, as well as in basic ones, over pH 9, but insoluble in physiological conditions and at low ionic strength. It can, however, be dissolved in concentrated urea solution. It is also pretty insoluble in the most common organic solvents, such as methanol, DMSO, DMF. Quantification of the polymer in cells is possible by means of HPLC of the amino acid components after isolation (Simon 1973), or by NMR of the crude product (Aboulgmad, 2000). On the structural point of view, cyanophycin is formed by aspartic acid and arginine in almost equimolar amounts; aspartic acid forms the polymer backbone, while arginine is bond to the aspartic β-carboxylic group. Its molecular weight may vary between (as was estimated by SDS polyacrilamide electrophoresis) 25 and 100 kDa, thus it is quite polydisperse (Simon 1973, 1976). Circular dichroism and Raman studies allowed to make hypothesis about the polymer's secondary structure, and it is commonly believed to assume a β-sheet conformation under acidic solution and likely, it was inferred, in the insoluble form (Simon 1980). Structural studies were also confirmed by ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy (Suarez 1999). Cyanophycin may be used, after chemical modification to reduce its arginine content (Joentgen, 1998) as a good biodegradable substitute of polyacrilate (Schwamborn, 1998); however, it is still not a practical material on the industrial point of view, since its biosynthesis, both from native cyanobacteria of from recombinant *E.coli* strains optimized for production, is too expensive to make it cost-effective (Hai, 2000; Ziegler, 1998; Oppermann, 1999; Aboumalgd, 2000).

Poly(lysine)

The only organism, among bacteria and eukaryotes, so far known to be able to produce poly (lysine) (PL), is the Gram-positive bacterium *Streptomyces albulus* ssp. *lysinopolymerus* strain 346. It was isolated from Japanese soil at the end of the 1970s.

This bacterium is constituently able to excrete the biopolymer in the medium at concentrations of up to 4-5 g/L (Shima, 1977, 1981).

Isolation of poly (lysine) is possible by means of ionic exchange chromatography of the culture filtrate, since it is a cationic polymer and neutral pH, and precipitation with ethanol/diethylether (Shima, Sakai, 1981). Polymer filaments are typically composed by 25-30 residues; therefore its molecular weight is not high.

Its physiological function is mainly that of an antibiotic; in fact, poly (lysine) can inhibit the growth of both Gram positive and Gram negative bacteria already at concentration around 1-8 mg/L (Shima, 1982, 1984) and it can also inactivate bacteriophages.

In view of these properties, poly (lysine) has been proposed as a preservative for animal feed. It is also able to form hydrogels highly capable of water adsorption (Choi, 1995, Kunioka, 1995) and, if crosslinked it can be used in pharmaceutical applications as a cationic adsorbent (Hyrayama, 1999). Poly (lysine) is nowadays produced biotechnologically by means of mutant strains optimized for the polymer yield (Hiraki, 1999).

Poly-γ-glutamic-acid

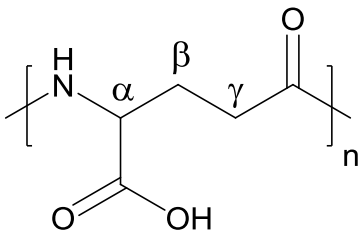


Figure 2: Poly-γ-glutamic- acid

Poly-γ-glutamic- acid, also known as γ-PGA, is an anionic homopolyamide formed by D- and L- glutamic acid units, unusually connected by amide bonds between α-amino and γ-carboxylic acid groups. It was first isolated by Ivanovics and coworkers (Ivanovics, 1937a; 1937b) in 1937 as the constituent of the capsule of *Bacillus*

anthracis; it is also known to be an important part of the mucilage of several soybean-fermented traditional Eastern foods, such as the Japanase *natto* (Sawamura, 1913, Fujii, 1963) and the Korean seasoning *chungkookjang* (Ashiuchi, 2005) (Figure 3).

Figure 3: Natto, a traditional Japanese dish naturally containing γ-PGA.

To date, it is known to be produced by some Archea, such as *Natronococcus occultus* (Niemitz, 1997) and *Natrialba aegypytiaca* (Hezayen, 2001), and several Gram positive bacteria strains, all belonging to the genus *Bacillu*s, and so phylogenetically related. Recently, also the Gram negative *bacterium Fusobacterium nucleatum* was found to be a γ-PGA producer (Candela, 2009). The only eukaryote organisms in which it is known to be present are some *Cnidaria*, animals armed with stringing cells called nematocysts (Weber, 1990).

Γ-PGA possesses many peculiar properties; among these, to be water-soluble (in its anionic, salt-form), biodegradable, non-toxic, non immunogenic, and even edible. As a polyamide, it is also quite robust with regard to hydrolytic degradation, both in acid and basic conditions. Therefore, the potential applications of this material cover a broad

range in industrial fields such as food, cosmetics, medicine, waste-water treatment, and will later be discussed.

Physiological function of γ-PGA

Poly-γ-glutamic- acid has several physiological functions, which may differ a lot according to the specific needs of the producer species; what is constant in all cases, as it will be illustrated, is the role of the biopolymer as an adaptation agent in hostile environments (Ashiuchi, 2002).

B. anthracis (a Gram-positive, sporulating bacterium) is notoriously the causal agent of the lethal and infectious human disease called anthrax. Its virulence has been found to be correlated with the presence of an anchored capsule composed by poly-γ-D-PGA (Makino, 1989); this capsule (which is, on the stereochemical point of view, composed by only D-glutamic acid monomers) enables the bacteria to avoid phagocytosis (Zwartouw, 1956), and makes it particularly non-immunogenic. Moreover, it prevent antibodies from accessing the bacterium (Mesnage, 1998), and, acting as a passive barrier, protect *B. anthracis* against phage infections (Mc Cloy, 1951).Poly-γ-D-PGA is, in this case, an essential virulence factor as well as a structural component.

Other bacterial strains release poly-γ-glutamic- acid in the environment, with different purposes.

Some *B. subtilis* and *B. licheniformis* strains, in fact, take advantage of high-cryoprotectant properties of the polymer to survive in the cold (Mitsuiki, 1998); other Bacillus soil-strains release the polymer to sequestrate toxic metal ions, to increase their resistance (Mc Lean 1990, 1992). Poly-γ-glutamic- acid is also used as a protective agent against high-saline conditions, thanks to its prominent water binding capacity, by halophilic Archea such as *Natrialba aegyptica* (Hezayen, 2001). Finally, the polymer

can be used as a source of glutamate in starvation conditions (Kimura, 2004), or as a storage compound for carbon and nitrogen precursor (Schreier, 1993). A peculiar case is that of the animal called *Hydra* (belonging to the phylum *Cnidaria*), in which γ-PGA is a secretory product of stringing cells which serves as an adhesive to adhere to the surface of the aggressor, or prey, while *Hydra* injects toxic venoms in it, as well as to regulate internal osmotic pressure necessary for explosive evagination (Weber, 1990).

In summary, currently known data support the hypothesis that γ-PGA, when is released by the bacterium, subjected to external stress, in the environment, serves to increase the producer's survival chances when (Gross, 1998; Hezayen, 2000), acting as a persistence agent, while anchored γ-PGA is considered a bacterial virulence factor (Candela and Fouet, 2006).

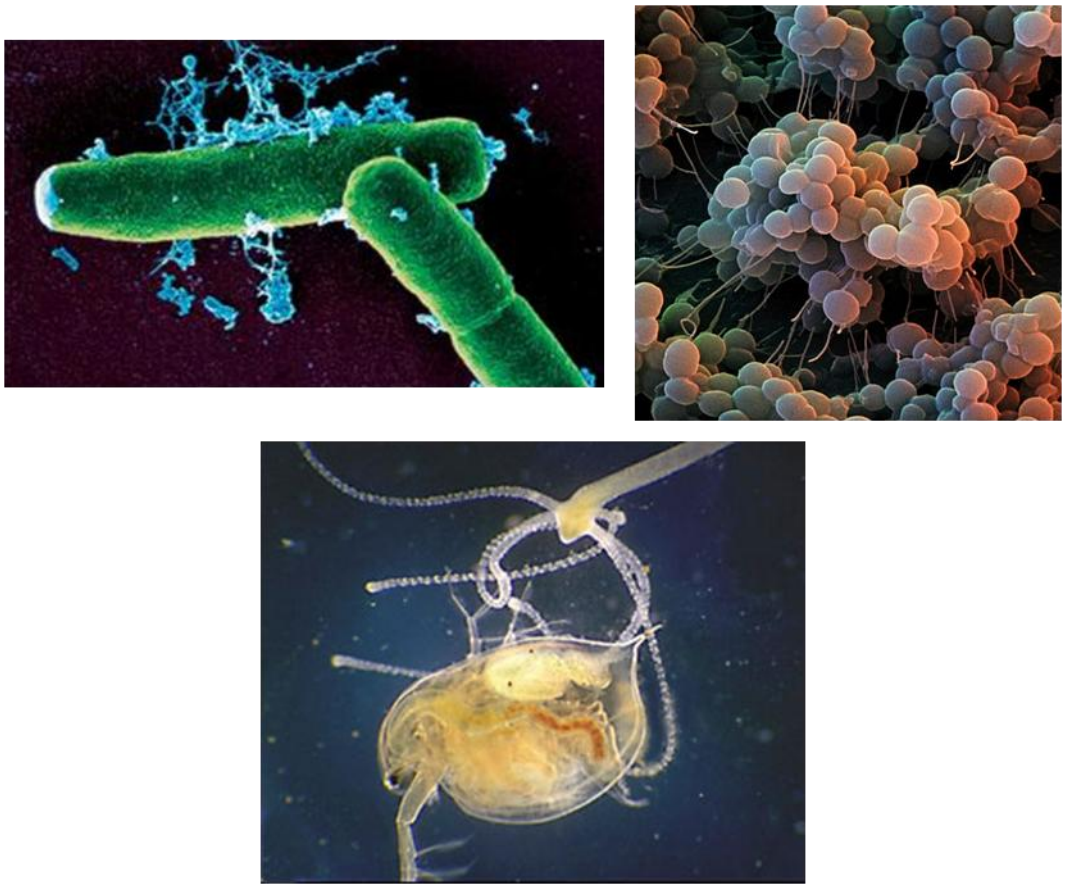


Figure 4: γ-PGA *in vivo*: anchored (upper left), released (upper right) and secreted by stringing cells (bottom centre).

Chemical properties of γ-PGA

The main chemical peculiarity of γ-PGA, with differentiates it from other polypeptides and proteins both structurally and functionally, is that glutamic residues are non-ribosomally polymerized *via* the γ-amino bond instead of the α-amino one.

This property, common to all the polypeptides produced by bacteria such as *B. anthracis*,(Bruckner, 1953) *B. subtilis* (Chibnall, 1958), *B. licheniformis*,(Troy, 1973) *and B. megaterium* (Torii, 1973), has been historically demonstrated by a variety of methods, going from enzyme assay using trypsin, which is able to cleave only and just α-amino bonds, and to which γ-PGA proved to be stable (Haurowits, 1949), to infrared spectroscopy (Hanby, 1950) to proton and carbon nuclear magnetic resonance spectroscopy applied to structural analysis (Birrer, 1994; Borbély, 1994; Pérez-Camero, 1999). Another method of practical importance is chemical degradation in which γ-PGA will yield γ-amino-δ-hydroxyvaleric acid as a monomer, while α-PGA would instead result in α-amino-δ-hydroxyvaleric acid (Nitecki, 1971).

The stereochemical properties of γ-PGA obviously depend on its enantiomeric content, which can be studied by means of both chemical and enzymatic methods; the former are the most easily available. In particular, a convenient and simple method is represented by total hydrolysis of the polymer followed by chiral chromatography (Kunioka, 1995), or derivatisation with Marfey's reagent (1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide) and analysis of the resulting diastereoisomers by reverse-phase HPLC (Cromwick, 1995).

As a matter of fact, three stereochemically different types of poly-γ-glutamic- acid have been found out among products of bacterial origin (Tanaka, 1997; Ashiuchi, 2003): homopolymer composed only by D-glutamic acid (as in the case of the capsule of *B.*

anthracis), or L-glutamic monomer (as it happens in *Hydra*); copolymer in which D- and L- momomers are lined up in different ratios (as it is found in the poly-γ-glutamate which is part of extracellular mucilage in many) *B. subtilis* strains). Some representative data are listed in Table 3.

Table 3. Enantiomeric content of γ-PGA produced by some different organisms

Organism	Content (%)		Reference
	D-Glutamate	L-Glutamate	
B. anthracis	100	0	Hanby (1946)
Planococcus halophilus	100	0	Kandler(1983)
Sporosarchia halophila	100	0	Kandlar (1983)
B. licheniformis	10-100	90-0	Pérez- Camero (1999)
B. subtilis natto	50-80	50-20	Kubota (1993)
B. subtilis (chungkoojang)	60-70	40-30	Ashiuchi (2001)
Staphylococcus epidermidis	50	50	Kocianova (2005)
B. megaterium	50	50	Toril (1956)
B. halodurans	0	100	Aono (1987)
Natrialba aegyptiaca	0	100	Hezayen (2001)
Hydra	0	100	Weber (1990)

As it can be seen, the enantiomeric content may vary a lot, depending on the producer species, strain and even on the culture conditions, as it will be shown; this variability has been representing a longstanding problem, bearing both theoretical as well as practical relevance, for the study and development of bacterial produced γ-PGA.

In most cases the natural gamma-PGA is composed of a mixture of D- and L-glutamic acid residues, whose ratio can affect its conformation and the orientation of biopolymer functional groups, with clear consequences on its reactivity. Conflicting reports have been published on cultivation parameters that control both polymer molecular weight and stereochemistry, but no conclusive assumptions can be drawn; in fact, every wild

isolate which has been utilized as gamma-PGA producer behaves differently and no optimisation protocol can be simply adapted passing from one strain to another.

Nevertheless, the large amount of scientific literature available is useful in defining the variables to be tested, which appear to be the addition of L-glutamic acid, the carbon sources (citric acid, glucose, glycerol, fructose), the nitrogen sources (ammonium salts, urea), mineral ions (K_2HPO_4, NaCl, $CaCl_2$, $FeCl_3$, $Mg/MnSO_4$); minor parameters that affect productivity and quality of gamma-PGA include the aeration and pH of the medium composition (Cromwick, 1995; Cromwick, 1996; Ashiuchi, 2001; Jung, 2005; Bajaj, 2009). It is not surprising that the investigation of the parameters for controlling gamma-PGA molecular weight and composition is not only of fundamental interest but of practical importance for the exploitation of this biopolymer as an efficient enzyme carrier and for its commercial development thereof.

An exemplar case may be considered that of γ-PGA formed by *B. licheniformis* strain ATC9945A. Conflicting opinions have been existing for decades (Leonard, 1958; Troy, 1973) whether the stereochemical content of the formed biopolymer was affected by the concentration of Mn^{2+} salt in the culture medium, or not; first collected data proved to be ambiguous or inconclusive, but as a matter of fact, the enantiomeric proportion of the polymer could vary from 38 % to 86% in the D-glutamic acid monomer from culture to culture, in a seemingly unpredictable way. The issue was finally solved in 1995 by Cromwick and Gross, who were able to definitely establish that, for this particular strain, enantiomeric composition is sensitive to the presence of $MnSO_4$; precisely, L-glutamic monomer content ranged from 59 to 0 % for a $MnSO_4$ concentration between 0 to 615 μM, respectively. These results were later confirmed by Pérez-Camero (1999).

Γ-PGA production by microorganisms

Table 4 Poly-γ-glutamic acid (γ-PGA) producing bacteria

Strain	Major nutrients	Culture conditions	Productivity (g/L)	Molecular weight	Reference
		Glutamic acid-dependent γ-PGA producer			
B.licheniformis ATCC 9945A	Glutamic acid (20g/L), glycerol (80 g/L), citric acid (12g/L), NH_4Cl (7g/L)	30 °C, 4 days	17-23	$1,4\ 10^5$-$9,8\ 10^5$	Troy (1973) Cromwick (1996)
B. subtilis IFO3335	Glutamic acid (30g/L), citric acid (20g/L)	37 °C, 2 days	10-20	$1,0\ 10^5$-$2,0\ 10^5$	Kunioka (1994)
B. subtilis F02-1	Glutamic acid (70g/L), glucose (1g/L), veal infusion broth (20g/L)	30 °C, 2-3 days	50	$1,20\ 10^5$	Kubota (1993)
B. subtilis (natto) MR-141	Sodium glutamate (30g/L), maltose (60g/L), soy sauce (70 g/L)	40 °C, 3-4 days	35	n.d.	Ogawa (1997)
B. subtilis (chungkoojan)	Glutamic acid (20 g/L), sucrose (50 g/L), NaCl (0,5-5,0 g/L)	30 °C, 5 days	13,5-16,6	$>2,0\ 10^5$	Ashiuchi (2001)
B. subtilis NX-2	Glutamic acid (30 g/L), glucose (30 g/L)	37 °C, 1 day	30,2	n.d.	Xu (2005)
		Glutamic acid independent-PGA producer			
B. subtilis TAM-4	Fructose (75 g/L), NH_4Cl (18 g/L)	30 °C, 4 days	20	$1,6\ 10^5$	Ito (1996)
B. licheniformis A 35	Glucose (75 g/L), NH_4Cl (18g/L)	30 °C, 3-5 days	8-12	$3,0$-$5,0\ 10^5$	Cheng (1989)

Several *Bacillus* bacterial species are known to secrete γ-PGA, as a product of fermentation, into their growth medium (Bovarnick, 1942; Thorne, 1954; Housewright, 1962, Murao, 1969; Troy, 1973; Hara, 1982; Cheng, 1989; Kunioka, 1992; Kubota, 1993). Since *B. anthracis* is not suitable for industrial production of the polymer because of its pathogenic nature, developers' attention has focused on *B. subtilis* and *B.licheniformis* strains, which belong to the Generally Regarded as Safe (GRAS) bacteria category, and which are easily cultivated.

Table 4 lists some significant examples, reporting the principal features of the most useful strains in terms of industrial application, paying attention in particular to their productivity and to the best culture conditions developed so far.

Extensive investigation has been performed on these strains, with the aim of optimizing nutrient requirements and culture conditions to boost cell growth and γ-PGA production. It must be noted that the overall approach has been, until recent times, heuristic. Many questions remain open about genes and enzymes, as well as the metabolic pathways involved and related to γ-PGA synthesis. Many aspects such as variation in molecular weight and enantiomeric composition of the final product still have to be clarified, too.

In fact, it has been found that nutrient requirements (carbon source, nitrogen sources, metal ions) required for cell growth and γ-PGA production depend on the particular bacterial strain used; factors such as ionic strength, aeration, pH of the medium also play an important role and affect productivity and quality of the resulting polymer. Culture conditions, specific for each strain, have in fact a strong impact on its amount, molecular weight and enantiomeric composition (Sung, 2005; Jung, 2006).

Moreover, evidence suggests that also synthetic mechanism involved differ between each strain.

According to nutrient necessity, bacteria capable of producing γ-PGA are divided in two principal categories: the former requires the addition of L-glutamic acid to the medium in order to let the cells grow and produce the polymer; the latter does not require its addition for γ-PGA production.

The former group, exogenous L-glutamic acid dependent bacteria, include *B. anthracis* (although, as already said above, this species has no applicative interest) (Thorne,

1953), *B.licheniformis* ATCC 9945A (Troy, 1973), *B. subtilis* IFO3335 (Kunioka, 1994), *B. subtilis* F02-1 (Kubota, 1993), *B. subtilis* (natto) MR-141 (Ogawa,1 997), *B. subtilis* (chungkoojan) (Ashiuchi 2001), *B. subtilis* NX-2 (Xu, 2005).

The latter group, namely exogenous independent L-glutamic acid bacteria, comprise *B. subtilis* TAM-4 (Ito 1996), *B. licheniformis* A 35 (Cheng 1989), as well as *B. subtilis* 5 E (Shih, 2001) and *B. licheniformis* S173 (Kambourova, 2001).

It is worth noting and remarked here that the study of carbon source utilization demonstrated that in most γ-PGA producers glutamic acid monomers incorporated in the polymer derive even, in part, for the exogenous L-glutamic acid dependent strains, from a *de novo* biosynthetic pathway *via* tricarboxylic acid cycle, starting from citric acid or glucose.

Intracellular L-glutamic acid is presumed to be produced from citric acid via isocitric acid and subsequently α-ketoglutaric (2-oxoglutaric) acid, which, on his turn, may be converted to the desired product in two different ways.

As a matter of fact, this hypothesis is consistent with experimental data: metabolic investigation performed by Cromwick and Gross on the *B. licheniformis* ATCC 9945 strain (Cromwick, 1995) by means of NMR spectroscopy and ^{13}C labeled citrate showed that an amount equal to 10 to 30 %, depending on culture conditions such as pH, of the monomers incorporated in the polymer derived from citrate rather than directly from L-glutamic acid present in the growth medium. Similar observations were made for other γ-PGA producer strains.

Biosynthesis of γ-PGA

Although extensively studied, the biosynthesis of γ-PGA is still not totally understood.

Since both L- and D- monomers of glutamic acid may be included in the polymer backbone, the racemisation of L-glutamic acid has been an intriguing and debated topic. It has been supposedly explained by three distinct mechanism: the indirect action of an aminotransferase (Thorne, 1954), the direct one of a glutamic acid racemase Glr (Ashiuchi, 1998) and the direct action of a different glutamic acid racemase YrpC (Ashiuchi, Misono, 2002), too. Evidence shows that the indirect process is probably not adopted *in vivo* since the activity of the aminotransferase decreases during polymer production (Shih, 2005a-b); it was also shown that Glr racemase, a cytosolic enzyme highly selective for L-glutamic acid (Thorne, 1955; Ashiuchi, 1998; 2002) is the only agent involved in monomer racemisation (Ashiuchi, 2002).

Polymerization happens in a ribosomal-independent way, by means of a membrane-associated complex requiring L-glutamate, ATP and Mg^{2+} as cofactors. Two mechanisms have been proposed. According to the first, proposed by Troy (Troy, 1973; Gardner, 1979) who first identified a membrane associated enzymatic complex responsible for PGA synthesis in 1973, the polymer is formed in four subsequent steps: first, glutamate is activated by an ATP molecule (1) forming a γ-bond with AMP, which is bond to the amino acid *via* a γ-linkage; then, activated glutamate is transferred to a S-protein, generating a thioester (2); isomerisation may occur (3) and finally the glutamyl residue is transferred to the growing PGA chain (4).

$$\text{L–glutamic acid+ATP}\rightarrow \gamma\text{–L–glutamyl–AMP+PPi} \quad (1)$$

$$\gamma\text{–L–glutamyl–AMP+SH–enzyme}\rightarrow \gamma\text{–X–glutamyl –S–enzyme+AMP} \quad (2)$$

$$\gamma\text{–X–glutamyl–S–enzyme}\rightarrow \gamma\text{–D–glutamyl–S–enzyme} \quad (3)$$

$$\gamma\text{–D–glutamyl–S–enzyme+[}\gamma\text{–D–glutamyl]}_n\rightarrow[\gamma\text{–D–glutamyl]}_{n+1}\text{+SH–enzyme} \quad (4)$$

This mechanism is often referred to as the thiotemplate mechanism.

One of the alleged evidences in favor of this mechanism was the fact that the incubation of bacterial membranes, which the enzymatic complex is bound to, resulted, in presence of hydroxylamine, in the formation of the respective γ-glutamyl derivative.

More recently, Ashiuchi proposed an entirely different mechanism, starting from the observation that hydroxamate was actually not found after incubation of hydroxylamine with the synthetic enzymatic complex prepared *in vitro* (Ashiuchi, 2001b, 2004). According to this second mechanistic proposal, it is γ-PGA itself to be activated by an ATP molecule; formation of an activated PGA species is then followed by glutamate monomer transfer, in what has been defined as an amide ligation fashion. The driving force of the process is provided by the cleavage of ATP to ADP.

The two mechanisms are incompatible, and the latter hypothesis seems to be more in accordance with experimental data. Still, conclusive studies have to be performed.

Much still lies ahead to be elucidated on the topic; what has been ascertained, however, is that the synthesis is catalyzed by an enzymatic complex with 4 subunits. The membrane-bound γ-PGA enzymatic synthetic complex, called PgsBCA, has been also studied in fine detail, but so far its isolation in the active state was impossible due to its high unstable and hydrophobic nature (Ashiuchi, 2004). What has been ascertained is that it is able to accept both D- and L- glutamic acid monomers as substrates and that it has no racemase activity; the catalytic site is formed by both its subunits PgsB and PgsC, while subunit PgsA is responsible for the removal of the chain from the active site, allowing its elongation step by step. The most peculiar property of this enzymatic complex is that it is not stereospecific, a unique feature among transpeptidases

(Ashiuchi, 2001; 2004). Presence of Mg^{2+} seems to exert an action on the enzymatic complex activity.

Finally, it has to be noticed that little is known about how γ-PGA filaments are released from the cell.

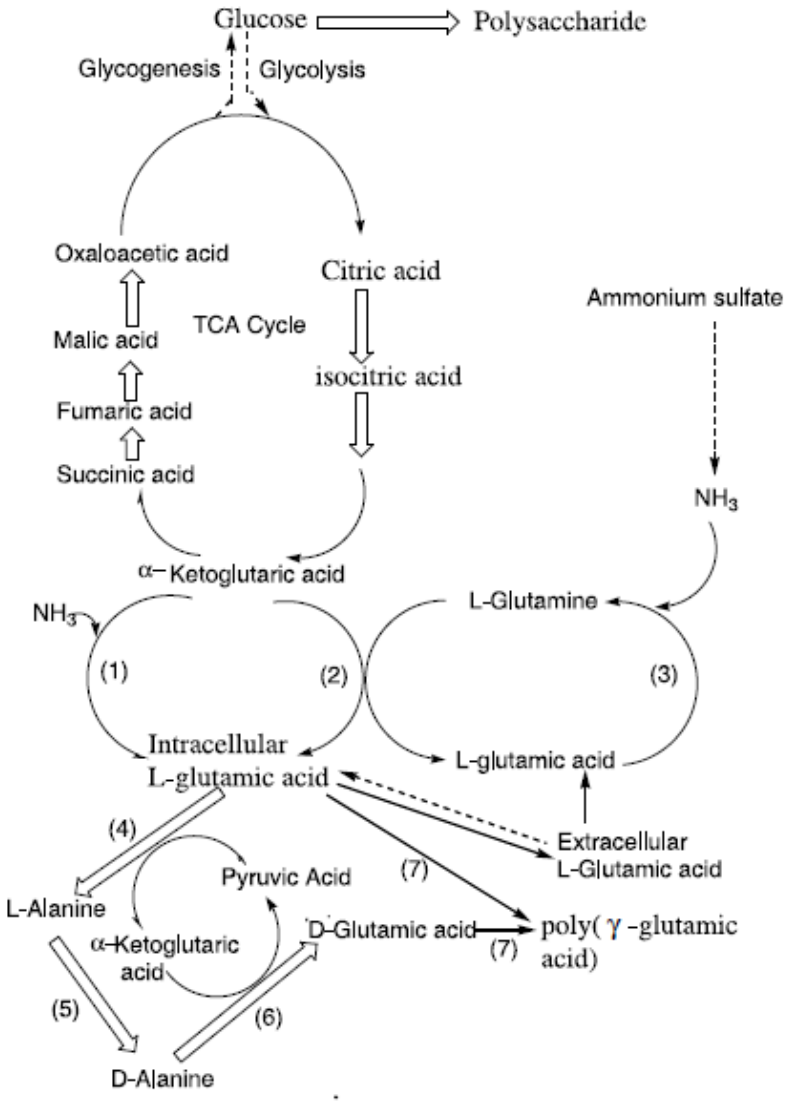


Figure 5: glutamic acid biosynthesis *via* tricarboxylic acid cycle and polymerization to give γ-PGA; the enzymes involved are (1) glutamate dehydrogenase (GD), (2) glutamate 2-oxoglutarate(α-ketoglutarate) aminotransferase (GOGAT), (3) glutamine synthetase (GS), (4) L-glutamic acid: pyruvic acid aminotransferase, (5) alanine racemase, (6) D-glutamic: pyruvic acid aminotransferase, (7) PGA synthetase. Source: Shih, 2001.

Finally, it also has to be remarked that the enantiomeric ratio between D- and L-glutamic acid monomers may be influenced by a number of factors which are still not fully understood. Strong differences seem to occur between natural strains; bivalent

metal cofactors such as Mn^{2+}, Co^{2+}, and Zn^{2+} (Leonard, 1958; Gardner, 1979) seem to influence racemisation.

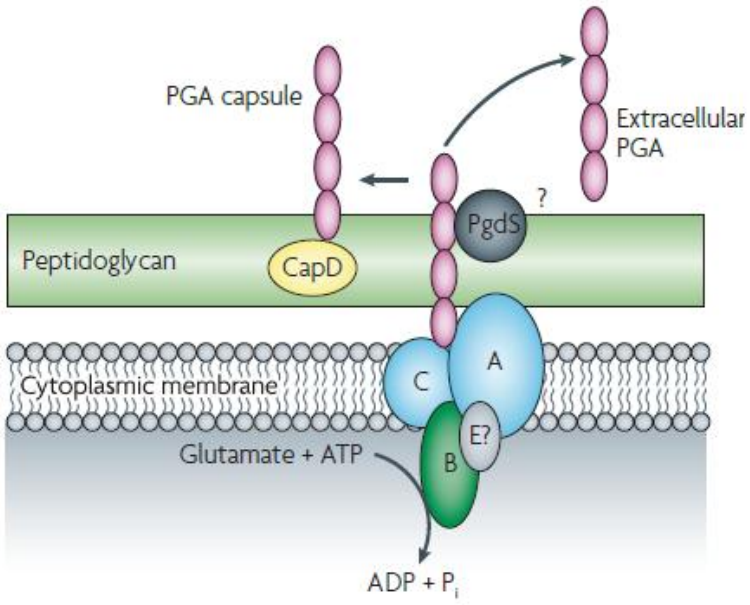


Figure 6: synthetic pathway of γ-PGA in *Bacillus* species; A, B, C, E represent the enzymes involved in the synthesis of released γ-PGA; CapD the enzyme responsible for the formation of capsular γ-PGA in *B. anthracis*.

Degradation of γ-PGA

Bioproduced γ-PGA has generally an average molecular weight between 10^5 and 10^6 Da and polydispersities between 2 and 5. Generally speaking the control of the molecular sizes of a polymer is particularly significant in order to tailor its proprieties and so it is important to know how γ-PGA can be degraded.

γ-PGA is stable below 60°C and in the presence of ordinary proteases (Oppermann-Sanio, 2002); in order to degrade it harsh conditions, such as prolonged exposure to extreme pH, high temperature or ultrasonic irradiation, or specific enzymes are necessary.

As a matter of fact, γ-PGA is synthesized by producers in the early stationary phase, and later degraded in the late stationary phase.

Producing bacteria express two different proteases able to degrade γ-PGA: endo-γ-glutamyl-peptidase and exo-γ-glutamyl-peptidase.

Production of endo-γ-glutamyl-peptidase was shown to occur in various *Bacilli* (Kunioka, 1993; Makino, 1988; King, 2000; Obst, 2004) and in some other bacteria such as the bacteriophage ΦNIT1 (Kimura, 2003). This enzyme usually is secreted in the medium and cleaves high molecular weight γ-PGA into fragments as small as 10^5 Da (Goto, 1992; Kunioka, 1993; Kimura, 2004).

Nowadays, is not clear if this enzyme targets γ-DL (Ashiuchi, 2003) or γ-DD (Suzuki, 2003) bonds, however it is known that it strictly splits endo-γ-glutamyl-bonds. This fact has been demonstrated by Suzuki (2003) working with a clonated endo-γ-glutamyl-peptidase from *B. subtilis* IFO16449. In this work the authors show how the enzyme is able to degrade high molecular mass γ-PGA giving a 490 kDa mass product and an 11 kDa one. Neither free glutamic acid nor γ-glutamyl oligomers were detected indicating that such enzyme cleaves only endo-bonds. Furthermore the authors found out that the enzyme is stable and active between pH 4 and 11 and in a temperature range from 4°C to 45°C. Its activity is not inhibited by the treatment with 5 mM EDTA, divalent cations nor 1mM phenylmethylsulphonyl fluoride while 1mM 4-(hydroxymercury) benzoate, a sulphydril inhibitor, remarkably slow the activity suggesting that the enzyme mechanism may be cysteine based, such as in DL-endopeptidase II (Smith, 2000). It was also reported that in this hydrolysis there is a linear correlation between the decrease in molecular weight and time (Tayal, 1999).

Exo-γ-glutamyl-peptidase, also known as GGT, is a key enzyme in glutathione metabolism (Ogawa, 1991; Xu, 1996) but it does not seem to be involved in γ-PGA synthesis in vivo (Williams, 1954; Ashiuchi, 2003). However there are some evidences

that it is involved in formation of *B. anthracis* capsula, binding γ-PGA to membrane peptidoglycanes (Candela, 2005), and in bioproduced polymer cleavage in *B. subtilis* (Kimura, 2004).

Degradation of γ-PGA can be carried out chemically by hydrolysis at extreme pH and high temperature (Goto, 1992; Kubota, 1996) or physically using ultrasonic irradiation. While hydrolytic methods are not deemed satisfactory because of the poor control cleavage obtainable in the process and the high polydispersity of the resulting products the use of ultrasonic waves seems to be an interesting alternative to enzymatic hydrolysis. Pérez-Camero and co-workers (1998) have shown how exposure to 20000 Hz for 2 hours cleaves γ-PGA of 2100 KDa into 60 to 90 KDa fragments that exhibit a narrowed polydispersity compared to the starting material. This method could be useful to control the molecular weight of the bioproduced polymer optimizing its proprieties for specific applications.

In conclusion, it has to be mentioned that, as it was reported by Muñoz-Guerra and coworkers (Portilla-Arias, 2007), γ-PGA and its ester derivatives depolymerize when heated over 200 °C in nitrogen atmosphere, releasing pyroglutamic acid and the corresponding esters as volatiles; the residues, analyzed by NMR, were spectroscopically indistinguishable from the polymer.

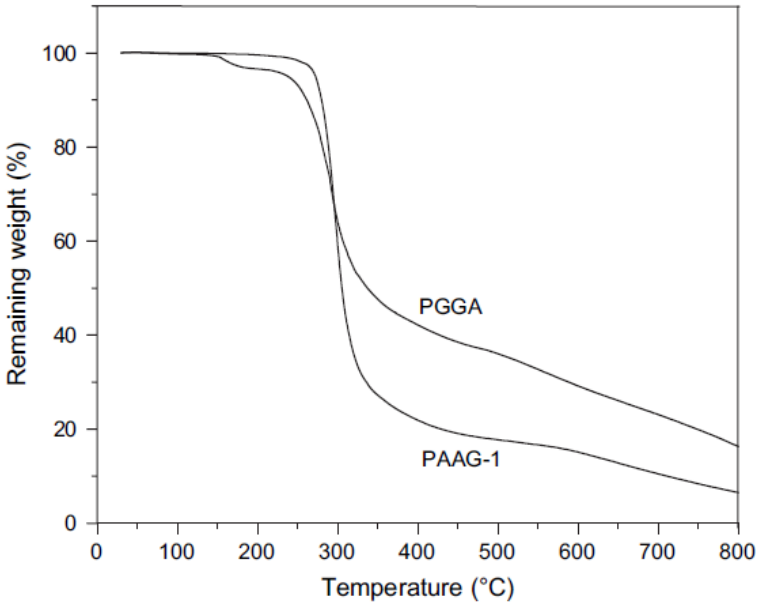


Figure 7 TGA profiles of γ-PGA (PGGA) and its methyl ester (PGGA-1), acquired at the heating rate of 10 °C min^{-1} under nitrogen atmosphere. The first modest weight loss under 200 °C is due to the release of water absorbed by the polymer during the preparation of the sample; polymer decomposition occurs in the range 250-300 °C, resulting in a loss of weight about 50 and 70 % of the initial mass, respectively. Source: Portilla-Arias, 2007.

Genetic aspects of γ-PGA synthesis

Poly-γ-glutamic acid is unusual and it is different structurally and functionally from proteins in that glutamate is polymerized via the γ-amide linkages, and thus should be synthesized by a ribosome independent manner. Identification and analyses of genes responsible for the synthesis of this polymer are extremely important to understand the biosynthetic mechanism. Nowadays all the genes responsible for the three stages of γ-PGA metabolism (racemisation, polymerization and degradation) have been identified (Ashiuchi, 2002).

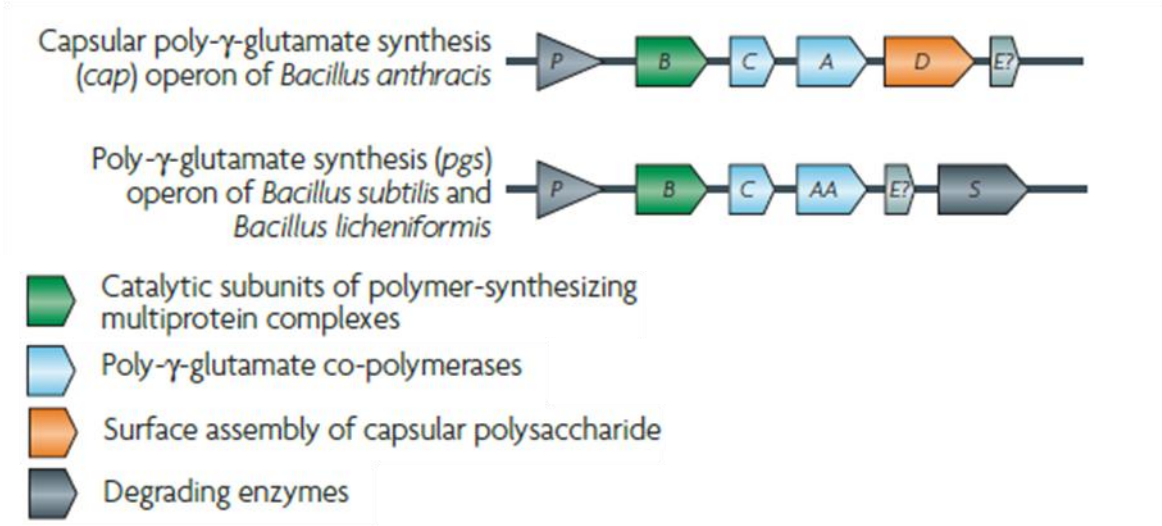


Figure 8: Genetic organization of genes and operons essential for γ-PGA biosynthesis. The triangle labelled P is the key regulatory promoter for biosynthesis; genes with unconfirmed functions are indicated by a question mark. Source: Rehm, 2010.

Many bacteria, including *Escherichia coli*, have a unique gene that encodes glutamate racemase. This enzyme catalyzes the formation of D-glutamate, which is necessary for cell wall peptidoglycan synthesis. However, *Bacillus subtilis* has two racemase genes, named *racE* (also called *glr*) and *yrpC*. Kada and co-workers (2004) found *racE* to be essential for growth as well as responsible for supplying D-glutamic acid for γ-PGA production and peptidoglycan synthesis. Furthermore, they suggested that primary function of *yrpC in vivo* is not glutamic acid related (Kada, 2004). Contradictorily, other authors (Kimura, 2004) showed that neither *racE* nor *yrpC* was required for *B. subtilis* cell to synthesize γ-PGA. On the other hand, the location of the two genes on the chromosome does not imply a connection to γ-PGA synthesis and so the point is yet to be clarified.

Only a few bacteria, mostly from the genus *Bacillus* (*B. subtilis 168*, *B. subtilis natto*, *B. licheniformis*, *B. anthracis* and *S. epidermidis*) have been reported to posses the genes required for γ-PGA synthesis and only two of these species have well characterized loci encoding their polyglutamate synthesis complex. The nomenclature for the genes

involved in γ-PGA synthesis is one of two types, depending on whether the synthesized γ-PGA is retained or released (Candela, 2005). If the γ-PGA is associated with the bacterial surface and form a capsule, then the corresponding genes are named *cap* (for capsule), whereas the corresponding genes are named *pgs* (for polyglutamate synthase) if the γ-PGA is released.

In *B. anthracis* the cluster of genes encoding the γ-PGA synthesizing complex is located on a plasmid (Uchida, 1987; Makino, 1988) while in other organism, the homologues genes are located on the chromosome (Nagai, 1997; Ashiuchi, 2001). It is widely accepted that all three *pgsBCA* genes are necessary and sufficient for γ-PGA production in vivo (Ashiuchi, 1999; Ashiuchi, 2001); however it has also been observed that if one of the three genes is disrupted, it can be complemented in a trans manner (Ashiuchi, 2004). Candela (2005) also outlined how even the previously overlooked *capE* is essential for the production of γ-PGA in *B. subtilis* and *B. anthracis*. In opposition to the findings of Ashiuchi, Urushibata and co-workers (2002) claimed that only *pgsB* and *pgsC* genes are essential for the in vivo γ-PGA production by *B. subtilis*. A conclusive study on this topic has yet to be published.

ComPA, degSU and degQ regulate γ-PGA production at transcriptional level in response to *quorum sensing* (which is defined, according to Rehm, 2010, as "the regulation of bacterial genes expression in response to fluctuation in cell population density, mediated by release of chemical signal molecules"), osmolarity and phase variation signals. The *comPA* system activates the transcription of the *pgsBCA* operon at high cell density, while *degSU* has the same effect in response to an increase in salinity and/or osmolarity (Ruzal, 1998). The mode of action of *degQ* has yet to be determined

even if it is known that its mutation severely compromises γ-PGA production (Msadek, 2002).

The gene encoding endo-γ-glutamyl-transpeptidase (*ywtD, dep, pgds)*, is located directly downstream from the *pgsBCA* operon in *B. subtilis*, as it happens in the majority of γ-PGA produced bacteria. It also shares the same orientation of the *pgsBCA* operon.

The gene product is similar to the DL-endopeptidase (II) family.

The gene encoding exo-γ-glutamyl-peptidase *(ggt* or *capD)* is instead usually located on the chromosome distant form the *pgdsBCA* cluster; in *B. anthracis* it is found on a plasmid downstream of it (Uchida, 1993). This enzyme is required in order to anchor γ-PGA to the cell wall, but it is not involved in the polymer synthesis (Candela, 2005). The expression of *ggt*, which occurs in the stationary phase, is reportedly controlled by the ComQXPA quorum sensing system in *B.subtilis* (Kimura, 2004).

Applications of Γ-PGA

Among its other properties, γ-PGA is water soluble, biodegradable, edible, independent of oil resources and non-toxic to humans and to the environment. Therefore potential applications of γ-PGA and its derivates have been rising in interest during the past few years for a broad range of industrial fields such as food, cosmetics, medicine, water treatment and for other purpose (Shih, 2001; Gardner, 1979; Buescher, 2007). A summary of the main ones is offered by Table 5.

Table 5: applications of γ-PGA

Field of application	Applications	Details
Water and wastewater treatment	Metal chelates or absorbents	Removal of heavy metals and radionucleus
	Bioflocculants	Substitutes for non-biodegradable and toxic flocculants as PAMA and PAC
Biodegradable materials	Bioplastic	Substitution for chemically synthesized non-biodegradable plastics
	Hydrogels	Excellent water absorbent; potential application in bioseparation, controlled drug release, biosensors, diagnostics; desert greening; substitutes for polyacrylate in napples
Food industry	Thickener	Viscosity enhancemecent for fruit juice beverages, sport drinks
	Cryoprotectant	Cryoprotectant for frozen foods
	Bitterness-relieving agents	Relief of bitter taste by amino acids, peptides, quinine, caffeine, minerals, etc.
	Ageing inhibitor or texture enhancer	Ageing prevention and texture improvement of bakery products and noodles
	Mineral absorbents	Promote mineral absorption in humans and animals, increase eggshell strength, decrease body fat, prevent osteoporosis in humans
Cosmetics	Humectant	Use for skin care in cosmetics
Medical	Drug and gene delivery	Use as carrier for the improvement of anticancer drugs in gene and cancer therapy
	Vaccine	Antigen for elicit antibody against anthrax; adjuvant for antigen
	Medical adhesive	Substitutes for fibrin in use as curable biological adhesive and haemostatic or medical bonding kit, sulture thread
Others	Dispersant	Dispersing pigment and minerals in detergent cosmetics and paper making
	Tissue engineering	Scaffolds with good mechanical strength and cytocompatibility for tissue engineering

Biopolymer flocculant

Treatment of wastewater, both in industrial downstream process, and after domestic use, usually makes use of several flocculants of synthetic origin. (Nakamura, 1976; Kurane, 1986).

These conventional materials are often not easily biodegradable; moreover, their constituents, such as acryloamide, are recognized as neurotoxic and even carcinogenic agents, thus representing a problem both on the environmental and on the health side (Vanhorick, 1983; Dearfield, 1988).

On these grounds, γ-PGA has been proposed as a bioflucculant for wastewater treatment, thanks to its constitutive biodegradability and to the innocuous nature of its degradation products toward humans and the environment. Evidence proves that its high flocculating activity can be tuned by the addition of multivalent cations (Ca^{2+}, Mg^{2+}, Fe^{2+}, Al^{3+} and Fe^{3+}) as well as by pH adjustment: in fact, various inorganic (solid soil, acid clay, active carbon, calcium and magnesium, among the others) and organic suspensions (cellulose, yeast, etc) could be flocculated by preparations based on γ-PGA. γ-PGA cross-linked by γ-irradiation properties as a flocculant agent were also positively assessed when it was tried in order to clarify a suspension of kaolin, bentonite, and *E.coli*, as well as turbid pond water (Kunioka, 2004; Taniguchi, 2005a; 2005b; 2005c). γ-PGA also qualified for use in processing water suitable for human consumption or for downstream treatment in food and fermentation industry.

Metal and radionuclide binding

Dedicated studies on known polymers of bacterial origin as γ-PGA have proved that it is able to bind many metal ions such as Ni^{2+}, Cu^{2+}, Mn^{2+}, Al^{3+} and Cr^{3+} (Mc Lean, 1990; 1992); it also forms a binuclear, bidentate complex with the ionic radionuclide U^{4+} (He, 2000). Therefore, it has been proposed as a binding agent suitable for the remediation of contaminated soils, sediments and waters. This is a topic of dramatic interest, given the rising challenge of reducing the risks associated with heavy metals and radionuclides spread as pollutants in the environment taking advantage of a biogeochemical process (Macaskie, 1998).

Bioremediation

The use of γ-PGA has also been envisioned as a nitrogen reservoir which, together with microbial biomass, could help in minimizing the drawback pollution effects of intensive breeding (Pötter, 2001; Höppensack, 2003). In fact, the cultivation of *B.licheniformis* strain S2 in swine manure and an optimised mineral salts medium had successfully converted 28% (W/W) and 0.1% (W/W) of the total nitrogen into cellular biomass and γ-PGA, respectively. This makes possible the conversion of the high quantity of ammonium and other nutrients present in animal manure into γ-PGA and biomass, preventing the eutrophication of soil and surface water, and the pollution of atmosphere, due to the spread of excessive amounts of biological waste, endowed with high quantities of nitrogen in the form of ammonium, in the environment. The resulting biomass, as well as biopolymer, could be used as slow-release fertilizers, also capable of storing and subsequently delivering cations precious for plants rizosphere (Kinnersly, 1994).

Drug carrier

In pharmaceutical technology, the use of γ-PGA has been proposed as a drug carrier or as a scaffold for the construction of drug conjugates with increased efficacy, as well as for the implementation of sustained release devices.

Taking advantage of its ability to form nanoparticles, γ-PGA has been tried in studies on controlled drug release as a biocompatible and not cytotoxic matrix material. For instance, nanoparticles of γ-PGA-L-fenylalanine esters were shown to prevent release of an entrapped protein drug for ten days under physiological condition. The reported lack of cytotoxicity of these nanoparticles represents a remarkable strongpoint (Akagi, 2005a; 2005b).

The conjugation of γ-PGA to cancer drugs, such as camptothecins (Singer, 2000) and cis-diclorodiammine-platinum (II) (CDDP) (Avichezer, 1998); has also been tried, resulting in good improvements in effectiveness and reduction of toxicity.

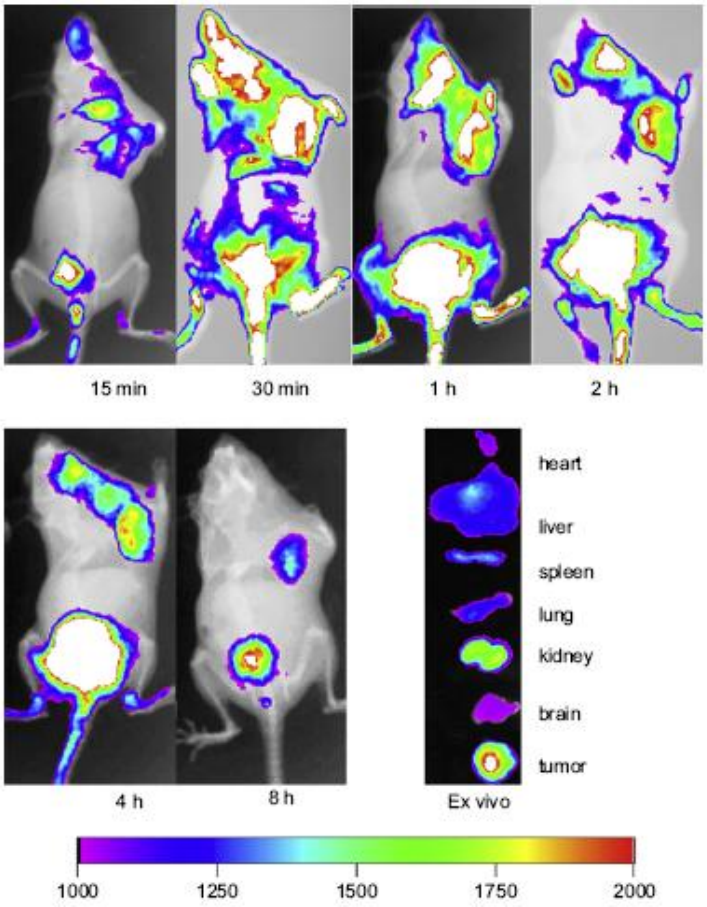


Figure 9: in this particolar elegant example, *in vivo* non-invasive NIR fluorescence images of real-time distribution of γ-PGA-Citric acid-CDDP conjugate carryng a NIR fluorescent dye obtained at different time point on mice liver cancer cell H22 mice and ex vivo images of dissected tissues after 8 h are illustrated (Xiong, 2012).

The most significant example of this strategy is perhaps represented by the construction, by means of covalent bonding, of a PGA-taxol system. In fact, the use of water soluble α- or γ-PGA makes possible to overcome the major difficulty given by the insolubility in water of the famous and potent anti-tumoral Paclitaxel (also known as taxol, TXL), a natural anti-microtubule agent which is naturally found in the needles and bark of the Pacific yew tree (*Taxis brevifolia*). It is effective, as chemotherapeutic agent, against various forms of human neoplasm, including breast and ovarian cancer (Rowinsky,

1995; Holmes, 1995), the main limitation being its scarce solubility in water. The use of PGA-TXL conjugates has, among its positive consequences, a significantly improved anti-tumor activity, as well as fewer side-effects, and the increase of the maximum tolerated dosage (MTD) as it was ascertained in preclinical studies. Phase I/II/III human clinical trials are under way in the USA and Europe (Holmes, 1995). Γ-PGA-based nanoparticles able to target liver cells (Liang, 2006a; 2006b) have also been conjugated to TXL.

Biological adhesives

Modern surgery often recurs to adhesive material of synthetic or semi-synthetic origin in all cases in which suturing, the most common technique for wound closure and control of severe bleeding, is not possible, *e.g.* to seal aortic dissections, to stop fluid leakage from the body, to prevent air to reach and contaminate tissues torn apart after a traumatic event, and when effective haemostasis and control continuous blood drain is needed.

These materials, however, may be cytotoxic and tend to degrade at low rate; moreover, their degradation products can cause a chronic inflammatory response (Tseng, 1990; Toriumi, 1996).

A new biological adhesive formed by cross-linking of gelatin and poly (L-glutamic) acid has been proposed as a substitute for fibrin glue, the most widely used material as surgical adhesive and haemostatic agent. With respect to the latter, the γ-PGA based material has better haemostatic capability and better bonding strength to soft tissue, and it is slowly degraded in the body without inducing any inflammatory response. On the contrary, fibrin glue has bad mechanical properties, weakly adheres to tissues, and may lead to a dangerous viral infection, being obtained from human blood (Otani, 1996; 1998a; 1998b).

Γ-PGA has also been used in odontology, in combination with glass powder, as self-curing glass ionomer cement for dental restoration and implant fixation (Ledezma-Pérez, 2005).

Vaccine

As it was mentioned above, a capsule made up of γ-D-PGA is a virulence factor in *B. anthracis*, making the pathogen able to elude the host's immune system. Thus, γ-D-PGA has been suggested as an antigen to elicit antibodies against anthrax, but, since it is non-immunogenic itself, for it to generate antibodies a protein carrier is requested (Schneerson, 2003; 2005; Wang, 2005). An anti- anthrax vaccine that includes γ-D-PGA as an active component has actually been prepared (Wimer-Mackin, 2005). The antibody titer has been improved thanks to a novel triazine-based conjugation strategy that could prevent fractioning of high molecular mass γ-D-PGA (Joyce, 2006).

Thermoplastics and hydrogels

Derivatives of γ-PGA could replace potentially polluting oil-derived polymers (such as polyacrylates) for many technological purposes. In particular, some esters of γ-PGA can be successfully used as thermoplastic (Kubota, 1993a; Borbély, 1994); they are able to form fibers and films which are totally biodegradable. For instance, γ-PGA benzyl ester can be manufactured in the form of a fibre or a membrane possessing good mechanical strength, elasticity and transparency (Kubota, 1992; Yahata, 1992; Gross, 1995).

Γ-irradiation of a γ-PGA aqueous solution is one of the possible methods enabling the formation of a hydrogel characterized by an impressive capability of water absorption, up to a ratio between water weight and polymer weight of roughly 3500 (Kunioka 1993; Choi, 1995a).

Combining γ-PGA with convenient concentrations of PEG-methacrylate thanks to a photo-induced reaction makes possible the preparation of hydrogels characterized by the proper kinetics for the release of particles of various size (Yang, 2002).

These constructs will be of great utility in agriculture as well as for the environmental and biomedical fields; in fact, they will be possibly used as water reservoirs in agriculture (Hara, 2002), as fertilizer, to prepare biodegradable diapers, as drug controlled release devices (Park, 2001) and as scaffold for tissue engineering (Matsusaki, 2005a; 2005b).

Food preservation and enrichment

Freezing is a well established and simple technique for preservation of materials of biological origin, such as food; however, the freezing process, and subsequent thawing, frequently causes unwanted deterioration biologically active substances and foods. The addition of cryoprotectants has been widely used to protect them.

Differential scanning calorimetry (DSC) studies (Mitsuiki, 1998; Shih, 2003) showed that oligo- and poly-γ-glutamic acids have an antifreeze activity that is higher than that of glucose, a highly antifreeze substance, in the molecular weight range below 20,000. Moreover, γ-PGA a weaker taste than the commonly used lower molecular weight cryoprotectants, such as saccharides, inorganic salts and amino acids, so it can be added to foods in larger quantities without a dramatic change in taste. Γ-PGA increased Ca^{2+} solubility *in vitro* and *in vivo* as well as intestinal Ca^{2+} absorption (Tanimoto, 2001). Addition of γ-PGA to a food increases the uptake of minerals and other active substances such as vitamins, polyphenols and carotenoids in the small intestine (Kashima, 2006). Naturally occurring γ-PGA and foods enriched with it might, on these basis, contribute to prevent disease like osteoporosis (Ashiuchi, 2004). Several other applications of γ-PGA in food industries have been reported; for example it has been

used to relieve bitter taste caused by amino acids, peptides, quinine, caffeine, minerals, etc (Sakai, 2000). The addition of the biopolymer, or of its edible salts, to the manufacture of starch foods, such as bakery products and noodles, results in the prevention of aging and the improvement of textures and shape retaining. Γ-PGA is also industrially used as an ice cream stabilizer and a thickener for fruit juice and sport drinks.

Other applications

Γ-PGA, in combination and a vegetable extract of aloe Vera, *Matricariaa chamomilla*, or green tea is able to increase pyrrolidonecarboxylic acid on a skin epidermis, enhancing the humectanct effects without affecting the quantitative balance and constancy of a humectant ingredient essential to the skin. This preparation is considered to be useful as a medicine for external use, a quasi-drug, a cosmetic and a bathing agent.

In cosmetics, the addition of γ-PGA to skin care products containing water-soluble as well as insoluble vitamins increases the solubility of the latter, thus promoting their absorption and their sustained-release, without any irritation of the skin. (Sung, 2006a; 2006b).

A hydrogel obtained from medium sized γ-PGA (MW=300 kDa) and poly (vinylalcohol) (PVA) can be used to coat medical devices for improved blood compatibility; water resistance, mechanical strength; protein adsorption properties can be tailored by varying the γ-PGA to PVA ratio (Lin, 2006).

Finally, it has been demonstrated that γ-PGA can be a useful water-soluble template for the immobilization of biomolecules to give polyvalent bioactive conjugates (King, 1998).

Chemical modification of γ-PGA

The chemical modification of γ-PGA represents a crucial issue in order to modulate its chemical-physical properties and to shape new materials of practical use. In fact, derivatisation of γ-PGA is a common strategy to reliably improve the material thermal and mechanical properties. Taking advantage of the presence of a free carboxylic group on the polymer backbone, the formation of esters or amides seems to be an adequate and straightforward approach to the purpose. On the other hand, this is a quite challenging topic, given the peculiarities of γ-PGA and the difficulties encountered in its manipulation.

So far, γ-PGA derivatisation has been achieved by means of two main strategies.

According to the first, proposed by Kubota and Endo (Kubota, 1993b) the formation of an ester is carried out *via* carboxylates, since standard Fisher's conditions are not feasible, resulting in depolymerization. Γ-PGA is therefore dissolved in solvents such as 1-methyl-2-pyrrolydone (NMP) or DMSO in presence of an inorganic base such as sodium bicarbonate; an alkyl or benzyl halogenide (such as ethyl of benzyl bromide) is subsequently added, and the reaction proceeds at relatively high temperature (60 °C). The amphiphilic product is then isolated by precipitation in methanol, or in an acidic aqueous solution (Morillo, 2001).

This approach permits the formation of copolymers, and has been particularly exploited for the preparation of substances endowed with suitable handling and processing properties and improved thermal stability, at the same time maintaining the biodegradability proper of γ-PGA. In particular, a careful choice of the size of the side chains determines the structural behavior of the product. Short alkyl lateral chains help the crystallization of the copolymer, while long ones enhance the formation of two-

dimensional, periodic, biphasic layered structures in which the main chain is rodlike, while the flexible side chains may stay disordered or crystallize in a separated phase, according to temperature (Morillo, 2003). These materials, characterized by a very distinctive self-organization at the nanometric scale, are of extreme academic as well as applicative interest.

After the first derivatisation is performed, transesterification becomes possibly another effective approach to the formation of γ-PGA derivatives (Melis, 2001). The first generation derivative has to be dissolved, at high temperature (180 °C) in a strong excess (up to 50 times) of an alcohol, in presence of a catalyst such as Ti $(OtBu)_4$. The degree of transesterification can be controlled by careful assessment of reaction time.

Moreover, the solubility in water of γ-PGA derivatives may be tuned by esterification with biodegradable and biocompatible oligo(ethylene glycol)s (Pérez-Camero, 2001); these kind of compounds can help in the assembly of stimulus responsive hydrogels, able to predictably react to given changes of environmental conditions.

The drawbacks of the esterification strategy are that the reactivity appears to be strongly sensitive of the alkyl group steric hindrance; therefore, the reaction often has to be performed in successive steps to reach high conversion degrees. Transesterification products, in turn, tend to be insoluble in working conditions, thus becoming inaccessible to reagents and preventing complete conversion.

The second main strategy for γ-PGA chemical modification is represented by the formation of amide derivatives.

This approach has been used mainly for the preparation of nanoparticles, which can be prepared starting from an amphiphilic material such as a γ-PGA derivative endowed

with a hydrophilic backbone and a hydrophobic moiety such as L-phenylalanine ethyl ester (Akagi, 2005b).

In fact, nanoparticles are essential in the construction of delivery systems able to modify the distribution of molecules of biological relevance such as DNA; proteins or drugs in cells and organs (J. Panyam, 2003). Properties of nanoparticles, in particular their degradation rate (which affects the release rate) can be modulated according to the molecular mass and the composition of the graft or block copolymers which they are made of; among the materials commonly used to the purpose, we can mention poly(lactide-co-glycolide) (PLGA), poly(lactic acid) (PLA) and poly(caprolactone) (PCL) (Hans, 2002**)**. Another crucial aspect in drug delivery systems preparation is their entrapment efficiency, at the same time avoiding the denaturation or degradation of the entrapped species (O'Hagan, 1994; Li, 2000).

Γ-PGA, thanks to its hydrophilic nature, is particularly suitable to be used as a scaffold for the preparation, by means of covalent bonding to a hydrophobic species such as L-phenylalanine-ethyl ester, of an amphiphilic graft copolymer; in aqueous solution, this compounds tend to spontaneously form, in a self-assembled way, nano-sized aggregates of micellar nature in which the hydrophobic block forms the inner core, able to easily incorporate a drug, while the hydrophilic one forms the outer shell (Zhang, L., 1996); Liu, 2004).

An amide derivative is prepared by means of a condensing agent such as a water soluble carbodiimide (Kunioka, 1997). The degree of functionalisation may be controlled, in principle, varying the WSC stoichoimetry. The product can be finally isolated by dialysis (Matsusaki, 2002).

AIM OF THE THESIS

Although poly-γ-glutamic acid has been known for a long time, and considerable efforts have been spent in investigating its chemical and biological properties, much lies ahead to be elucidated about this polymer biosynthesis, reactivity and conformational aspects. In addition, its potential applications appear to be still almost unexploited in the Western world.

Aim of the present work, which was carried on in collaboration with the Genetics and Microbiology Department and Pharmaceutical Chemistry Department of the University of Pavia, was the production, isolation, characterization and chemical modification of γ-PGA in order to obtain novel biomaterials possibly useful for biopackaging, biomolecule immobilization, biocatalysis, and as drug carriers.

Moreover, the information acquired and the methodologies developed in the course of the project will be of great help for future investigations dealing with the so far unclear aspects of polymer biosynthesis and the influence of conformation on its peculiar macroscopic properties and chemical reactivity.

For the industrial application of γ-PGA it is necessary to enhance the productivity of this natural polymer and to find fermentation conditions innovative and optimal also from the economic point of view. γ-PGA cannot be efficiently produced by chemical synthesis; improving bacterial production represents the best and feasible method to obtain the amount of biopolymer necessary to meeting industrial needs.

Γ-PGA is freely secreted in the fermentation medium by diverse *Bacilli* strains that, although useful in terms of industrial application, are not well characterized from the genetic point of view. In those wild isolates, enormous efforts have been devoted to establish the culture conditions to obtain maximal productivity, which can vary between 8 to 50 g/l in function of the specific producer strain (Shih, 2009). On the other hand,

the *Bacillus subtilis* domestic laboratory strains, 168 and derivatives, as the PB5383 strain (Osera, 2009), offer the advantage of the deep knowledge of their genetic outfit, defined in 1997 by sequencing of the 168 genome; besides, all genes have been systematically analyzed from the functional point of view by a number of laboratories in Europe and Japan in a Systematic Functional Analysis concerted effort (Schumann, 2001). In particular, in the course of this project, we dealt with the improvement of the production yield of the identified γ-PGA producer strain PB5383 that was based on the deep genetic knowledge of this strain and on the positive preliminary results obtained by introducing selected mutations, already characterized in 168. A stable and well defined genetic outfit has addressed us and our collaborators in the choice of the best nutritional and cultural conditions, overcoming the heuristic approach thus far applied with wild producers. Cost containment has been taken into account in selecting the best fermentation conditions and the purification protocols. All of these aspects are crucial for innovation and improvement of polymer production and represent a model application of advanced genetic engineering technologies having a positive impact in large-scale industrial production for a sustainable development.

Great efforts have also been made to synthesize derivatives of γ-PGA chemically. Ester derivatives of γ-PGA have been investigated for their capability to form biodegradable fibers and films that can replace currently used non-biodegradable polymers. It was found that the esterified γ-PGA served as a good thermoplastic and that poly-(γ-glutamic acid α-benzyl ester) could be processed into fibers or membranes with good strength, transparency and elasticity by the standard methods used in polymer processing (Buescher, 2007; Shih, 2001). The formation of hydrogels from γ-PGA with or without additional polymers gives rise to many novel applications, as the physical properties of a gel can be tailored to meet different requirements. Possible crosslinkers

include PEG-methacrylate, dihaloalkane or diamino compounds. A convenient method for gelling γ-PGA by γ-irradiation without the addition of other polymers or crosslinkers has been reported. Hydrogels are simply gels that swell strongly in aqueous media, and are typically composed of a hydrophilic organic polymer component that is cross-linked into a network by either covalent or non-covalent interactions. It is the crosslinking that provides for dimensional stability, while the high solvent content gives rise to the fluid-like transport properties. Development of new hydrogels from natural polymers has been of interest in recent years because of their potential applications as materials for controlled release, enzyme immobilization, or as moisture absorbents. Considering that both biodegradability and biocompatibility are required for biomedical uses, the development of biodegradable nanoparticles is indispensable for these applications. It is known that amphiphilic block or graft copolymers consisting of hydrophilic and hydrophobic segments are self-assembling materials, and are capable of forming polymeric associates in aqueous solutions due to their intra- and/or intermolecular hydrophobic interactions (Akagi, 2007a). Aggregates of various morphologies have been observed in a number of self-assembled polymeric systems. The morphology of the nanoparticles produced from amphiphilic block/graft copolymers can be varied by changing the composition of the hydrophobic and hydrophilic blocks on the polymer chains. The commonly used biodegradable polymers are aliphatic polyesters, such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (ε-caprolactone) (PCL), and their copolymers. However, an important limitation in the use of these polyesters for biomedical applications is their lack of reactive functional groups to which biomolecules or drugs can be covalently immobilized. Recently, novel amphiphilic graft copolymers composed of γ-PGA as the biodegradable hydrophilic backbone and hydrophobic amino acid (HAA), such as L-phenylalanine ethyl ester, as the hydrophobic segment were successfully synthesized by grafting L-HAA to γ-PGA. Due

to their amphiphilic properties, the γ-PGA–graft-L-HAA copolymers were able to form nanoparticles in water. Using ovalbumin (OVA) as a model protein, OVA-loaded γ-PGA nanoparticles were prepared by surface immobilization and encapsulation methods. It was found that these OVA-encapsulated γ-PGA nanoparticles could be preserved by freeze-drying process. Moreover, cytotoxicity tests showed that the γ-PGA and γ-PGA nanoparticles did not cause any relevant cell damage. These results demonstrated that γ-PGA nanoparticles have a great potential as protein carriers and could have application in various technological and biomedical fields (Akagi, 2005, 2007b).

Another topic of extreme interest is the immobilization of enzymes (such as lipases and Penicillin G acylase) and growth factors (such as the epidermal growth factor) to test the feasibility of the new biomaterials as protein carriers in biocatalysis and tissue engineering. Immobilized enzymes are bound to carriers, or are entrapped as soluble form in devices, such as microcapsules or membranes, that are impermeable to the enzyme, ensuring a continuous exchange of substrate or product. Immobilization on solid support is a non-biological way which confers added stability to proteins towards temperature and organic solvents, and provides a convenient mean to separate and reuse the biocatalyst to improve process economics. The latter properties, namely noncatalytic functions (NCFs), have to be performed by an immobilized enzyme by definition, regardless of its nature or preparation, and naturally complete the enzyme catalytic functions (CFs) (Cao, 2005). To design an immobilized biocatalyst, the main task is to select a suitable carrier (defined as the noncatalytic part of an immobilized enzyme, on which the catalytic part is constructed), experimental conditions (pH, temperature, nature of medium) and enzyme itself (source, nature and purity). If the selected method meets both the catalytic and the non-catalytic needs of the considered application, the

resultant immobilized biocatalyst can be labeled as "robust". The development of robustly immobilized enzymes is a major challenge in industrial biocatalysis and the subject has grown into an important research field. The properties of immobilized enzyme preparations are determined by the properties of both the enzyme and the carrier material. The interaction between these endows an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties (Tischer, 1999). The availability of a new material characterized by high chemical and physical versatility as well as biodegradability and safety, and not limited in supply (Shih, 2001), represents a promising biotechnological development in the biocatalysis field: it answers, in fact, the need to set up robust catalysts endowed both with positive CFs (activity, selectivity, stability) and NCFs (production, recovery, re-use of the catalysts and control of the process).

The application of γ-PGA and γ-PGA derivatives as innovative biomaterials is not limited to the biocatalysis field. In fact, due to the multifunctionality, safety and biodegradability of γ-PGA, it can also be used as scaffold for the immobilization of the human epidermal growth factor (EGF) to be used as medical device for tissue regeneration.

EGFs are produced by several different cell types and act on epithelial cells stimulating their migration and division. Several studies have demonstrated that members of the EGF-family and their cognate receptors (EGFRs) are important mediators of wound healing (Jost, 2000; Hardwicke, 2008). Local application of growth factors have met with limited success due to the inadequate delivery and persistence of the growth factor at the wound site; EGF and other growth factors can be rapidly washed off by exudates, adsorbed by the wound dressing or degraded, preventing their action. Growth factors

have been covalently tethered to biomaterials, partly overcoming some of the enunciated problems (Gu, 2004; Ehrbar, 2007).

It is therefore highly desiderable to develop novel scaffolds for EGF binding characterized by high activity and able to protect them from environmental denaturation. For tissue engineering applications, there are some special demands to biodegradable polymers. Γ-PGA and its derivatives may answer most of the above mentioned features (Matsusaki, 2002).

In spite of the potential of γ-PGA as protein carrier, only few reports have been published so far about its use as matrix for enzymes immobilization. This might be basically ascribed to three reasons: i) the still high costs of γ-PGA production since it cannot be easily achieved by chemical synthesis (Buescher, 2007); ii) the extreme variability in molecular weight and stereochemical composition of the naturally produced biopolymer, that can obviously affect its physical and chemical properties; and, finally, iii) the difficult handling of the natural biomaterial due to its high viscosity (Shih, 2001).

In view of using γ-PGA as an enzyme carrier, some of the above mentioned drawbacks have been partly overcome, but much effort is still needed to achieve an optimized, usable biomaterial. For the γ-PGA large-scale production, many attempts have been made to enhance bacterial productivity. The strategies were mostly based on the search for suitable culture conditions (Ogawa, 1997; Yoon, 2000), on the identification of better producer wild isolates (Kubota, 1993), or on both.

The above considerations contribute to justify our interest in the investigation of γ-PGA production and characterization on one side, and on its reactivity and chemical modification on the other; we devoted our efforts to the study of this material also in

view of its challenging applications in the field of tissue engineering, drug delivery, and biocatalysis.

RESULTS AND DISCUSSION

Production

One of the first targets of the project was the improvement of γ-PGA production applying biotechnological techniques to known *Bacillus* producer strains. This scientific approach itself represents a novelty over the heuristic, semi-empirical ones mostly used so far to the purpose.

In fact, most of γ-PGA producer strains are wild isolates, whose genetics and physiology are not well defined; their use requires laborious customization of strain-specific culturing conditions through heuristic approaches (Bajaj, 2009). This drawback can be overcome by using derivatives of *B. subtilis* strain 168 as γ-PGA producers. *B. subtilis* 168 is the model organism for Gram-positive bacteria: extensive scientific knowledge has accumulated in over one hundred years of research on this specific strain, and easy and standardized culturing conditions and genetic manipulation protocols are available (Earl, 2008; Harwood, 1992; Volker, 2005). In strain 168, γ-PGA biosynthetic genes are present but usually not transcribed (Urushibata, 2002), unless two genetic variations are introduced: i) the *degQ36* (Hy) or *degU32* (Hy) mutation (both leading to the same effect, i.e. an increased level of phosphorylation of the transcription factor DegU) (Ohsawa, 2009); ii) the functional *swrA* allele (Osera, 2009; Stanley, 2005), whose function is still unclear (Patrick, 2012).

Improvement of microbial γ-PGA production has generally followed two parallel routes: the identification of new and more efficient natural producer strains and the development of better fermentation conditions (Bajaj, 2009; Shi, 2006). A third potential route, so far poorly explored, is the control of the rate of γ-PGA post-synthesis degradation by inactivation of specific γ-PGA degrading enzymes.

Our collaborators at the Genetics and Microbiology Department of the University of Pavia could identify, among others, a mutant *B. subtilis* strain called PB5383, deriving

from the most common 169 *B. subtilis* laboratory strain and constitutively able to produce high molecular weight γ-PGA, as early as 2009 (Osera, 2009).

It was conveniently possible to take advantage of the detailed knowledge of the genetic asset and physiology of the producer bacterium as well as of the ease of manipulation of the laboratory strain, to target specific pathways related to the biopolymer production.

Yields as good as those of the best natural known producers (Bajaj, 2011), were achieved.

Advanced genetic engineering techniques made possible the construction and subsequent selection of several mutant strains, with the aim to enhance productivity of the biopolymer.

In particular (Scoffone, 2013), deletion of the genes related to the expression of polymer- degrading enzymes endo-γ-glutamyl peptidase (***pgdS***) and exo-γ-glutamyl hydrolase (γ-glutamyltranspeptidase GGT; ***ggt***) was performed.

Comparison between the productivity of the parental PB5383 strain, and three mutants in which the *pgdS* gene (PB5513, *ΔpgdS*), *ggt* gene (PB5518, *Δggt*) and both of them (PB5522, *ΔpgdS, Δggt*) were respectively deleted, demonstrated that the effect of the double deletion on the increase of polymer production is of substantial importance. In fact, for the double deletion mutant, yield was reached about 40 g/L in 48 hours, almost doubling the parental strain's one, and above the average of known producer strains (Bajaj, 2011). Quantification was performed drying polymer aliquots, extracted from at least three independent cultures, to a constant weight.

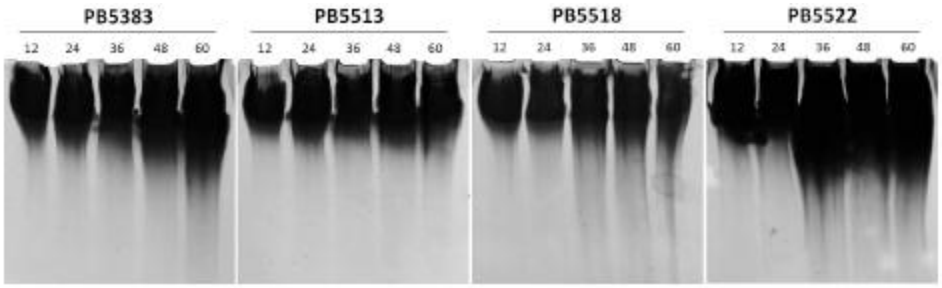


Figure 10: electrophoretic separation of purified γ-PGA on SDS-page. Numbers above each lane correspond to the time points of sample collection, in hours. The yield increase in the case of the double deletion mutant (PB5522) is qualitatively evident.

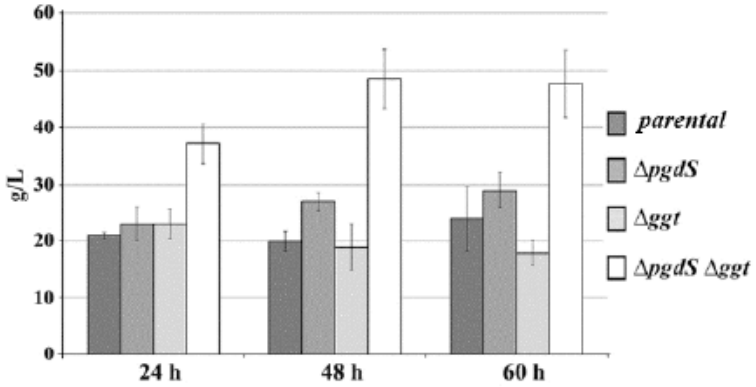


Figure 11: comparison between *B.subtilis* PB5383, PB5513 (*ΔpgdS),* PB5518 (*Δggt),* PB5522 (*ΔpgdS Δggt)* strain productivity; samples collected at time cultures of 24, 48 and 60 hours, respectively.

Observation of the growth curves associated to each strain also showed that growth rate between the mutants is comparable.

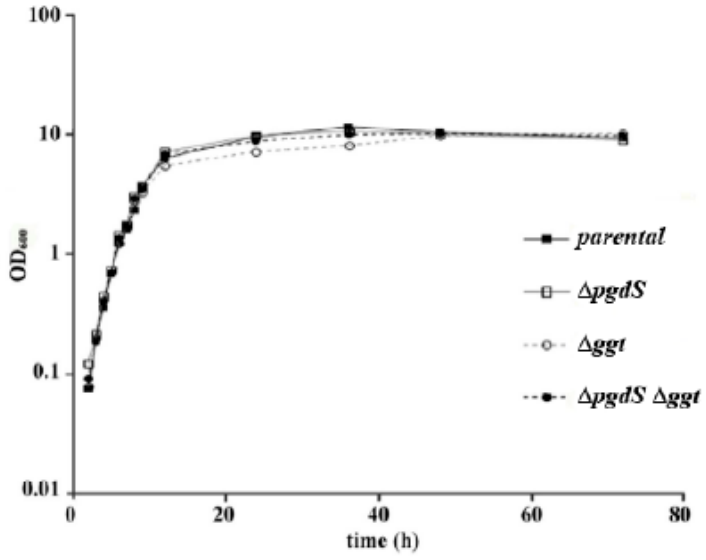


Figure 12: growth curves in E medium for *B.subtilis* PB5383, PB5513 (*ΔpgdS),* PB5518 (*Δggt),* PB5522 (*ΔpgdS Δggt)* strains; time is measured against Optical Density (OD) at 600 nm.

To make sure that the excellent improvement in yield for the double mutant was not due to a second site mutation, the mutant strain itself was reconstructed starting from mutant strains PB 5509 (*ΔpgdS)* and PB 5516 (*Δggt)* which were transformed by a plasmid carrying the lacking deletion. Independent experiments performed in duplicate confirmed the beneficial effect of the double gene deletion on γ-PGA productivity.

Purified biopolymer samples were analysed by SEC (Fig. 13A), and number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity (M_w/M_n) were calculated. Data in Table 6 show that the *_pgdS _ggt* double mutant produced γ-PGA with lower M_n and M_w values and higher polydispersity than the other samples; analysis of variance carried out on the independent samples collected at 24, 48 and 60 hours confirmed this observation ($P<0.05$). The *_ggt* mutant, impaired in the end-chain degradation, that has a minor effect on polymer distribution, produced a polymer that was similar to that produced by the *wt* strain in terms of M_n and M_w (Kimura, 2004). The *_pgdS* mutant, missing the endo-hydrolase activity, produced γ-PGA characterized by values of M_n and M_w higher than the *wt* sample, particularly at earlier time points, although polydispersity did not notably vary.

Table 6: SEC measurements for γ-PGA produced by PB5383, PB5513, PB5518, PB5522 B. subtilis mutant strains

Sample		M_n (10^6 Da)	M_w (10^6 Da)	Polydispersity (M_w/M_n)
PB5383 *parental*	24h	1,66± 0,075	2,23±0,098	1,34±0,032
	48h	1,31 ± 0,066	1,87±0,110	1,43±0,012
	60h	1,36 ± 0,111	1,92±0,221	1,41±0,067
PB5513 Δ*pgds*	24h	1,93 ± 0,205	2,49±0,168	1,29±0,071
	48h	1,46± 0,127	2,06±0,146	1,41±0,025
	60h	1,40±0,108	2,00±0,158	1,43±0,012
PB5518 Δ*ggt*	24h	1,58±0,096	2,22±0,074	1,40±0,037
	48h	1,35±0,046	1,94±0,023	1,44±0,037
	60h	1,28±0,026	1,90±0,040	1,48±0,006
PB5522 Δ*pgds* Δ*ggt*	24h	1,36±0,138	1,87±0,178	1,38±0,013
	48h	1,01±0,152	1,55±0,172	1,54±0,063
	60h	0,92±0,061	1,45±0,069	1,59±0,039

SEC traces (Fig. 13A) were plotted against the molecular weight calculated from the standard calibration curve, in order to obtain the molecular weight distribution profiles in the 0.1 - 5 MDa range (Fig. 13B); molecules lower than these values co-elute with impurities and are below the resolving limit of our system (Yao, 2009). The distribution curve showed that the increase in productivity of the *_pgdS _ggt* mutant was due to accumulation of shorter polymer chains (0.1 - 2 MDa), whereas very high molecular weight molecules (>3.5 MDa) were less abundant than in other strains.

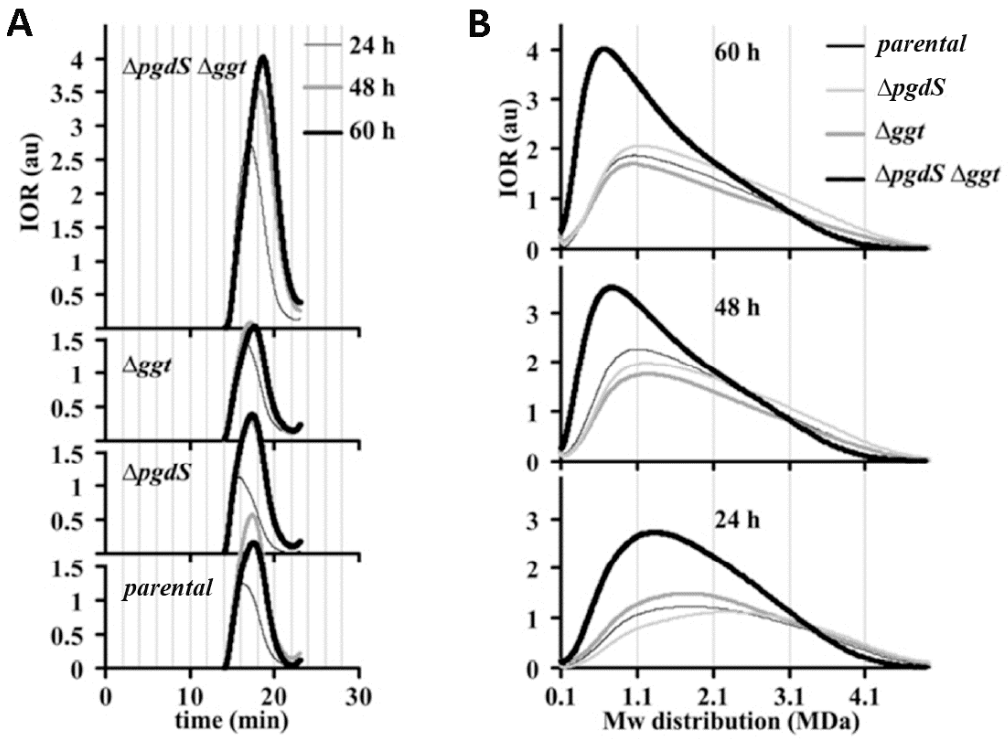


Figure 13: SEC traces (A) and MWD profiles (B) for γ-PGA produced by PB5383, PB5513, PB5518, PB5522 *B. subtilis* mutant strains

Isolation

The first challenge encountered in the manipulation of γ-PGA was the isolation of the polymer from its culture broth.

In order to solve the issue without recurring to chromatographic techniques (which may represent a bottle neck for a possible scale-up of the process), several protocols were tested, each of them based on the precipitation of the product, in its scarcely water-soluble acidic form, with a cheap alcohol, such as methanol.

Best results, confirmed by NMR, were obtained by the removal of cells by centrifugation or filtration, followed by a first precipitation of the product from the culture broth with methanol; then acidification to pH 2-3 with an aqueous mineral acid such as HCl, dialysis against water to remove the remaining impurities (mainly represented by polysaccharides) and finally precipitation with *i*-propanol.

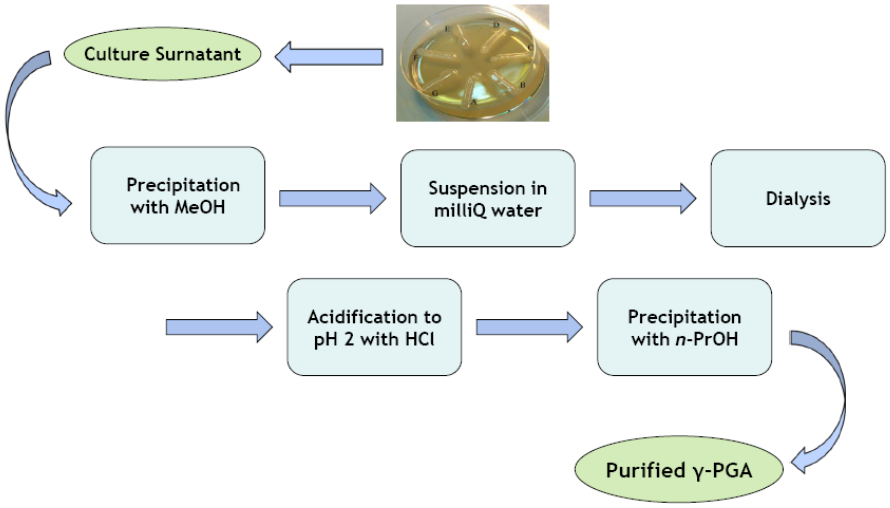
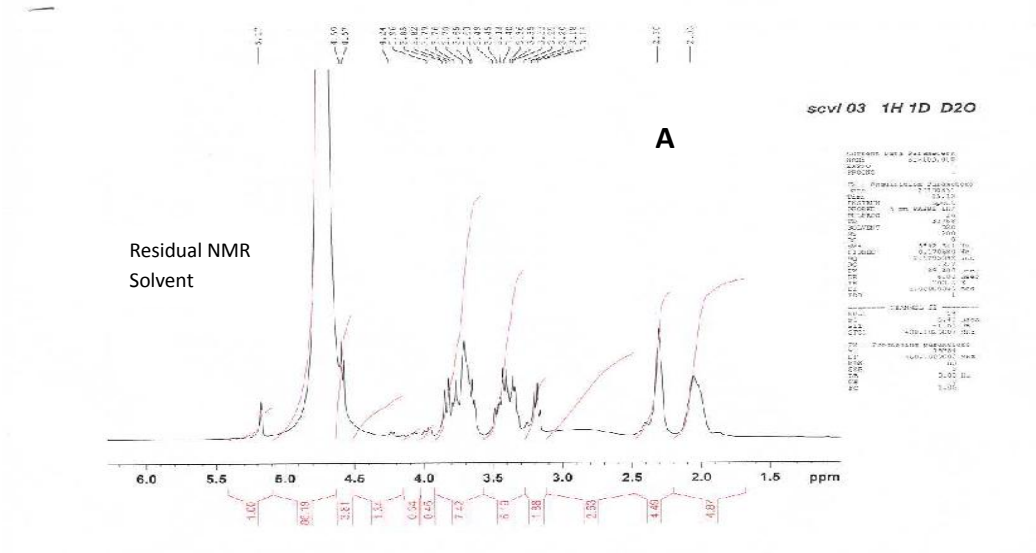


Figure 14: graphic representation of γ-PGA purification protocol by successive precipitations.

As an alternative, ultrafiltration with an Amicon device equipped with a 30 kDa cut-off membrane, under Nitrogen atmosphere, was also successfully attempted.

This latter methodology proved, however, to be quite more time-consuming than the former one, and to deliver reduced yields (less than 50% of those obtained by the precipitation method).



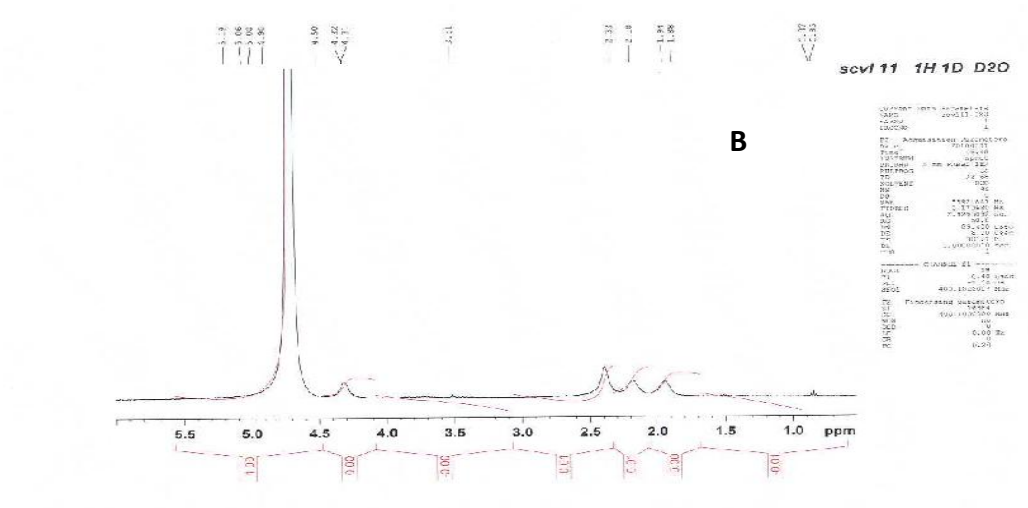


Figure 15: Comparison between two γ-PGA samples, before (A) and after (B) purification; polysaccharides present in the crude have been eliminated.

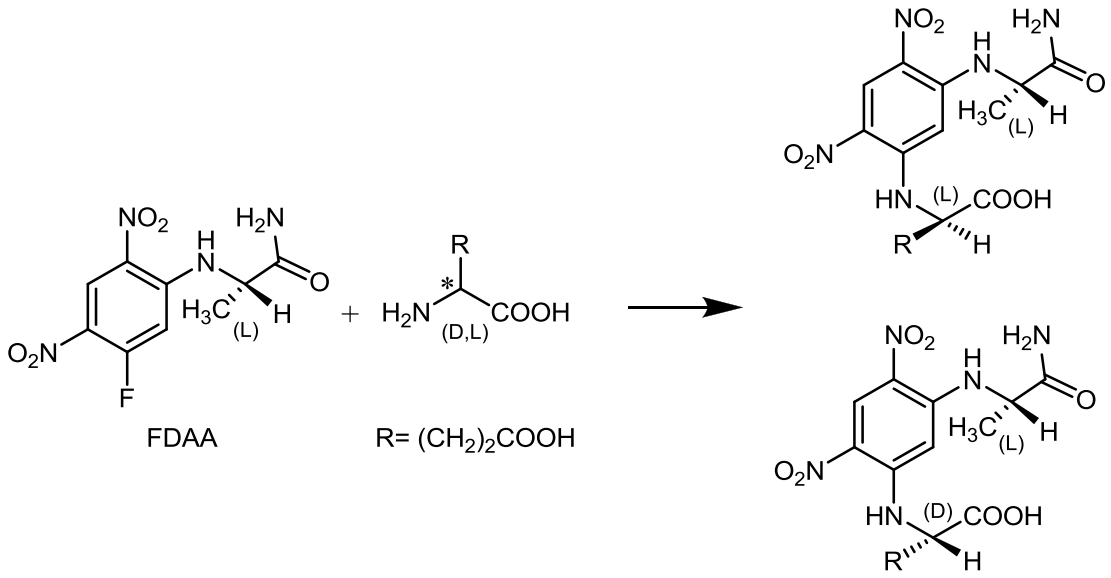
Stereochemical L/D composition

In the introduction it has been discussed how the stereochemical composition of the biopolymer, which affect its conformational as well as macroscopic properties, depends on the bacterial strain responsible for its production.

Therefore we considered this to be a crucial part of our investigation, and decided to analyze the stereochemical composition of the bioproduced (PB5383) as well as of that a commercial available sample (Natto Bioscience, low molecular weight γ-PGA), for comparison. The study could be possible thanks to our collaborators at the Pharmaceutical Chemistry Department of the University of Pavia.

To the purpose, we followed the methodology proposed for γ-PGA by Cromwick e Gross in 1995. According to this method, a sample of the biopolymer must be completely hydrolyzed in acidic conditions; the resulting monomers are derivatized with the so called Marfey's reagent (1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide, FDAA) which can be regarded as a chiral version of the notorious Sanger's reagent. The

so formed diastereoisomers can be finally separated and analysed in reverse phase HPLC.



FDAA R= $(CH_2)_2COOH$

Scheme 1

Marfey's reagent methodology

Derivatisation of D, L-glutamic acid was performed following the protocol reported by Bhushan and Brückner in 2004, itself an adaptation of the original one described by Marfey in 1984.

The reaction between Marfey'reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA) and a D,L amino acid results in the formation of L,L and L,D diastereoisomers of the amino acid itself (Scheme 1), which can be separated and quantified in reverse-phase HPLC.

The methodology was first developed for a in-house prepared mixture of L- and D-glutamic acid, and then applied to the analysis of a commercial (Natto Bioscience, low molecular weight) and a non-commercial (Genetics and Microbiology Department, University of Pavia, mutant strain PB5383, purified with Amicon, cut-off 30kDa, and lyophilized) sample. The diastereoisomers of D, L glutamic acid were analysed in

HPLC under conditions, which were properly adapted from those proposed by B'Hymer in 2001.

All analyses were repeated twice.

The results obtained are shown in the following chromatograms (Fig. 16 and 17).

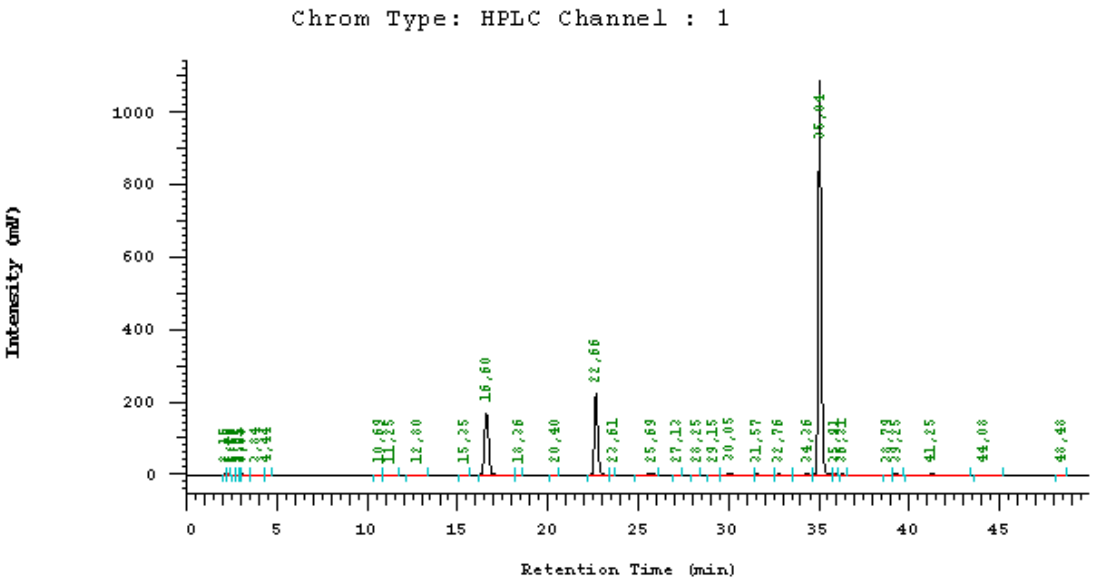


Figure 16: stereochemical composition of commercial γ-PGA: L-glutamic acid represents 48 % of total composition, D-glutamic acid 52%.

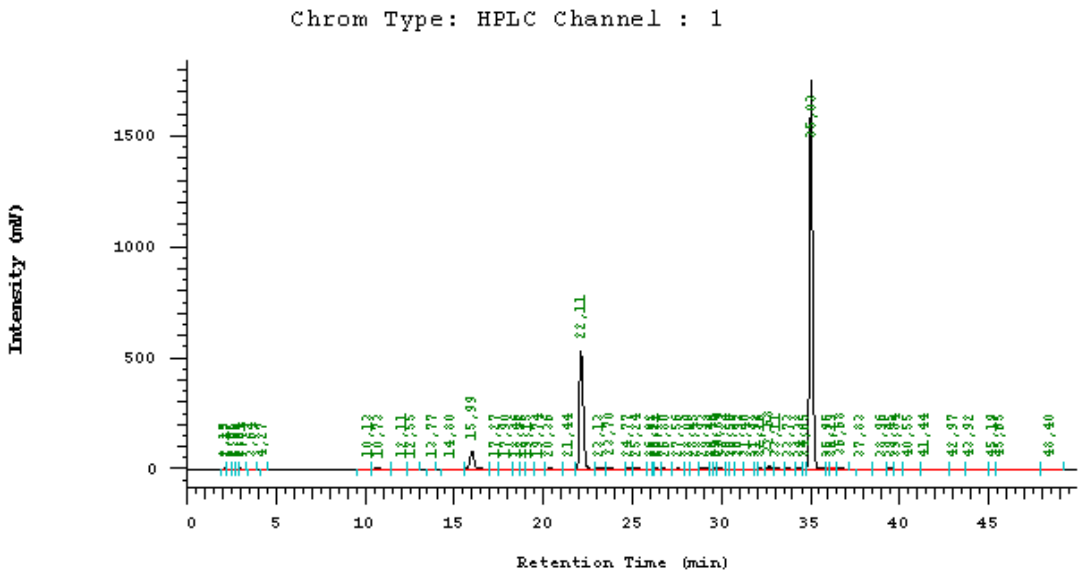


Figure 17: stereochemical composition of in-house polymer: D-glutamic acid isomer is prevalent (84% of total composition).

The order of elution, as was confirmed also by literature (B'Hymer, 2001), is the following: derivatized L-glutamic acid, derivatized D-glutamic acid, Marfey's reagent.

We could conclude that, while the commercial sample is composed by L- and D-isomers of glutamic acid in 48:52 ratio respectively, on the contrary the polymer produced by the *Bacillus* mutant strain at the University of Pavia is composed mainly by D-glutamic acid (86%).

B. subtilis is known to be a poly-γ-DL-producer, but the prevalence of the D-isomer is a peculiar characteristic of this particular mutant strain, which should make it non-immunogenic -as it is the case, for instance, for the completely D-PGA produced by *B. anthracis* (Keppie 1963), which contributes to the severity of its virulence. This feature, together with its total atoxicity, may possibly make it interesting from a pharmaceutical technology point of view, for the preparation of drug carrier and controlled delivery constructs.

Molecular weight determination

Another fundamental parameter for the characterization of a polymer is its molecular weight. The necessity of its measurement also arises from the inherent characteristics of biosynthesis, which may bring a strong variability between each culture product.

To determine it, the technique of election is size-exclusion chromatography, coupled with an in-line multi-angle-laser-light-scattering detector (MALS).

It is known from polymer chemistry that this technique is able, without the necessity of any standard, to deliver a state-of-the art-accurate measurement of molecular weight (M) and molecular weight distribution (MWD).

The response of the light-scattering detectors is in fact directly proportional to the molecular weight of the polymer, following the equation (Mendichi, 2001):

$$LS_{signal} = K_{LS} \times \left(\frac{dn}{dc}\right)^2 \times MW \times c$$

Where K_{LS} is an instrumental constant, c is the concentration, and dn/dc is the excess of the refraction index of the macromolecule with respect to the solvent. This value is available in literature for γ-PGA in aqueous solution (Wyatt, 1993).

It is also possible, from the measurement of angular variation of scattered light (generally known as radius of gyration, R_g) to get information about the macromolecular size distribution (RGD), as well as about the polymer conformation state.

In collaboration with ISMAC-CNR (Milan), this kind of characterization was carried out on the biopolymer obtained at Pavia, and also on a low-molecular weight commercial sample (both in the acidic and sodium salt form), for comparison.

The results are summarized in Table 7.

Table 7: molecular weight determination

Sample	**M_p (kg/mol)**	**M_n (kg/mol)**	**M_w (kg/mol)**	**M_z (kg/mol)**	**M_w/M_n**	**M_z/M_w**	**Rec. Mass %**
University of Pavia (**PGA 12**)	592.5	348.8	620.9	888.5	1.61	1.43	91.3
Natto Bioscience (**PGA Acid)**	13.4	12.7	16.1	20.9	1.27	1.30	84.1
Natto Bioscience (**PGA Na Salt**)	22.6	20.6	28.3	39.8	1.37	1.41	92.1

Table 7 reports:

- the molecular weight of the maximum of the chromatographic peak (M_p);
- the number average molecular weight (M_n), describing the statistical average molecular weight of all the polymer chains in the sample;

- the weight average molecular mass (M_w), which, compared to M_n, takes into account the molecular weight of a chain in determining contributions to the molecular weght average;
- the z average molecular mass (M_z),a higher molecular mass average which is very sensitive to the higher molar mass species and therefore useful to describe properties which strongly depend on them;
- the polydispersity indexes Mw/Mn and Mz/Mw, which describe the amplitude of the distribution of molecular mass;
- percentage amount of sample mass recovered at the exit of the column with respect to that injected is also shown.

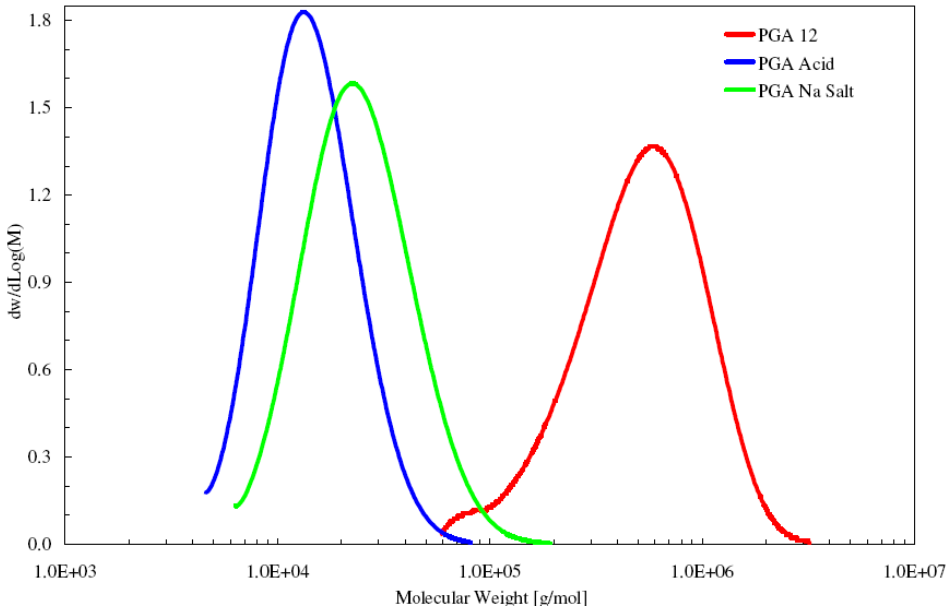


Figure 18: superposition of the differential MWD of the samples

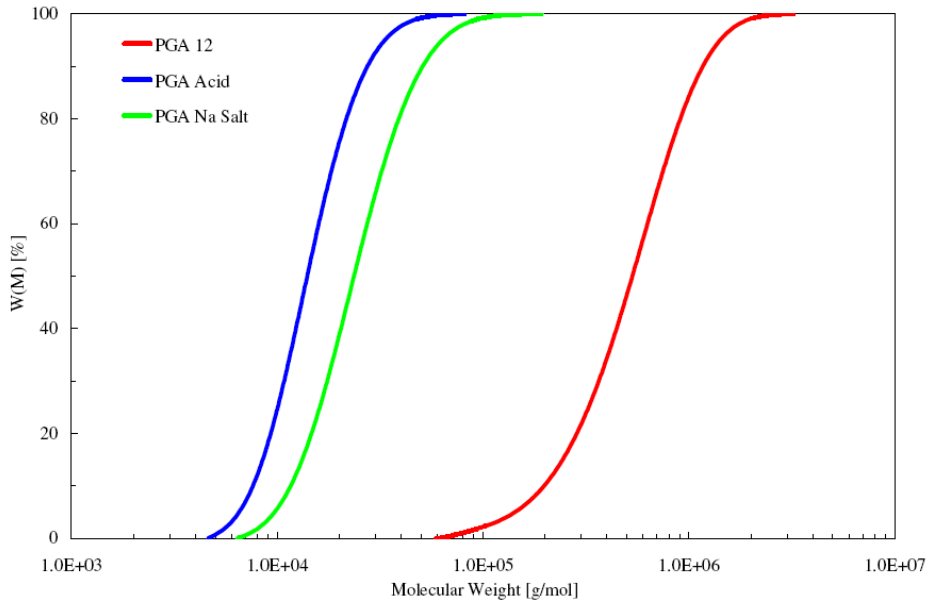


Figure 19: superposition of the three cumulative MWD of the samples

In Figures 18 and 19 the molecular weight distribution (MWD) of the polymer is expressed in the differential and cumulative ways commonly used in the field of polymer chemistry.

A brief analysis of the data let us make the following observations:

- the biopolymer produced by the mutant *B. subtilis* PB5383 (PGA12) has a very high molecular weight, around 620 kg/mol, and a wide MWD (Mw/Mn ≈1,6);
- the commercial sample, on the contrary, has a low molecular weight (≈16 kg/mole in the acidic form, ≈28 kg/mole in the sodium salt form) and a relatively narrow MWD (≈1,3 in the acidic form, ≈1,4 in the sodium salt form).

These data are in contrast with those claimed by the producer, who stated the molecular weight to be around 100 kg/mol.

This strong difference in molecular weight, as well as the different stereochemical composition, may contribute to explain the difference between macroscopic properties of the commercial and in-house produced biopolymer.

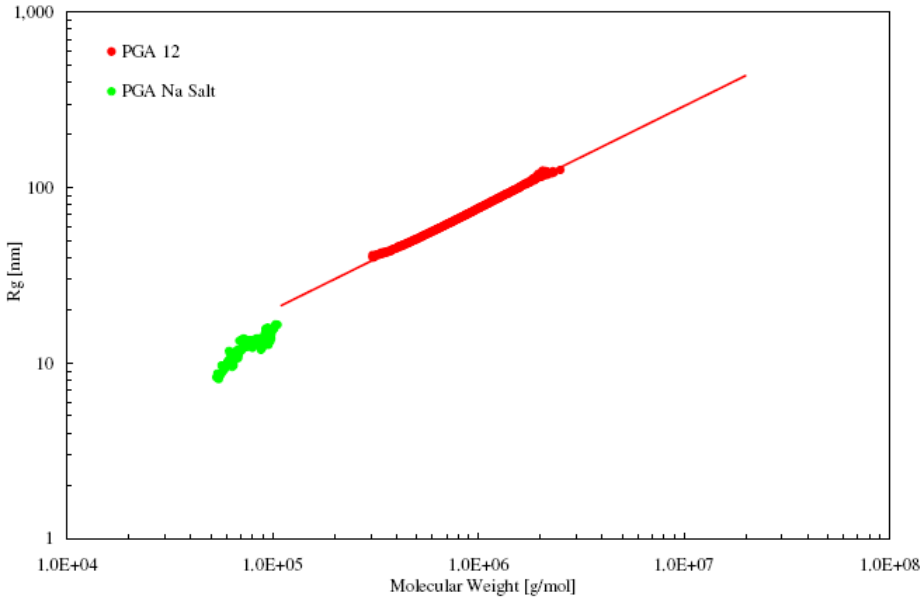


Figure 20: Conformation Plot

Figure 20 illustrates the Conformation Plot of the *B.subitilis* mutant- bioproduced polymer and that of the Natto Bioscience commercial one, in the sodium salt form. The Conformation Plot (analogous to the Mark-Houwink plot, which can be derived from intrinsic viscosity measurements in function of molecular weight and which is fundamental for polymer structure analysis) is constructed plotting radius of gyration data acquired by means of a light scattering detector against MW data. Its slope represents α parameter in the equation:

$$R_g = K \times M^{\alpha}$$

where R_g is the radius of gyration and M the molecular weight. The α parameter itself is related with the conformation of the macromolecule. For the sample PGA12, its value is ≈ 0,58 which is typical of a stiff random coil conformation. The commercial sample molecular weight is too low to permit a reliable estimation.

After measuring γ-PGA molecular weight by means of SEC-MALS both on commercial and on laboratory produced samples, we directed our attention to other possible methodologies able to provide us with an *in-house* means for molecular weight measurement. Finding and developing a quick and easy method to the purpose, not requiring high-specialized equipment and/or particularly expert and skilled personnel, would compensate for the loss of analytical accuracy, and provide us with a first evaluation ability, useful for routine work.

To reach this goal, we addressed the works reported by Park (2005) and Paraskevas (2002). These authors developed an assay based on the use of Sanger's reagent to evaluate γ-PGA molecular weight. In fact, noticing that every polymeric filament has a terminal amino group, it is possible to determine the numerical ratio between the amino groups and the γ-glutamyl residues present in the polymer's backbone. This ratio is, obviously, related to the filament molecular weight. In order to estimate the amino group number, the functionality can be, in principle, easily derivatized with Sanger's reagent. Thus, measuring the UV absorbance of Sanger-γ-PGA hydrolysates and comparing it with an appropriate UV absorption standard curve of Sanger-glutamic acid, it is possible to calculate the molecular weight of a polymeric sample.

Operatively, after the construction of the Sanger-glutamic acid standard UV absorption curve, the polymeric sample must be derivatized, hydrolyzed in acidic conditions and then measured at the UV spectrophotometer.

We therefore approached this methodology, trying the evaluation of commercial samples whose molecular weight had been previously determined by means of SEC-MALS, as well as of laboratory bioproduced samples. The method proved to be highly sensitive, but not robust enough for our purposes. Our results were in good accordance with those obtained thanks to more advanced methods when we analyzed commercial

samples, but this did not happen in the case of laboratory bioproduced ones. In fact, the measurement of laboratory produced samples proved to be dramatically affected by the presence of interferents, particularly glutamate monomers and oligomers. Park and coworkers actually (Park, 2005) show how this method becomes extremely fragile when the sample is not characterized by high purity grade; this analytical grade, according to the authors, can be obtained only by the use of expensive, costly and time-consuming purification techniques such as ion exchange chromatography, which is able to separate oligomeric and monomeric fractions from high molecular weight ones. Therefore, this method is unfit to be applied to our laboratory produced biopolymer samples, as long as they reach just technical grade purification after having undergone a protocol based on subsequent precipitations.

The application of the protocol on low molecular weight γ-PGA of commercial origin provided us with an estimated value (*ca* 30 000 Da) in reasonable accord with the value measured by SEC-MALS.

On the contrary, interferents heavily affected the protocol when we tried it on samples produced by our strain, thus affording misleading results and confirming the limits of this technique reported by literature.

Sonication

In collaboration with the Microbiology Department of the University of Pavia, we also developed an effective method to control the polymer molecular weight. In fact, it was found that sonication at fixed frequency (around 30 kHz) is able to degrade high molecular weight polymer filaments to a limit value, in very brief times (≈ 200 s).

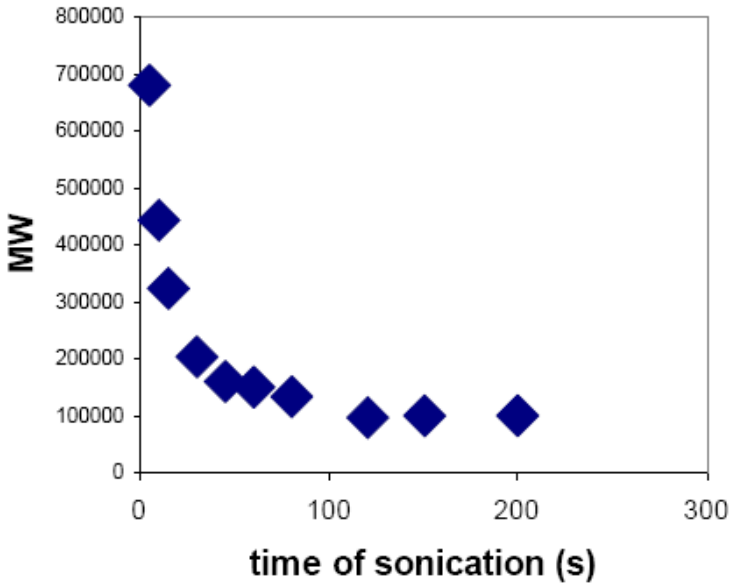


Figure 21: sonication of a sample of in house- produced γ-PGA; the MW drops from 700 kDa to less than 100 kDa in around 200 s.

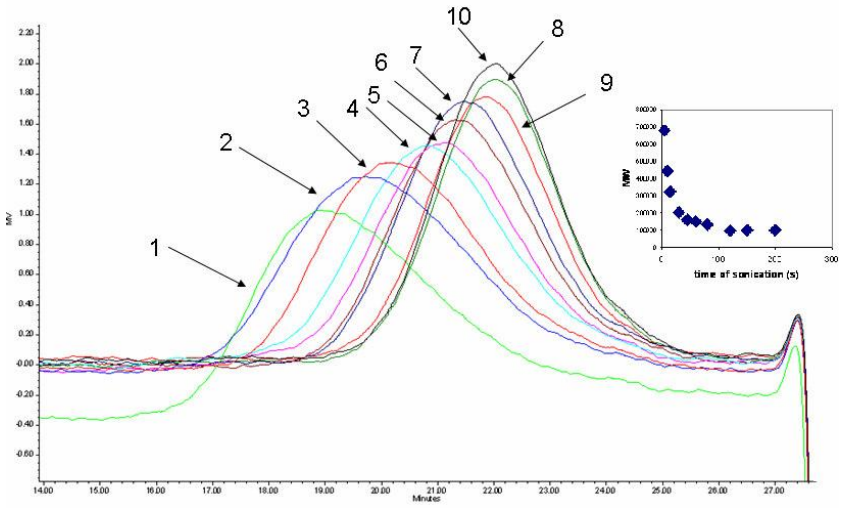


Figure 22: SEC analysis of the sonicated sample.

Rheology

Another important aspect in the characterization of a polymer is the study of its rheological properties, both for elucidating the physical-chemical properties of the material and to provide a reliable estimation of its molecular weight (Irurzun, 2001). On this respect, it has to be noticed that viscosimetric studies on γ-PGA itself are scarce (Kubota 1996); comparison between SEC-light scattering measurements and viscosimetric ones has been performed, but a numerical correlation between these data has not been proposed so far.

In collaboration with the Pharmaceutical Department of University of Pavia, we were able to perform viscosimetric measurements, using a rotational rheometer, on three representative samples: two commercial available ones (purchased from Natto Bioscience and Biological Tech, Co), with low and high molecular weights, respectively, and a laboratory bioproduced (University of Pavia) one.

Analyzing these measurements, it is possible to make some considerations about the viscosimetric behaviour of our samples.

The low molecular weight (20 kDa, 100kDa declared by producer) commercial sample (Natto Bioscience) is characterized by Newtonian behavior, i.e. its viscosity remains constant with the increase of shear stress. Its viscosity is quite low (2,12 mPa s on average). This value is in good accordance with that expected for a low molecular weight polymer (Fig. 23).

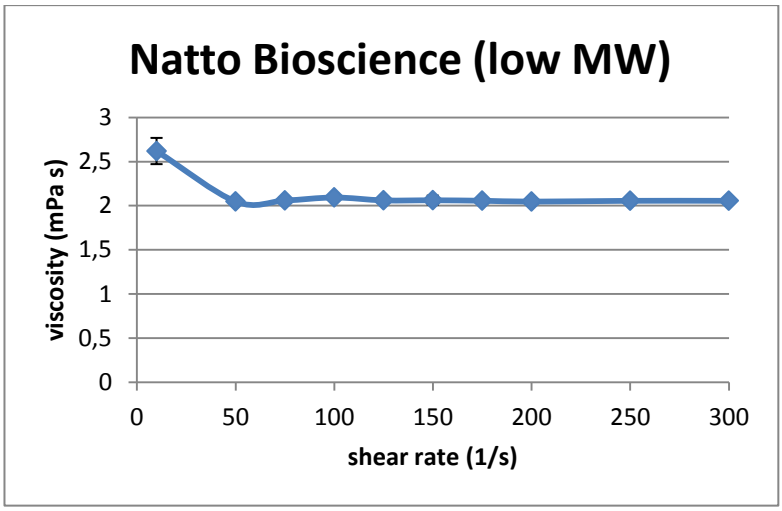


Figure 23

On the contrary, the high molecular weight (700 kDa) commercial sample (Biological Tech, Co.) is characterized by pseudo-plastic behavior, i.e. its viscosity tends to decrease with shear stress increase. This is typical of polymeric solutions, because under shear stress, the filaments tend to pass from a coiled conformation to a more ordered one in which they stretch in the movement direction, increasing their hydrodinamicity. For this sample, viscosity decreases from an initial value of 161 mPa s to 86 mPa s with a shear rate increase from 10 to 300 /s (Fig. 24).

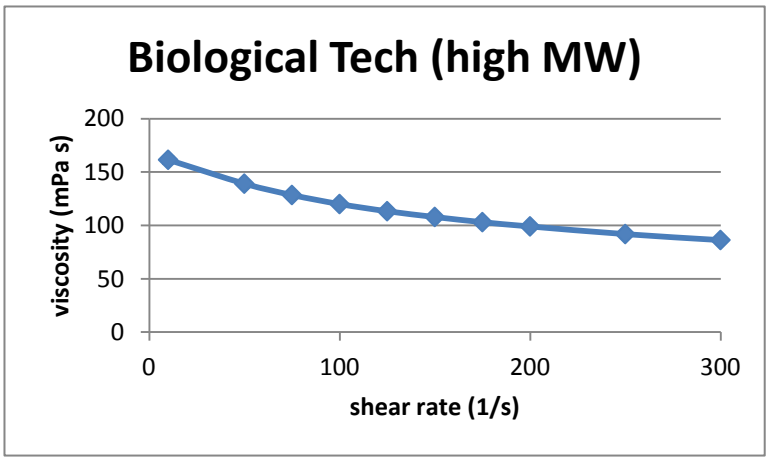


Figure 24

An analogous pseudo-plastic behavior is shown by the bioproduced at University of Pavia polymer, whose MW is also around 700 kDa (determined by SEC-MALS technique). Its viscosity decreases from 132 mPa s to 79 mPa s in the shear rate range 10-300 /s. This is consistent with the fact that the high molecular weight of this species and that of the commercial high molecular weight samples are similar (Fig. 25).

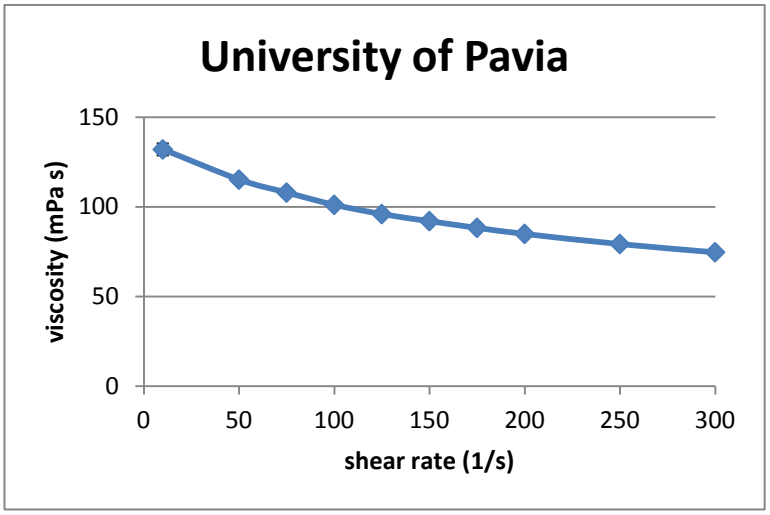


Figure 25

Conformational aspects

Γ-PGA conformation and its influence on its chemical-physical behavior and reactivity is still an open issue. In fact, the polymer filaments, can adopt several different conformations in solution, which on their turn depend on numerous factors, including the enantiomeric composition and the degree of ionization of the polymer, the pH conditions, the nature of the solvent. Although a systematic study on the subject is still lacking, some hypothesis have been formulated along the years.

First of all, a study performed on poly-γ-D-PGA conformation in aqueous solution showed that considerable differences exist depending on the degree of ionization of the polymer itself, with the un-ionized species preferably assuming an helical conformation stabilized by hydrogen-bonds, while, on the contrary, the ionized polymer tend to a

random-coil state. In 1964, Rydon, on the basis of optical rotator dispersion (ORD) measurements, proposed two possible structures for this kind of poly-γ-glutamate, namely 3_{17} helix and 3_{19} helix, both of them stabilized by intramolecular hydrogen bonds. More recently, molecular dynamics and quantum mechanical calculations led Zanuy (Zanuy, 1998; 2001) to indicate the 3_{19} left-handed helix as the most stable conformation for this kind of polymer.

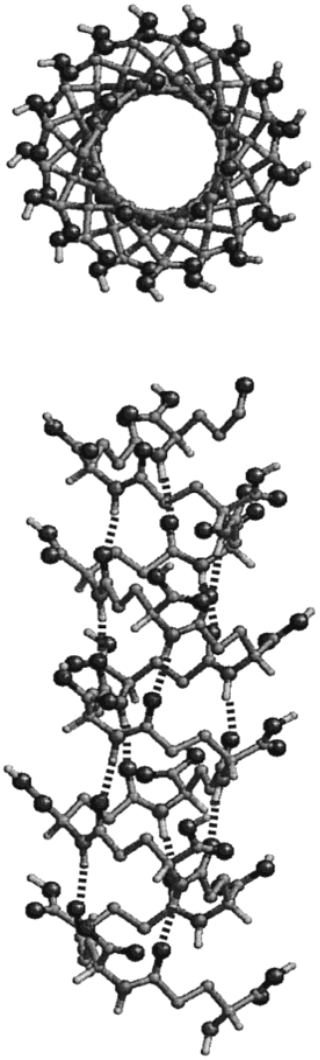


Figure 26: Equatorial and axial projections of the model conformationof un-ionized poly-γ-D-glutamic acid (Zanuy, 2001)

In 1973, Sawa and coworkers, after viscosity as well as ORD and IR spectroscopy measurements, proposed that γ-PGA produced by *B. subtilis* was in a parallel β-sheet in

acidic solution, shifting to a more and more contracted random coil conformation with the rise of pH to neutrality and then alkaline values.

In 2000, He and coworkers performed a conformational study on γ-PGA produced by *B.licheniformis* by means of attenuated total reflectance FT-IR spectroscopy.

It is in fact known (Cantor, 1980) that the α-helix, β-sheet and random coil structures of polypeptides and protein can be distinguished referring to the spectral bands at 1655 cm^{-1} (amide I stretch) and 1550 cm^{-1} (amide II stretch), which are diagnostic for α-helix, while a shift of the amide I stretch to 1630 cm^{-1} is typical for β-sheet; random coils are associated with a shift of the amide II stretch band to 1535 cm^{-1}.

The results collected by He's group show that conformation of *B.licheniformis* γ-PGA in aqueous solution is affected by pH, ionic strength, and concentration of the polymer itself. In fact, at low pH, a low concentrated (0,1 % w/v) γ-PGA solution is protonated and in helical conformation, as it is confirmed by the infrared bans at 1659 cm^{-1} (amide I stretch) and 1558 cm^{-1} (amide II stretch), respectively. Rising the pH to neutrality, the amide I shift to 1635 cm^{-1}, indicating a change of conformation to the β-sheet structure in parallel with deprotonation. At higher pH, the polymer gets a random coil structure. A similar trend is observed also with respect of ionic force, the increase of which makes the conformation shift from α-helix to β-sheet in the range 0,1-0,5 M; α-helix is also associated to polymer low concentrations; its increase to values as high as 25 mM as glutamate cause a predominance of β-sheet, presumably because the intramolecular interactions which maintain the helical structure are overwhelmed by intermolecular ones.

Lately, the pH dependency of PGA helix folding dynamics has also been confirmed and modeled by Krejtschi (2011) Gooding (2012) by means of CD and FTIR spectroscopy.

Moreover, in order to elucidate the stereochemical microstructure of the polymer, Martínez de Ilarduya (2002) performed the synthesis of γ-PGA according to Sanda (2001) in order to get standard sample suitable to conformational NMR studies.

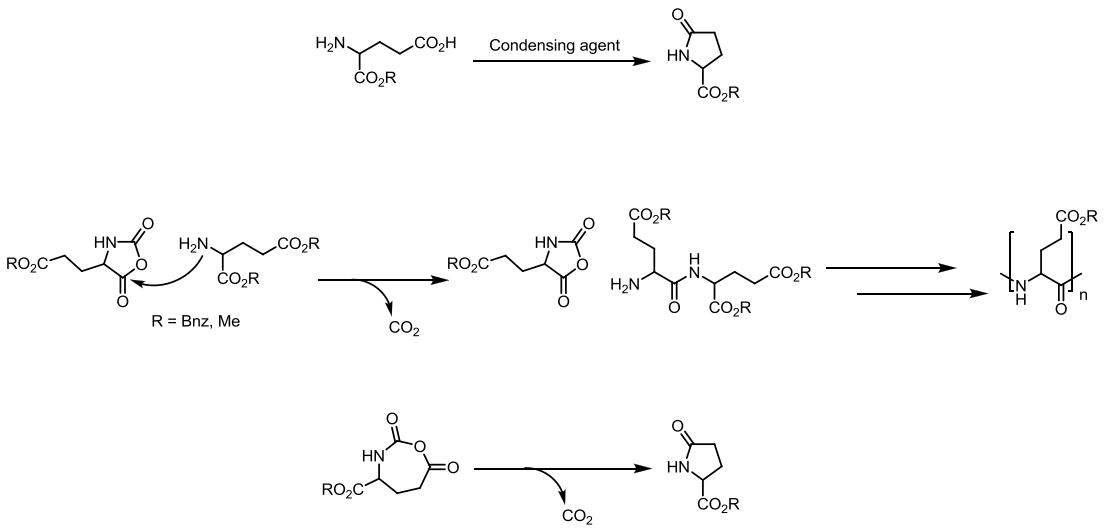
Chemical synthesis

During our efforts for completing the chemical-physical characterization of biopolymer, we realized that its peculiar macroscopic behavior (for instance its tendency to form gels, its erratic solubility in organic and aqueous media, its high hygroscopicity), as well as its chemical reactivity, should be related to its microscopic properties, and particularly to molecular weight and conformation (this related, on its turn, to the polymer stereochemical composition); studying these properties could help to elucidate their influence on the material, and provide useful suggestions for its manipulation, not to mention the possibility of future theoretical studies dealing with the so far unclear stereochemical aspects of γ-PGA biosynthesis.

On these grounds, we decided to try the chemical synthesis of the polymer, in order to obtain standard samples with a well defined enantiomeric structure and controlled molecular weight; these will serve as model compounds to perform conformational investigation on, taking advantage of circular dichroism spectroscopic techniques.

A preliminary study of the problem made immediately clear that direct polymerization of glutamic acid was out of discussion. In fact, it is known that coupling of two α-protected glutamic moieties only results in the formation of pyroglutamic acid derivatives (Kajtár, 1969).

Other approaches involving protected lactame intermediates end up with no exception in the formation of poly-α-glutamic acid or again in the formation of pyroglutamic esters (Sun, 2011).



Scheme 2: non productive lactame synthetic approaches for γ-PGA synthesis

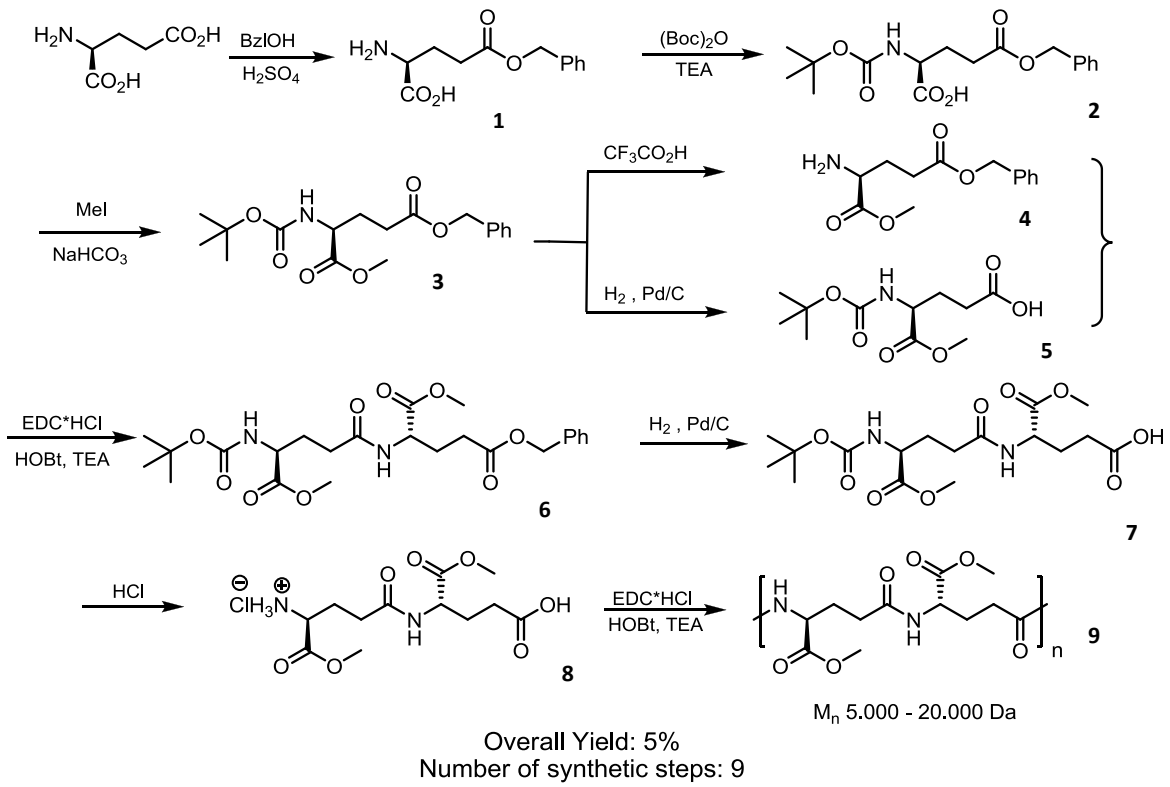
Synthesis in solution

We therefore decided to recur to the well established methods of functional group-protecting chemistry. As for this strategy, the only example so far published in literature is the one reported by Endo and coworkers in 2001 (Sanda, Fujiyama, Endo, 2001). Using standard protection reactions, the core idea of the synthesis is to form, making use of standard peptide chemistry methods, α-protected glutamic acid dimers which can be polymerized to obtain a low molecular weight poly-γ-glutamic acid.

To get the key intermediate, glutamic acid needs to be orthogonally protected on the whole of its functional groups, making it possible to selectively deprotect the α-amino group on one protected moiety, and, on the other side the γ-carboxylic group of a second moiety, in order to form a nucleophilic and an electophilic intermediate which can be condensed to the fully protected dimer.

This protected dimer can be subsequently deprotected, in two steps, to afford an intermediate prone to be condensed with N-(3-dimethylaminopropyl)-N'-

ehylcarbodiimide hydrochloride (EDC·HCl) and hydroxybenzotriazole (HOBt) as coupling agents (Scheme 3).



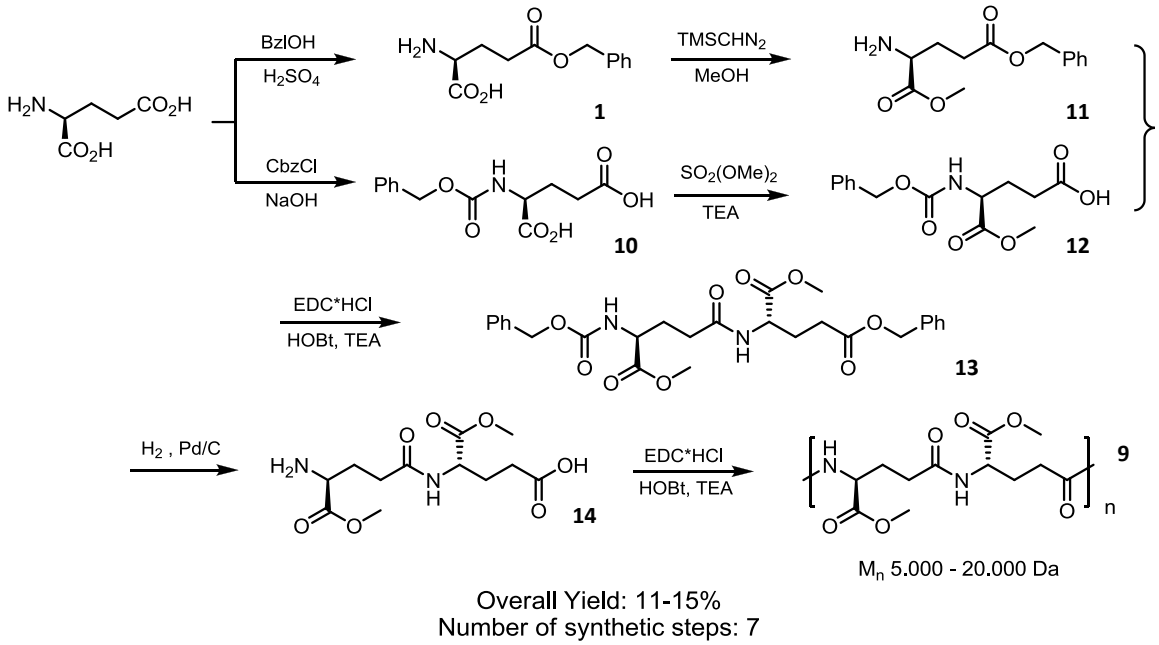
Scheme 3

We were able to successfully reproduce this protocol, but we also experienced it as being quite long and tedious. Nine steps are necessary, and the overall yield does not exceed 5 %.

Thus, we tried an improvement of the procedure, to obtain more quickly the formation of the key protected dimer intermediate. To reach this goal, we decided to avoid the protection of one of the three groups leaving the amino or the γ-carboxylic group free, so that we could spare one deprotection step before the formation of the dimer. A consequence of this choice was the change of the protective group used for the amino and γ-carboxylic functions of the electrophilic and nucleophilic units, respectively. Two

orthogonal groups were more conveniently substituted with ones which could be both removed in a single step; in particular, the benzyl ester protection at the γ-carboxylic group was maintained on the nucleophilic unit, while the Boc protection used for the amino group on the electrophilic one was replaced with the Cbz protective group, which could be cleaved by hydrogenation together with the benzyl moiety.

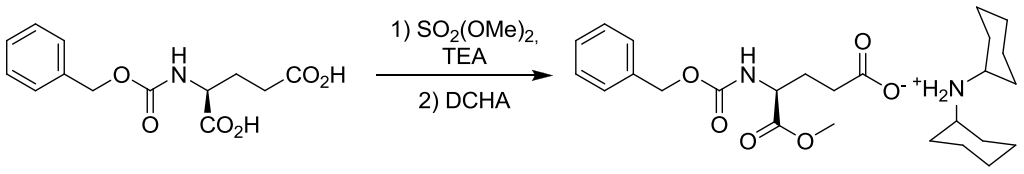
The new protocol is illustrated in Scheme 4.



Scheme 4

The seemingly banal methylation of the α-carboxylic group of the electrophilic intermediate for the dimer coupling proved to be a particularly tricky step.

We tried many protocols, the best of which proved to be treatment of N-Cbz protected glutamic acid with a stoichiometric amount of dimethylsulphate; in chloroform, only one of the two carboxylic groups reacted; a slight preference in the formation of the α-ester was found. Selective precipitation of α-ester with dicyclohexylamine afforded the electrophile with a 60% yield (Nefkens, 1964). Reaction is illustrated in Scheme 5.



Scheme 5

Our improved protocol resulted to be composed by 7 steps, in an overall yield between 11 and 15 %.

Solid phase synthesis

Having reached the goal of improving an existing protocol, we considered the option of a totally different strategy: producing poly-γ-glutamic oligomers by means of solid phase synthesis techniques.

Solid phase peptide synthesis methodology represents a total novelty in this field; most of all, it carries several advantages, both on the theoretical and the practical point of view.

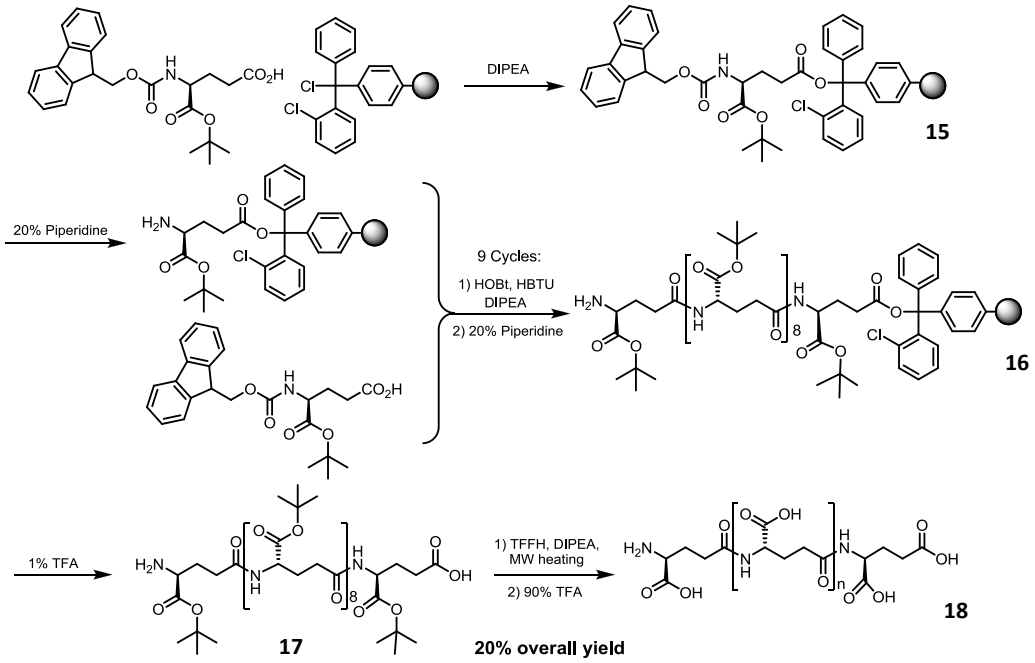
First, it displays far more stereochemical flexibility than Endo's one: in fact, using that approach, it is only possible to obtain either L-L, or L-D, or D-D dimers, depending on the choice of the configuration of the starting monomers; therefore, the final polymer can only result to be isotactic (if the starting dimers are only L-L or D-D) syndiotactic(L-D dimers), or atactic (composed by randomly coupled L-L, D-D and L-D dimers).

Starting from an oligomeric building block (for instance, in our case, a decamer) makes the creation of much more complex structures, and therefore, of a larger conformational variety of the final polymeric product, possible.

Moreover, solid phase protocol is relatively easy to implement and can be even partially automated: this makes the production of the polymer operatively simpler and simpler,

and quite faster, than in the case of solution synthesis, requiring several laborious protection, deprotection and purification steps.

We thus chose to implement a Fmoc procotol to generate a ten residue oligomer of γ-glutamic acid, which could be subsequently polymerized in solution in standard coupling conditions, working at high concentration to minimize the possibility of ciclyzation. The overall synthetic strategy is illustrated in Scheme 6.



Scheme 6

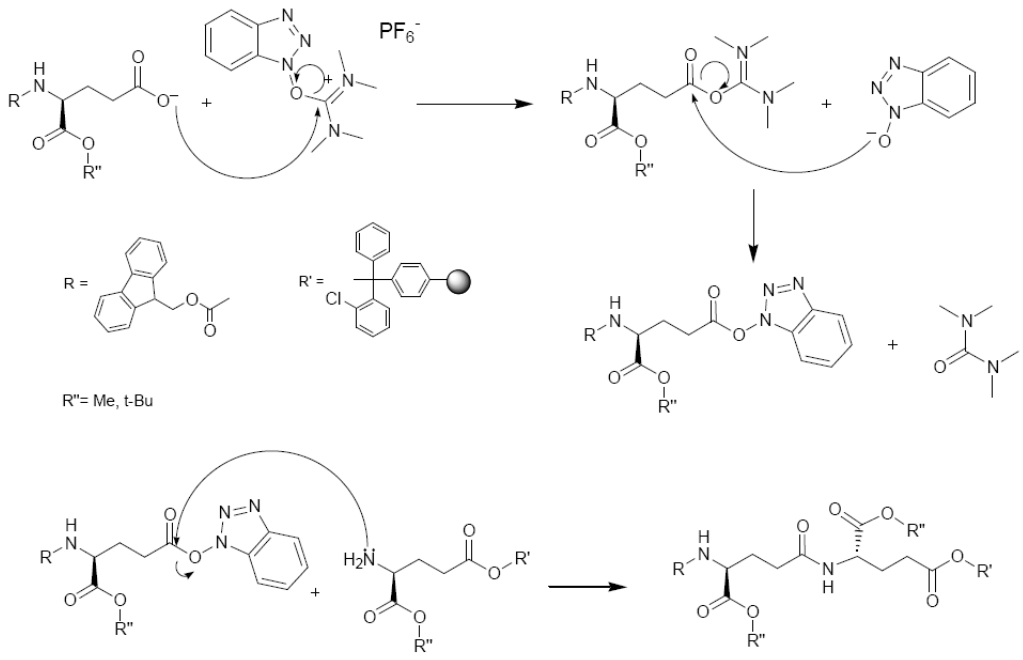
The choice of the Fmoc protocol is due to its ease of implementation as well as to the facile deprotection of the amino group in mild conditions, without affecting the most common protections suitable for the carboxylic position. In fact, as it is widely known, the Fmoc group can be removed by a piperidine solution.

The starting monomer was a commercial available amino acid, namely Fmoc-Glu-O-t-Butyl-OH.

As supporting material, 2-chloro-trytil chloride was selected, for its robustness towards several working conditions, commercial availability, and cheapness.

The first step of the process is the attachment of the N, α-carboxy-diprotected amino acid monomer to the resin, which is possible by simple treatment with a base such as di-isopropyl-ethyl-amine (DIPEA).

Once the first monomer has been attached to the resin, it can be put in an automatic synthesizer, an instrument capable of performing all the necessary steps for the condensation of amino acids, at each cycle deprotecting the amino group of the last residue on the growing chain, and then letting it couple with a new amino acid thanks to HOBt and HBTU or EDC or DCC reagents, in our case (Scheme 7).

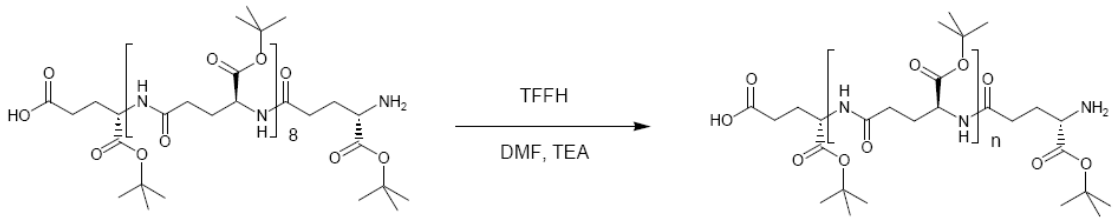


Scheme 7: solid phase polymerization.

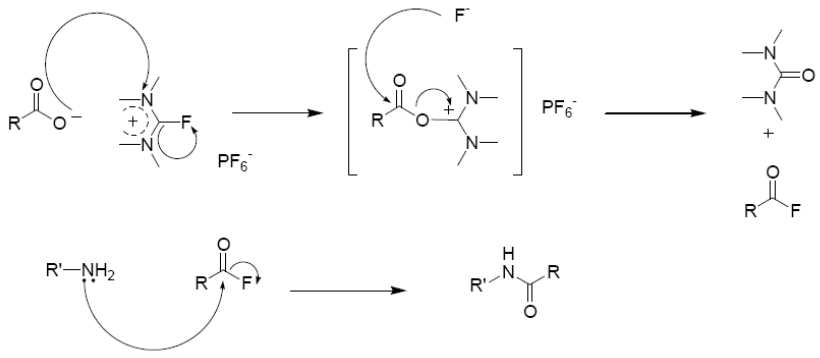
Once the oligomer of the desidered number of residues (a decamer in our case) has been formed, it can be easily cleaved from the resin by treatment with a 1% trifluoroaceticacid aqueous solution.

The final polymerization of the oligomers was performed in solution.

Many conditions and coupling agents were tested, the best results being obtained thanks to the use of N,N,N',N'-tetramethyl-fluoro-formamidinium-hexafluorophosphate (TFFH) a recently developed reagent which is particularly suitable for bulky substrates (Scheme 8-9).



Scheme 8: coupling reaction in solution



Scheme 9: TFFH reaction mechanism

We also took advantage of microwave heating, which proved to have a very positive influence on the outcome of the reaction. We suppose that this might be explained by

the fact that microwaves, a form of coherent heat electromagnetic radiation, interact with the peptide chain dipole moment, keeping the chains in a linear and therefore reagent-accessible conformation, differently from what normally happens in solution, when the chains tend to form secluded structures such as α-helix (thanks to hydrogen bonding) or random coils.

The effectiveness of this approach was confirmed by conventional Size Exclusion Chromatography (calibration operated with respect to Polystyrene standards) measurement of the molecular weight of a sample taken form a crude polymerization attempt. Although presented a relatively high polydispersity, a poly-γ-glutamic acid *t*-butyl ester was formed (although it is easily deprotected with aqueous TFA, the polymer was initially kept in the ester form to avoid the risk of contaminating the sample), its molecular weight being comparable to that of a commercial sample such as that acquired by Natto Bioscience Company.

Results are shown in the following table 8.

Table 8

Sample	M_p g/mole	M_n g/mole	M_w g/mole	M_z g/mole	M_w/M_n	M_z/M_w
Γ-PGA t-butyl ester	17,950	8,000	14,140	21,850	1.77	1.55

A possible drawback of this process is its relatively higher cost, compared with solution phase protocols, but since for our purposes only little amounts of polymer are necessary, this objection can be considered of marginal importance.

Derivatisation

The ability of chemically modifying γ-PGA is an essential requirement for the technological valorization of the material. Every attempt to reach this goal must of course start from the observation that the only functionality present in the

macromolecule is the α-carboxylic group pending from the polymer backbone. A careful choice of the substituting group (which will be likely linked to the polymer in the form of an ester, or an amide) will contribute to the modulation of its chemical-physical properties; for instance, increasing its hydrophobicity, and therefore, the possibility for it to be used as a biopackaging material, or as scaffold for the construction of nanoparticles for biotechnological and pharmaceutical use.

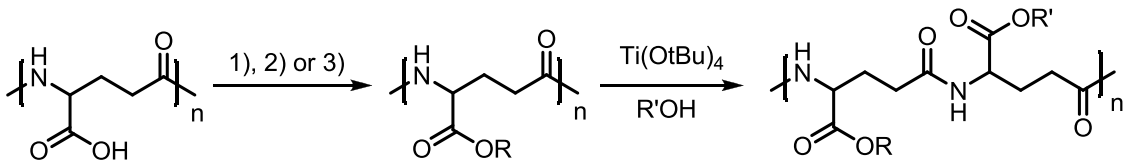
However, evidence indeed proved that the inherent tendency of this group to react is far from being excellent. In fact, the reactivity of the carboxylic groups pending from the polymer backbone is unusual, and quite cumbersome.

So far, our efforts have aimed at the esterification of the biopolymer, both directly and indirectly, *via* transesterification, as well as at the formation of amide derivatives.

Although the procedures involved may appear trivial at a first glance, their development proved to be difficult and tedious, due to three main obstacles: the scarce chemical reactivity of the material, and the difficulty encountered in isolation and purification of the products.

To date, three methodologies have been attempted and developed for γ-PGA esterification:

- Nucleophilic substitution on carboxylates;
- Direct esterification *via* previous acyl chloride formation;
- Use of a carbodiimide as condensing agent.



Scheme 10: 1) RBr, $NaHCO_3$, DMSO, 45 ºC, 5-8 days; 2) $(COCl)_2$ or $SOCl_2$, THF, DMF, ROH, 24-36 h, from 0 ºC to r.t.; 3) EDC or COMU or TFFH, DMSO, ROH, 24-36 h, r.t.

Our efforts made possible the preparation of the following derivates of γ-PGA:

- γ-PGA-α-methyl- ester
- γ-PGA-α-ethyl- ester
- γ-PGA-α-isopropyl- ester
- γ-PGA-α-*n*-butyl- ester
- γ-PGA-α-tetradecanoyl- ester
- γ-PGA-α-benzyl- ester
- γ-PGA-α-ethyl-α'-poly (ethylene glycol)-yl ester
- γ-PGA-α-benzyl-α'-poly (ethylene glycol)-yl ester

Nucleophilic substitution on carboxylates

The first method, reported by Kubota and Endo (Kubota, 1993b) and widely used by Muñoz-Guerra has proven to be effective, but quite inefficient. To achieve a degree of substitution exceeding 50 %, the procedure requires dissolving the polymer in a high-boiling solvent such as DMSO and letting it react with alkyl or benzylic halide in basic condition for times between 5 and 10 days, performing two subsequent derivatisations. Afterwards, the product must be isolated by precipitation in cold acidic water. This procedure is quite long and tricky, and skill is requested to get rid of solvent traces.

Following this approach, we have prepared benzyl- and ethyl- esters of γ-PGA.

The formation of the ethyl ester and its degree of functionalisation can be assessed by ^{1}H-NMR; in particular, the signal belonging to the amide of the derivative falls at higher

fields than that belonging to the starting material; therefore, by the ratio of the two signals, it is possible to evaluate the progress of the reaction.

In our case, after a first derivatisation in presence of a 5-fold excess of ethyl bromide, which lasted 5 days, we were able to obtain a sticky, viscous product, with a degree of functionalisation about 15-20%. Its isolation was possible by precipitation of the reaction mixture, drop by drop, in a 15 fold quantity of acid water, whose pH was carefully maintained around 1,5-2 by addition of HCl. This procedure is straightforward, but slow, and the filtration of the product is quite tedious; in fact, its gluish nature makes its manipulation difficult. Moreover, appreciable amounts of DMSO tend to adhere to this first generation product; rinsing with acid water, and/or organic solvents, are of little help in getting rid of solvent traces. Also a long (several hours) run at the rotary evaporator, connected to an acetone/dry ice trap, did not success.

We had therefore the material react a second time, in the same conditions. Second generation product showed a quite higher degree of functionalisation (almost 70%), after 3 days of reaction. Complete conversion was not reached even by further prolonging reaction times, or increasing the reagent excess, or reaction temperature; however, it is reported in literature that steric effects become significant with the increase of functionalisation, affecting the reaction outcome. Moreover, the possibility of a reduction in molecular weight after prolonged reaction in these conditions has also been proposed by Muñoz-Guerra. The isolation of the second generation ester was possible by the same protocol previously used; the material was quite easier to handle, and less prone to contamination by DMSO.

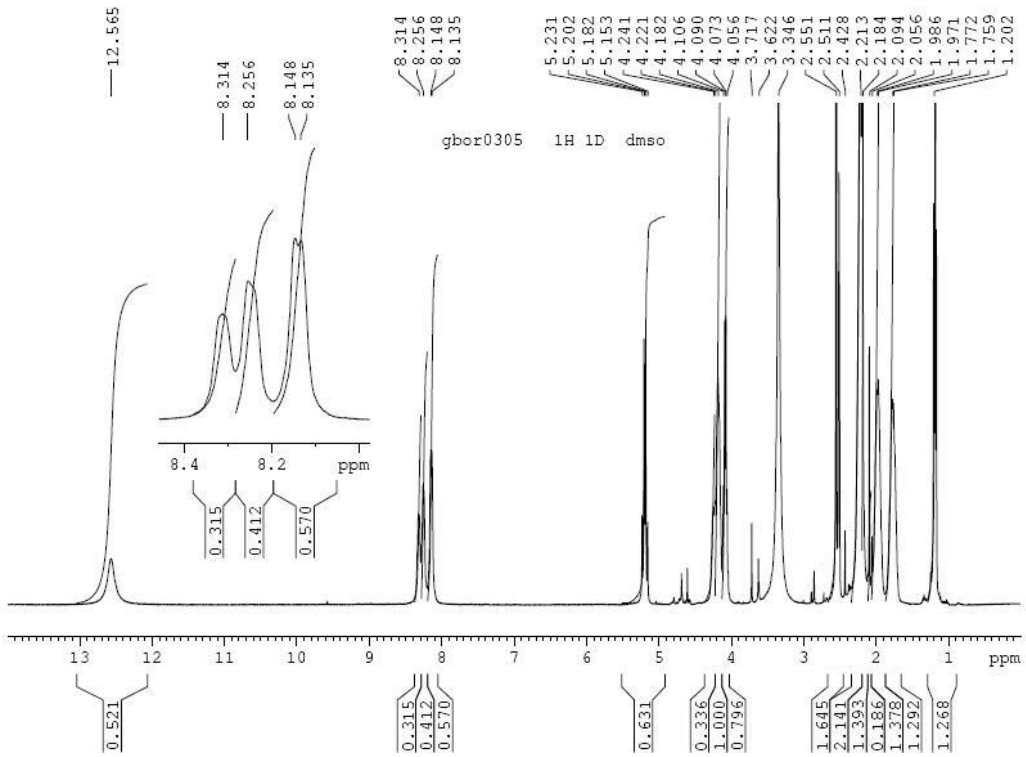


Figure 27: first generation ethyl ester of γ-PGA

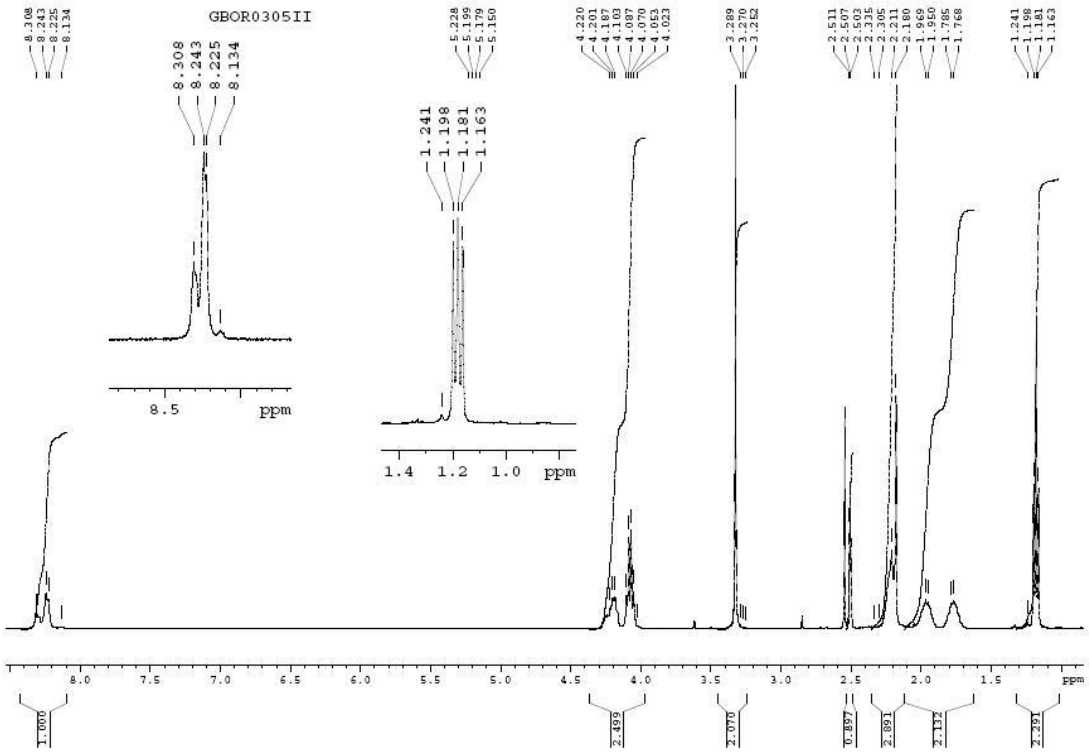


Figure 28: second generation ethyl ester of γ-PGA

Similar considerations are possible in the case of benzyl ester of γ-PGA; this material is quite easier to handle and isolate; in fact, it is known in literature for its favorable mechanical properties, which make it suitable for the preparation of films. It must be noticed, however, that even for second generation products it is difficult to reach a functionalisation degree much higher than 50 %.

In both cases, the use of an alkyl of benzyl iodide instead of the corresponding chloride gave no improvement with respect to time required for reaction, work-up of the reaction, or overall yield.

Transterification

First generation derivatives can undergo transesterification in presence of a catalyst such as $Ti(OtBu)_4$; still, the reaction requires high temperatures (around 100 °C), the use of extremely high excess (up to 50 times) of alcohol, and the isolation of the product is often not easy.

We decided to perform this reaction in presence of poly (ethylene glycol) with average molecular weight around 550, using the ethyl and benzyl ester as starting materials. The functionalisation with poly (ethylene glycol) of a first generation derivative of γ-PGA is in fact reported as an effective way to modulate its properties, with respect to thermal stability and solubility in water, for instance. Also in this case, we had to notice that it was extremely difficult to reach complete functionalisation. In the case of the ethyl ester transesterification product, we could easily get a product which had a functionalisation degree of 59% in the ethyl ester and 27% in the poly (ethyleneglycoyl) ester; 14% of the carboxylic functions remained unreacted. A similar behavior is observed when the benzyl ester undergoes transesterification with (ethylene glycol).

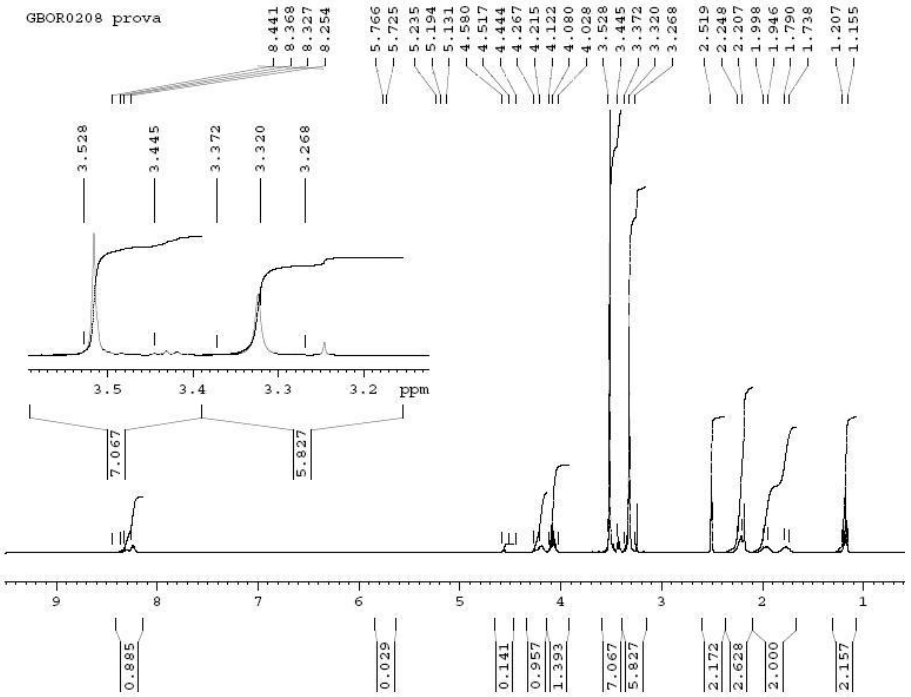


Figure 29: transesterification of the diethyl ester of γ-PGA with poly (ethylene glycol, M_n ≈ 550)

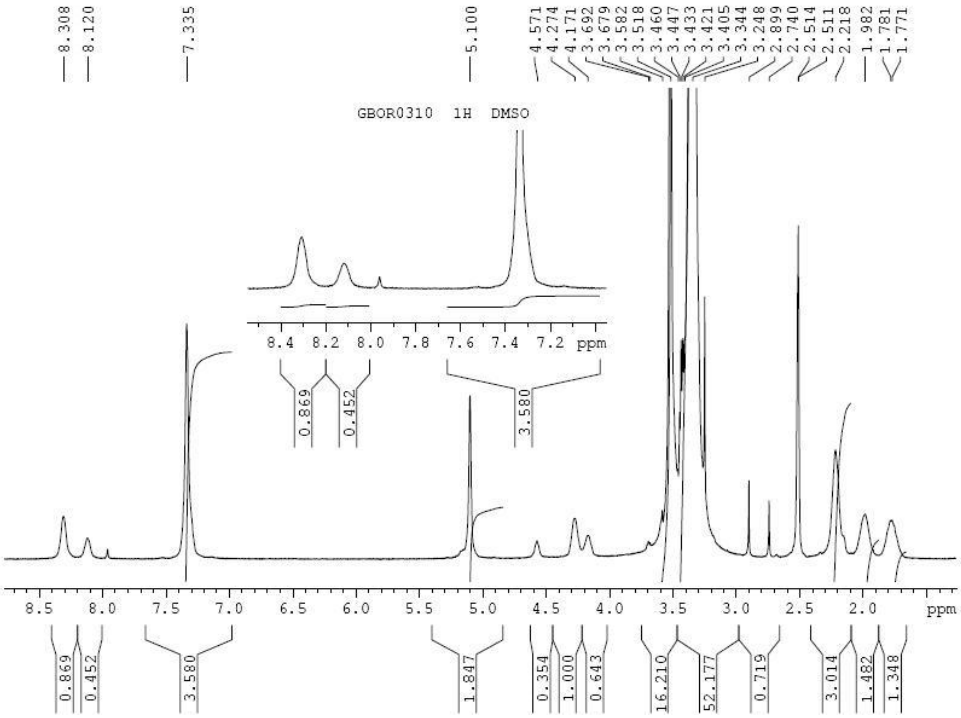


Figure 30: transesterification of the benzyl ester of γ-PGA with poly (ethylene glycol, M_n ≈ 550)

In an attempt to improve the scope of the nucleophilic substitution on carboxylates approach, we also tried the well established methodology of phase transfer catalysis, making use of tetraalkylammonium halides and dissolving γ-PGA in basic water and the

halide in dichlorometane, respectively. Unfortunately, our attempts were not successful. Future investigation, however, should be devoted to a deeper exploration of this effective and simple methodology, maybe concentrating the attention on the choice of the proper catalyst to the purpose.

Direct esterification

The second methodology, which to the best of our knowledge has not been proposed before, consists in the formation, facilitated by a catalytic amount of DMF, of an acyl chloride *in situ*, followed by direct esterification, in an etheric solvent such as THF, or directly in the alcohol corresponding to the desired ester. The reaction is quite faster (24-36 hours), and the product is relatively easily isolated by precipitation in a cold alcohol such as methanol and filtration. The drawback of the procedure is that also in this case, the material has to react twice in a row to achieve some functionalisation in the second generation product. Moreover, the functionalisation degree is not always excellent.

It must be noted that an inherent limit in the chemical manipulation and modification of γ-PGA lies in its scarce solubility in most organic solvents, and in their mixtures; this fact negatively affects yields and purification, and strongly narrows down the possible applicable reaction conditions.

Taking advantage of this novel methodology, we were able to synthesize some short and long chain aliphatic esters of γ-PGA, namely methyl-, ethyl-, isopropyl, *n*-butyl- and tetradecanoyl- esters.

The preparation of the methyl ester gave the better results in terms of product yield, reaction time, easiness of the procedure and of the work-up. In fact, in 24 hours we were able to obtain a degree of functionalisation higher than 95%, using a moderate excess (6

times) of oxalyl chloride; the work-up simply consists in neutralization of the excess acid, and evaporation of the alcohol, resulting in the straightforward precipitation of the product, which is very pure and not contaminated by high-boiling solvents such as DMSO (a drawback of the carboxylates approach) or by other additives.

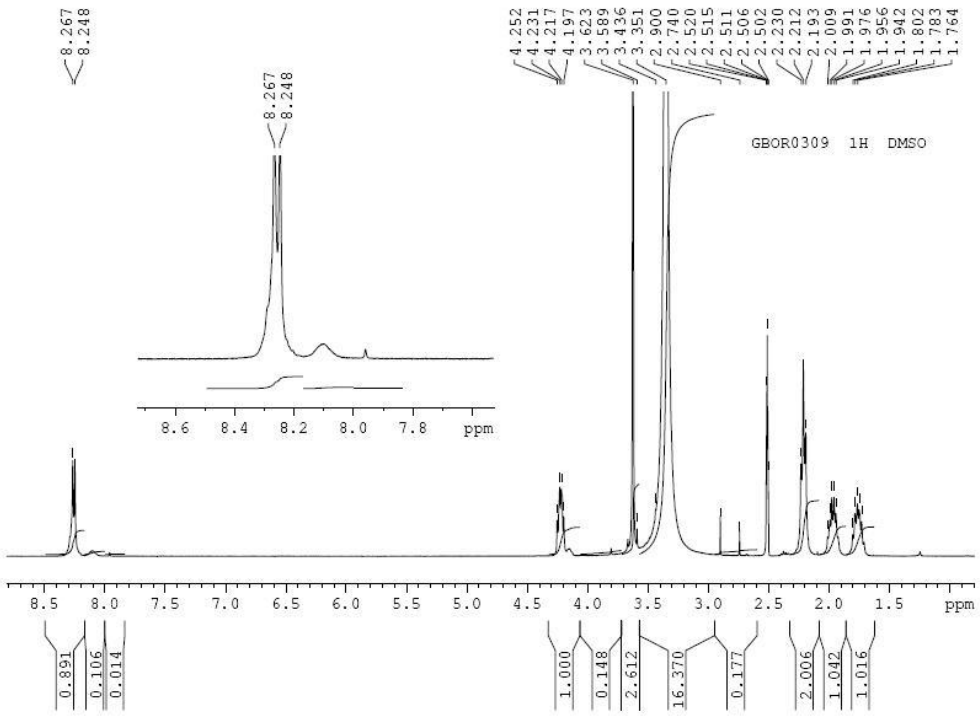


Figure 31: methyl ester of γ-PGA *via* direct esterification. The magnification shows the signal of the amide, which permits the calculation of functionalisation degree.

As for the other esters, we also had good results, but the functionalisation degree of first generation products, in some cases, tends to remain stable around 30 % (except in the case of the tetradecanoyl esters, when, it tends to drop to less than 20 %). In the case of the isopropyl as well as in that of the *n*-butylester, the product smoothly reached 70% functionalisation, while this curiously didn't happen for the ethyl derivative; we suppose that sterics may play a role, as well as, the solubility issues which deeply affect the reactivity of the polymer. The latter factor is probably crucial in determining the outcome of the reaction. The increase of oxalyl chloride excess (up to 20 times the substrate) is not always beneficial, as well as the raise of the quantity of alcohol or the

use of polar co-solvents such as MeCN, able to help the polymer dissolution, gave mixed and sometimes even erratic results.

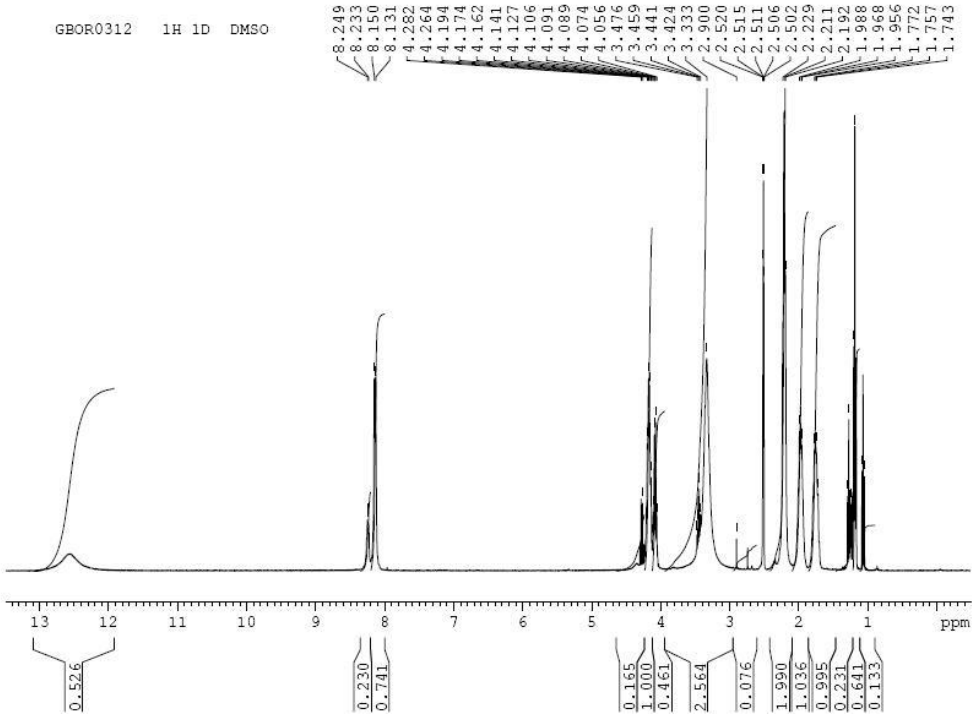


Figure 32: ethyl ester of γ-PGA *via* direct esterification.

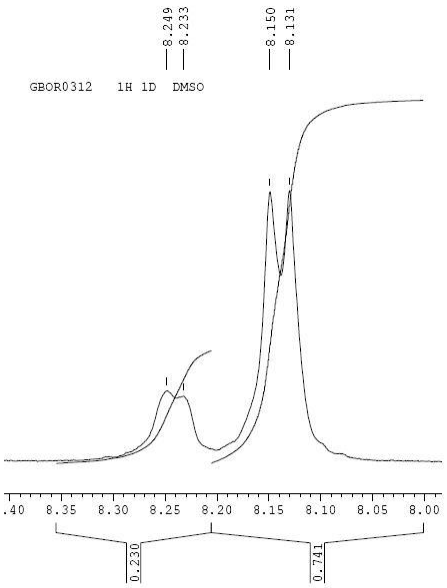


Figure 33: magnification shows the signal of the amide belonging to the ethyl ester of γ-PGA, which permits the calculation of functionalisation degree.

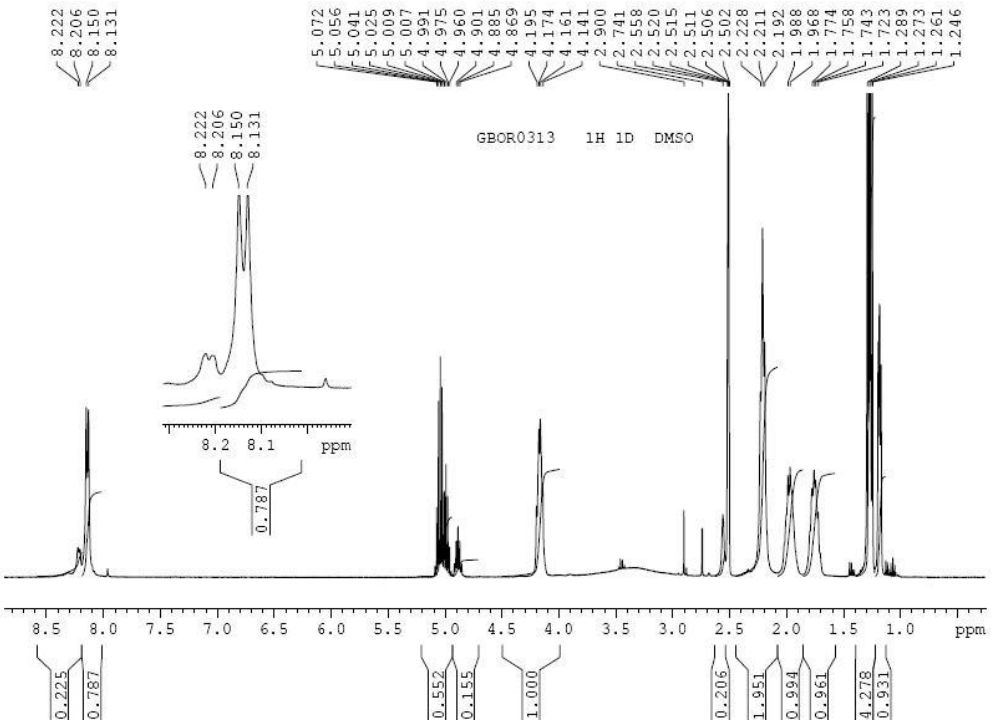


Figure 34: isopropyl ester of γ-PGA *via* direct esterification.

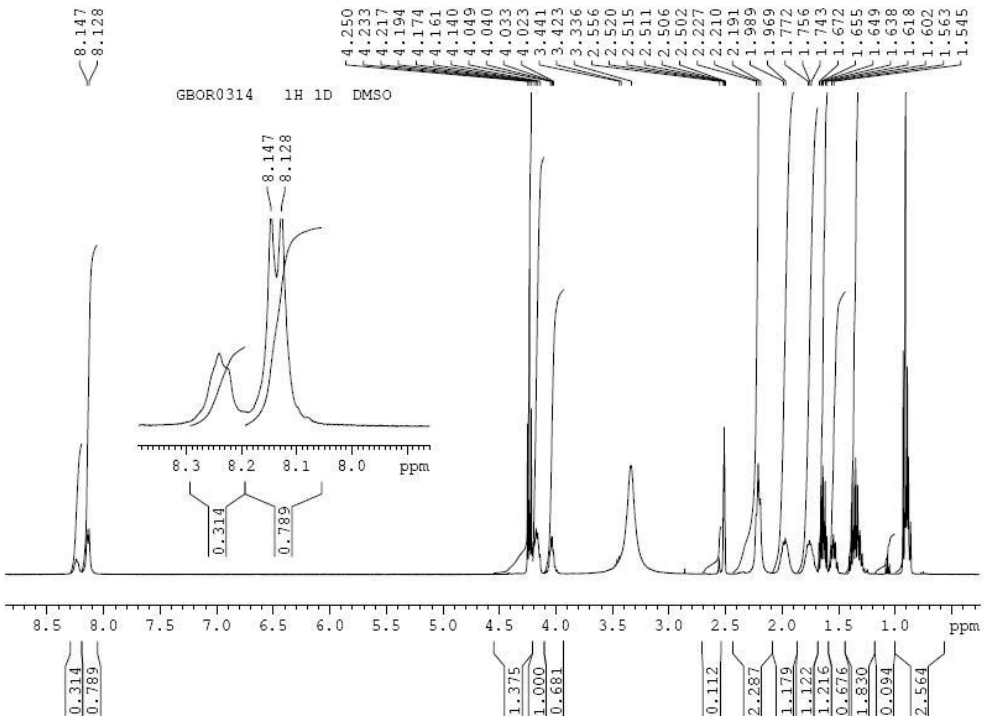


Figure 35: *n*-butyl ester of γ-PGA *via* direct esterification.

Use of condensing agents

The third methodology (Akagi, 2005b) has its origins in peptide coupling chemistry, and makes use of condensing agents such as a carbodiimide (usually a water-soluble one) or a stronger reagent such as COMU or TFFH. Operatively, the polymer is dissolved or suspended in DMSO, and reacts for 24 -36 hours in presence of an organic base (such as TEA, or DiPEA, or Py) and an alcohol, or an amine. The product has to be isolated by means of dialysis against distilled water, followed by lyophilisation. The bottle neck of the procedure is represented by the inherent slowness of the dialysis procedure, and, more importantly, also by the difficulty in getting rid of the byproducts of the coupling agent used. However, the method, when the proper conditions are developed, has some effectiveness, permits the preparation of amides besides esters, and opens many important possibilities, especially for biomedical and pharmaceutical applications, where γ-PGA is conjugated to species such as amino acids and peptides.

Novel methodologies in course of development

We have also tried other methodologies, in particular the formation of an active ester *in situ*, such as a pentafluorophenyl- or p-nitro-phenyl ester, as well as the activation of the carboxylic group in the form of the corresponding succinimide. So far these approaches have given mixed results, mainly because of the difficulty in product isolation. In fact, the precipitation of the material of interest from the reaction solvent may be cumbersome, and require days and sometimes even weeks, although efforts have been put in screening the best precipitation procedures and conditions. Still, these methodologies are currently in course of development. Our exploration in this field is far from being over.

Γ-GLUTAMYL TRANSPEPTIDASE (γ-GGT) of *B. subtilis*

Γ-glutamyl transpeptidase (GGT, E.C. 2.3.2.2) is a widely distributed, heterodimeric enzyme, found in organisms from bacteria to plants and mammals (Tate, 1981).

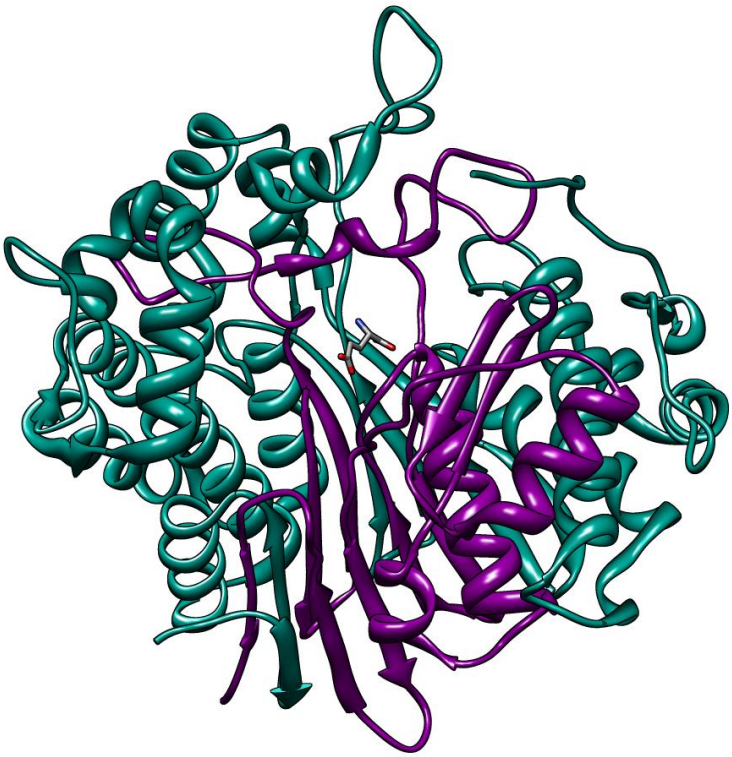


Figure 36: B. subtilis γ-GGT secondary structure (PDB id =3A75)

It cleaves glutathione, a "γ-glutamyl donor", releasing the cysteinylglycine portion and forming a covalently bound γ-glutamyl-enzyme intermediate through an ester linkage between the γ-carbon atom of the glutamyl group and the oxygen atom of the catalytically active, conserved *N*-terminal threonine residue of the small enzyme subunit (Inoue, 2000).

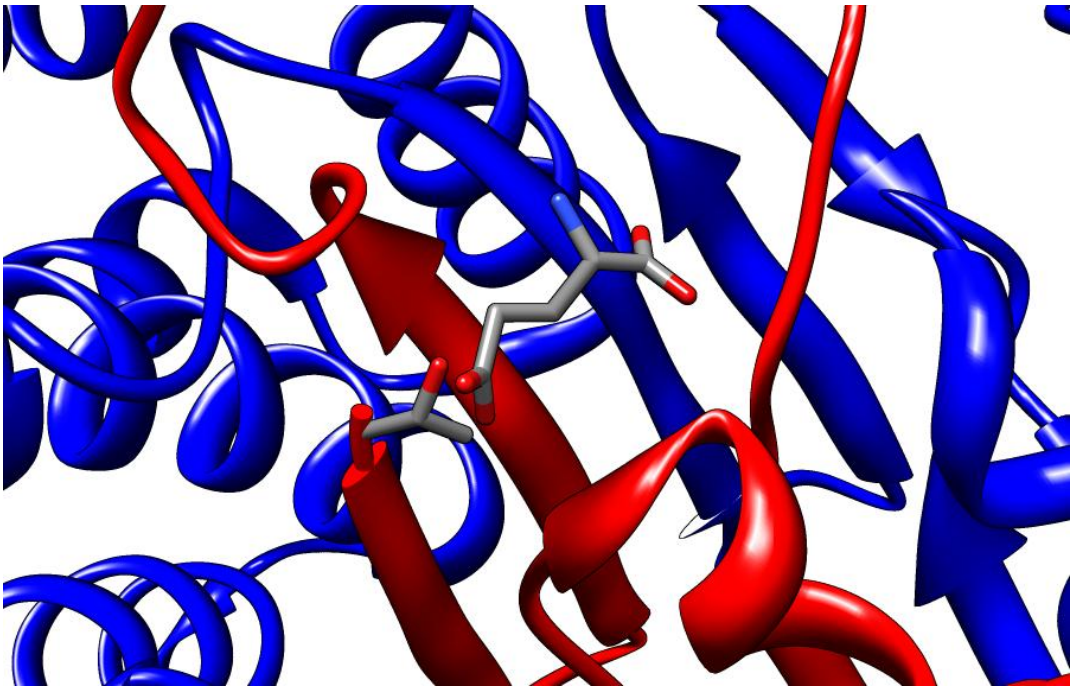
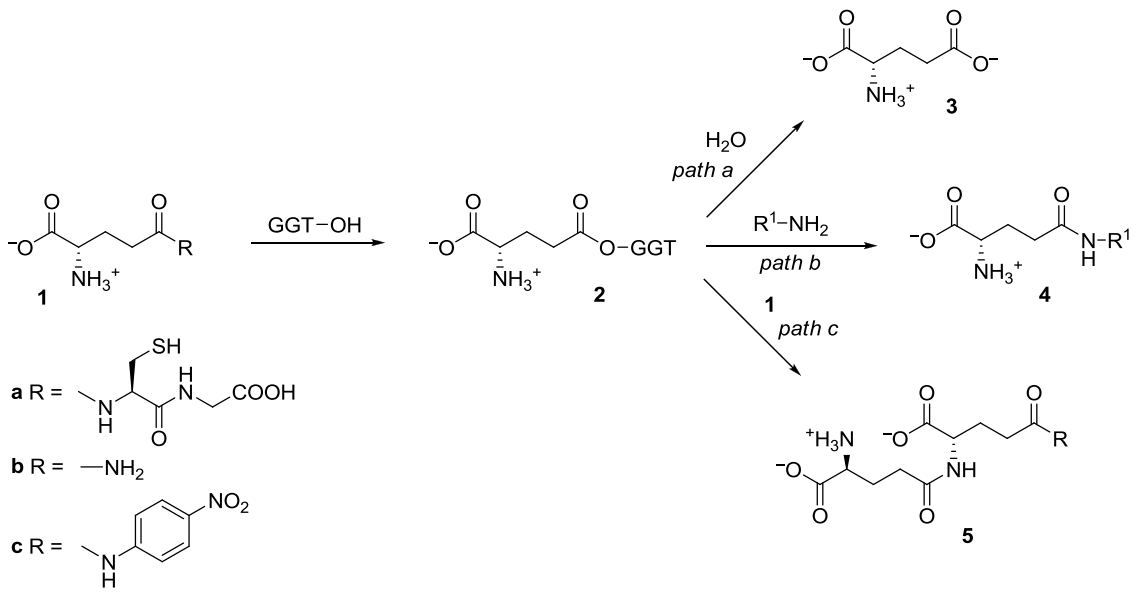


Figure 37: detail of *B.subtilis* γ-GGT active site (pdb id: 3A75); the catalytically active *N*-terminal threonine residue and a ligand (glutamic acid) are in evidence.

The γ-glutamyl moiety can then be transferred to an acceptor nucleophile in a second step. If the acceptor is a water molecule, the hydrolysis of the γ-glutamyl-enzyme adduct occurs with the release of free glutamic acid (Thomson, 1976; Allison, 1985; Taniguchi, 1998). On the other hand, if the acceptor is the amino group of a second species, a transpeptidation reaction ensues, affording the γ -glutamyl derivative of the acceptor (Tate 1974; Thompson, 1977; Meister, 1981). A third reaction, namely an autotranspeptidation, in which the same species behaves both as the donor and as the acceptor, could also take place in vitro in the presence of a high concentration of the donor substrate (Allison, 1985) (Scheme 11).



Scheme 11: a prospect of the γ-GGT possible reactive paths, hydrolysis (a), transpeptidation (b) and autotranspeptidation (c). Source: Speranza, Morelli, 2012.

GGTs belong to the *N*-terminal nucleophile (Ntn) hydrolase superfamily, a group of different enzymes able to cleave an amide bond. All the enzymes in this superfamily are auto catalytically activated through a proteolytic cleavage, which liberates an *N*-terminal nucleophilic, catalytically active residue (Oinen, 2000).

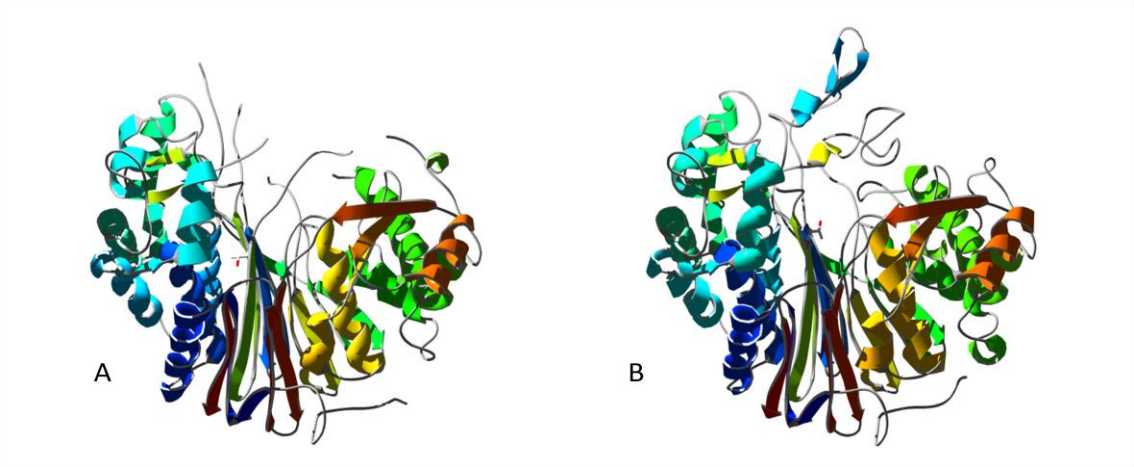


Figure 38: E.coli γ-GGT before (A) and after (B) autocatalytic activation.

Mammalian GGTs are membrane-bound glycoproteins, found in tissues and organs where transport phenomena are especially significant, e.g. renal proximal tubules,

jejunum, bile duct, epididymis, choroid plexus, ciliary body, liver, pancreas, bronchioles. The enzymatic activity found in these tissues can vary over a broad range, depending also on species. For example, almost no detectable enzymatic activity is found in the blood serum of rats and mice, whereas the enzyme is detectable in human serum, where its level is of clinical importance in the detection of liver diseases (Rosalki, 1975; Goldberg, 1980).

In mammals, GGT is mainly involved in glutathione metabolism and cysteine salvage (Tate, 1981; Taniguchi, 1998). In humans, GGT is also implicated in a number of pathological processes, such as tumor progression (Hanigam, 1995) and neurodegenerative deseases (Owen, 1996). Some mechanisms of drug resistance seem to be also dependent on GGT activity (Godwin, 1992).

The observation that mammalian GGTs catalyze the transpeptidation reaction ca 180 fold faster than the hydrolysis reaction (Ikeda, 1995a, 1995b), together with the before mentioned enzyme localization studies, is in favour of the physiologically significant function of the GGT-catalyzed transpeptidation reaction in vivo through the so-called γ -glutamyl cycle (Griffith, 1978; 1979). The γ-glutamyl cycle pictures the conversion of amino acids into their γ-glutamyl derivatives in a GGT-catalyzed reaction using glutathione as the γ-glutamyl donor. The γ-glutamylamino acids are then translocated inside the cells, where the cyclotransferase enzyme catalyzes the intramolecular reaction of the γ-glutamyl derivatives releasing the free amino acids and affording pyroglutamic acid.

The occurrence of the γ -glutamyl cycle is however under debate, as the concentration of some amino acids in vivo is well-below their K_M values with respect to GGT (see for example Elce, 1976).

The role of GGT in microorganisms is often even less clear, as microbial GGTs can differ considerably in substrate specificities with respect to the mammalian counterpart; also the enzyme localization is different for the various microorganisms.

From the periplasmic fraction of E. coli K12, a GGT (EcoGGT) was isolated which accepts glutamine in addition to glutathione as a γ -glutamyl donor (Suzuki 1986). The stereochemical specificity towards the acceptor amino acids of EcoGGT is reminiscent of that of mammalian GGT, both accepting L- but not D-amino acids. However, differently from

Human GGT, EcoGGT has a preference for basic and aromatic acceptor amino acids. The catalytically active residue of EcoGGT was identified in the N-terminal threonine residue of the small subunit (Inoue 2000). The crystal structure of EcoGGT was also solved (Sakai, 1993; Okada 2006) and, with the aid of structural information, light was shed on the autocatalytic process leading to the mature enzyme (Suzuki 2002) and on some insight of the reaction mechanism (Wada, 2008). Beside the N-terminal, other residues important for enzyme maturation and catalytic function were identified through site-directed mutagenesis studies (Hashimoto 1995; Ong, 2008; Hsu, 2009).

EcoGGT found use also as a biocatalyst for the synthesis of γ-glutamyl derivatives at a preparative level (see for example Suzuki, 2007 and references therein). Glutamine represented the γ-glutamyl donor but, due to its high propensity to act also as acceptor, important amounts of γ-glutamylglutamine byproduct were obtained in these reactions.

In Helicobacter pylori, GGT (HpyGGT) is an apoptosis-inducing protein (Shibayama, 2003) and it is essential for the establishment of the infection, thus being considered as a virulence factor (Chevalier, 1999). HpyGGT was selected as a model enzyme for the study of the autocatalytic cleavage of the inactive precursor (Boanca, 2006). Differently

from human GGT and EcoGGT, Hpy GGT shows only a limited transpeptidase activity. Structural studies based on x-ray crystallographic data demonstrated the formation of a threonine-threonine catalytic dyad (Boanca, 2007) the involvement of the mobile C-terminus of the protein in catalysis (Williams, 2009) and the presence of a lid loop covering the glutamate-binding pocket of the mature enzyme during catalysis. In particular, a tyrosine residue (Tyr433) positioned in the middle of the lid loop shields the enzyme-bound glutamyl residue, allowing only the γ-carboxy group to be solvent-exposed (Morrow, 2007).

GGT from *B. subtilis* is an extracellular protein. The gene encoding GGT in *B. subtilis* was identified in 1996. It was expressed and the amino acid sequence was established (Xu, 1996). *B. subtilis* GGT is a 587 amino acids protein, carrying a 28 residues *N*-terminal portion signaling the extracellular localization. It shares 41% sequence homology with *E. coli* GGT. The physiological significance of the enzyme remained however unclear at that time.

The glutaminase activity and the salt tolerance of *B. subtilis* GGT were acknowledged (Minami, 2003) and a technological use of this enzyme was suggested in the manufacture of soy sauce, a traditional Japanese seasoning, the taste of which depends mainly on its glutamic acid content (Kijima, 2007). The enzymatic activity of the enzyme was investigated. pH, temperature optima and substrate specificities were defined for both hydrolase and transpeptidase activity. As the enzyme showed transpeptidase activity towards some acceptor amino acids, Authors proposed its use for the enzymatic synthesis of γ-glutamyl compounds, although this possibility was not experimentally demonstrated. Among the tested amino acid acceptors, glutamine and glutamic acid showed the lowest activities and no experiments were carried out using D-amino acids (Minami, 2003).

The GGT isolated from *Bacillus subtilis* 168 (BsubGGT) has some unique structural features. Compared to the GGTs of other species, it possesses an extra sequence at the *C*-terminus of the heavy subunit and it lacks the lid loop.

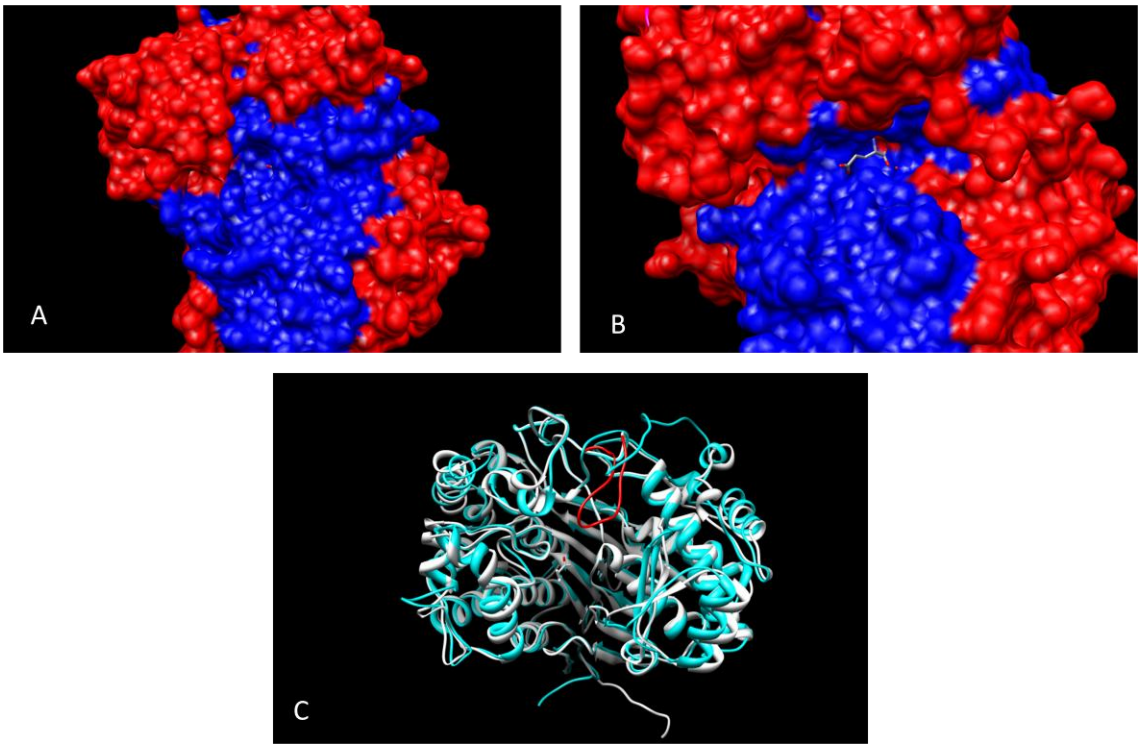


Figure 39: representation of *E.coli* γ-GGT (pdb id: 2DBX) (A) in comparison with *B.subtilis* one (pdb id: 3A75) (B); the superimposition of the two structures (C) clearly displays the difference represented by the absence, in the latter, of the lid loop (red).

Differently from other GGT structures, the bound glutamic acid in the active site of *B. subtilis* GGT is therefore fully solvent exposed (Wada, 2010). In this respect, *B. subtilis* is similar to the enzyme CapD (Wu, 2009), a recognized virulence factor from *Bacillus anthracis* (Scorpio, 2007) involved in anchoring and remodeling the poly-γ-D-glutamic acid capsule surrounding the bacterium (Candela, 2005; Richter, 2009). There is however little sequence homology between BsubGGT and CapD, as BsubGGT resembles much more other microbial GGTs. In particular, the catalytically active threonine residue at the *N*-terminus of the small subunit is highly conserved among all known GGTs, as are conserved the residues that bind the γ-glutamyl moiety through

hydrogen bonds and electrostatic interactions involving the α-amino and the γ-carboxyl groups. Differences in the active site architecture of *B. subtilis* GGT and *B. anthracis* CapD were ascribed to the different size of their natural substrates. A large PGA polymer was indeed recognized as the substrate of CapD, whereas the discrete, low-molecular weight glutathione was assumed to be the substrate of *B. subtilis* GGT (Wada, 2010).

However, also *B. licheniformis* and some strains of *B. subtilis* (Thorme, 1954) release a polymer of glutamic acid units linked through γ -glutamylic bonds extracellularly. As a consequence, the involvement of GGT in the biosynthesis of poly-γ-glutamic acid was in a first time proposed for *B. subtilis* (natto) (Ogawa, 1991). Evidences for a membrane-bound PGA-synthetase complex seem however to be more convincing, although the biosynthesis of PGA may vary in different microorganisms (Kunioka, 1997). It was therefore proposed that in *B. subtilis* (natto), GGT is more likely involved in PGA capsule hydrolysis for energy supply under carbon-limiting conditions, rather than in its biosynthesis (Ogawa, 1997). This view was supported by some other investigations carried out on *B. subtilis* GGT. It was indeed found that *B. subtilis* GGT is able to hydrolyse PGA generating D- and L- glutamic acid in amounts corresponding to their proportion in the PGA substrate. Using γ-glutamyltripeptides labelled with γ-D-glutamic acid or α-L-glutamic acid at either the amino or the carboxy terminal, it was demonstrated that the hydrolysis proceeds starting from the amino terminal, and the enzyme shows no preference for L-glutamic acid over the D-stereoisomer (Kimura, 2004).

Under nitrogen-limited supply, *B. subtilis* mutants lacking GGT sporulate more frequently than the wild type microorganism. Γ-PGA thus represents a source of nutrients, in that it can be degraded and the constituent glutamic acid residues are

internalized by cells and used as nitrogen source. PGA degradation requires the intervention of at least two enzymes. An endo-type PGA hydrolase generates PGA fragments of approximately 10^5 Da. These fragments become in turn substrates of GGT, which liberates one glutamic acid residue at a time starting from the amino terminal. The action of the endo-type hydrolase is a required condition for the intervention of GGT, in that it raises the molar concentration of the *N*-terminal ends of γ -PGA chains up to the KM value of GGT. This observation, together with the high activity of *B. subtilis* GGT towards γ -PGA, supports the hypotheses that γ -PGA is the natural substrate of *B. subtilis* GGT and that γ -PGA hydrolysis is its physiological role (Kimura, 2004).

Besides *B. subtilis* (natto), other GGTs have been isolated and characterized from various *B. subtilis* strains. From *B. subtilis* TAM-4 two isozymes of GGT were isolated with similar enzymatic properties. They showed glutaminase activity towards D- and L-glutamine. The transpeptidase activity was tested using L-amino acids and some glycinyl dipeptides as acceptors (Abe, 1997).

The GGT from *B. subtilis* NX-2, isolated from soil in China, revealed a broad substrate stereospecificity, showing the same activities towards L-amino acids and their racemic forms (Wu, 2006). This enzyme was used for the enzymatic synthesis of γ-D-glutamyl-L-tryptophan (Wang, 2008), a promising immune regulation compound in the treatment of tuberculosis (Orellana, 2002).

A recently discovered GGT from *B. subtilis* SK11.400 showed a maximum transpeptidase activity at pH 10 and maintained its activity also towards the few D-amino acids tested as acceptors (Shuai, 2011). The main goal of the studies about the GGTs from *B. subtilis* strains was the characterization of the enzyme properties and their possible role in the homeostasis of γ-PGA capsule of the bacterium was never

mentioned. Among the GGTs mentioned, the amino acid sequence is given only for GGT from *B. subtilis* SK11.400, and comparison with the sequences of other bacterial GGTs showed that it lacks the lid loop.

Enzymatic activity of *B. subtilis* GGT

The *Bacillus subtilis* 168 GGT gene, excluding the signal sequence, was PCR amplified from genomic DNA and cloned in a pET vector in front of a His-tag. The construct was transformed in BL21 (DE3) *E. coli* cells and gene expression was induced. The GGT protein is retained in the cell because it lacks the signal sequence, and undergoes autocatalytic cleavage producing the active large (L, 40 kDa) and small (S, 20 kDa) subunits. The complex was purified with Ni-conjugated agarose beads. Different amount of recombinant proteins (as indicated in the figure) were loaded on a 12% SDS-PAGE gel (fig. 40), together with an aliquot of the flow-through and remaining beads. The molecular weight markers (NEB, broad range) are loaded on the left.

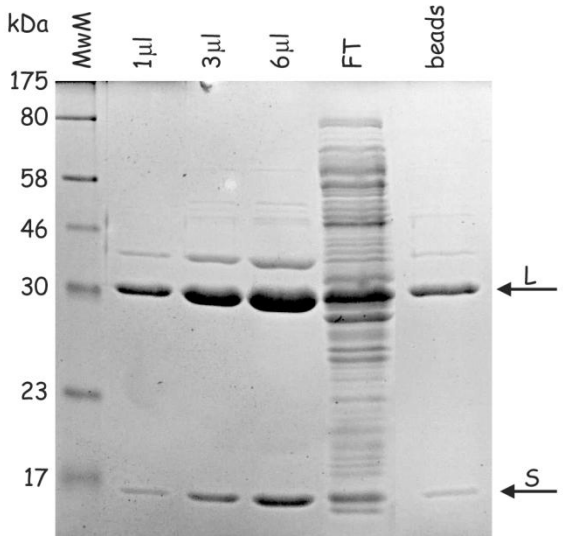


Figure 40: SDS-PAGE illustrating *Bacillus subtilis* 168 GGT mutant protein expression

Enzymatic activity of *B. subtilis* GGT was measured by standard methods using the

chromogenic substrate γ-glutamyl-p-nitroanilide. The release of p-nitroaniline in the GGT-catalyzed reaction can be determined spectrophotometrically at 410 nm. A calibration curve was constructed by determining the absorbances of buffered solutions of p-nitroaniline of increasing concentrations. This calibration curve was then used for the calculation of the concentration of the same compound released in a GGT-catalyzed hydrolysis of γ-glutamyl-p-nitroanilide carried out in standardized conditions. A unit (U) of enzyme is defined as the amount of enzyme which liberates 1 μmol of p-nitroaniline within 1 minute at 25 °C and pH 8,5. Because the enzyme shows two kind of activities, i.e. hydrolase and transpeptidase, two values can be determined, one in the presence of the standard acceptor compound glycylclycine (transpeptidase activity), and one in its absence (hydrolase activity). We determined in a first experiment the hydrolase activity, which was 16,4 U/mL of enzyme solution.

Hydrolase/transpeptidase ratio of B. subtilis GGT

Mammalian GGTs show a more marked transpeptidase activity with respect to the hydrolase activity. Human GGT catalyzes indeed transpeptidation ca 180-fold faster than hydrolysis (Ikeda, 1995). Moreover, the hydrolysis/transpeptidation rate is strongly pH-dependent. More basic pH values favor transpeptidation reaction, while acidic pH values are beneficial for the hydrolysis reaction. For bacterial GGTs the differences between the reaction rates seems to be less distinct. *E.coli* GGT, for example, catalyzes the transpeptidation reaction only two-fold faster than the hydrolysis (Boanca, 2006).

Evidences from the literature suggested that also for *B. subtilis* GGT the difference between the two reaction rates could be small (Minami, 2003). With the aim of measuring this difference more accurately, we compared the reaction rate of the GGT-

catalyzed reactions of γ-glutamyl-p-nitroanilide in the absence and in the presence of an excess of the acceptor compound, glycylglycine. Reaction progress was monitored spectrophotometrically at 410 nm for few minutes, during which time the liberation of p-nitroaniline in the reaction media was linear. From the slopes of the two curves, the ratio between transpeptidation and hydrolysis reactions was determined to be ca 1,3-1,5 at pH 8,5 and 25 °C (Fig. 41).

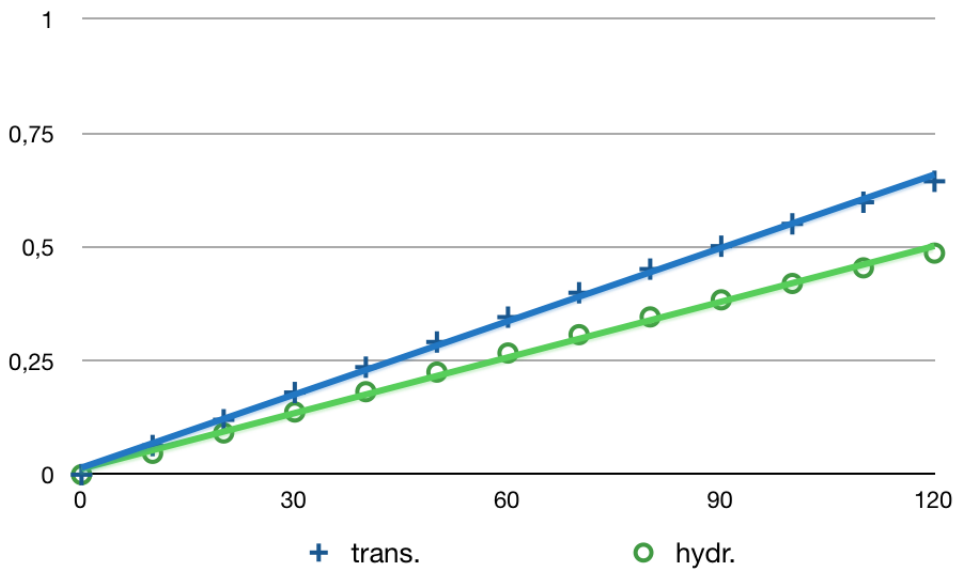


Figure 41: hydrolysis *vs* transpeptidation reaction effected by *B. subtilis* GGT

The experiment was repeated using L-methionine as the acceptor compound. In this case, no appreciable differences between the two reactions rates were noticed.

Hydrolase activity of B. subtilis GGT towards γ-PGA

The ability of *B. subtilis* GGT to degrade γ-PGA by removing one glutamic acid residue at time was also checked. Γ-PGA was dissolved in TRIS buffer at pH 8.5 and, after addition of GGT, the reaction mixture was shaken at 25 °C for 24 hours. Glutamic acid liberation was revealed by TLC analysis of the reaction mixture using an authentic sample for comparison. Attempt to use L-methionine in a transpeptidation reaction as

the acceptor compound for the γ-glutamyl moieties liberated from γ-PGA by the enzyme, gave however no results. In these conditions, the only detectable product in the reaction mixtures was free glutamic acid.

pH-dependence of Glutaminase activity of B. subtilis GGT

The glutaminase activity of *B. subtilis* GGT was early recognized (Minami, 2003), and the pH dependence of the enzyme-catalyzed glutamic acid liberation was already studied. These studies, however, focused on the amount of glutamic acid liberated, rather than on the reaction course. Therefore, we monitored the time course of the reaction by HPLC. Calibration curves were constructed for glutamine and glutamic acid using increasing concentrations ranging from 25 mMol to 100 mM and they were analyzed by HPLC after derivatisation with dabsyl chloride (Proveen, 1983). Three reactions were then carried out on 100 mMol solutions of glutamine at pH 7,4, 8,2 and 9,8. At fixed time intervals (1, 2, 3, 5, 7 and 24 hours) 100 µL aliquots were withdrawn from each reaction mixture, derivatised with dabsyl chloride and analyzed by HPLC for glutamine and glutamic acid contents.

At pH 7,4 a decrease of glutamine concentration in the reaction mixture was observed with time, which paralleled the increase of glutamic acid content (Fig. 42).

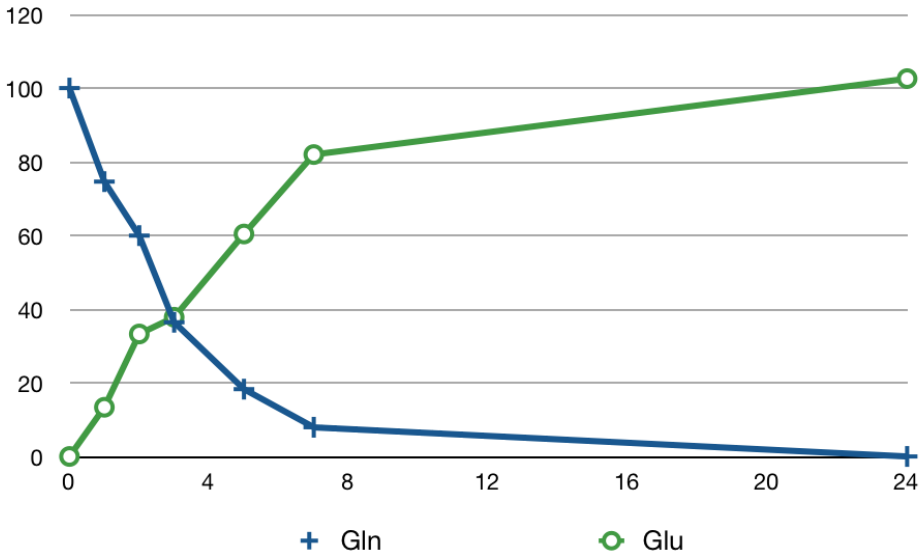


Figure 42: pH-dependence of Glutaminase activity of B. subtilis GGT- at pH 7,4, only hydrolysis occurs

Raising the pH to 8,2 a new peak in the chromatogram appeared transiently between 1 and 5 hours reaction time, which was attributed to γ-glutamylglutamine. HPLC-ESI MS experiments confirmed our hypothesis of an autotranspeptidation reaction, attributing to the new peak in the chromatogram a molecular mass of 562, corresponding to that of the dabsyl derivative of γ-glutamylglutamine. Further evidence came from the use of an authentic sample of γ-glutamylglutamine, on purpose synthesized and used for the construction of a calibration curve aimed at its quantitative estimation in the reaction mixture.

The most impressive result was however obtained at pH 9,8, in that glutamine disappeared very quickly within two hours, and a series of little peaks appeared in the chromatograms. The amounts of glutamic acid and γ-glutamylglutamine formed in the same time were insufficient to explain a so rapid disappearance of glutamine (fig 43).

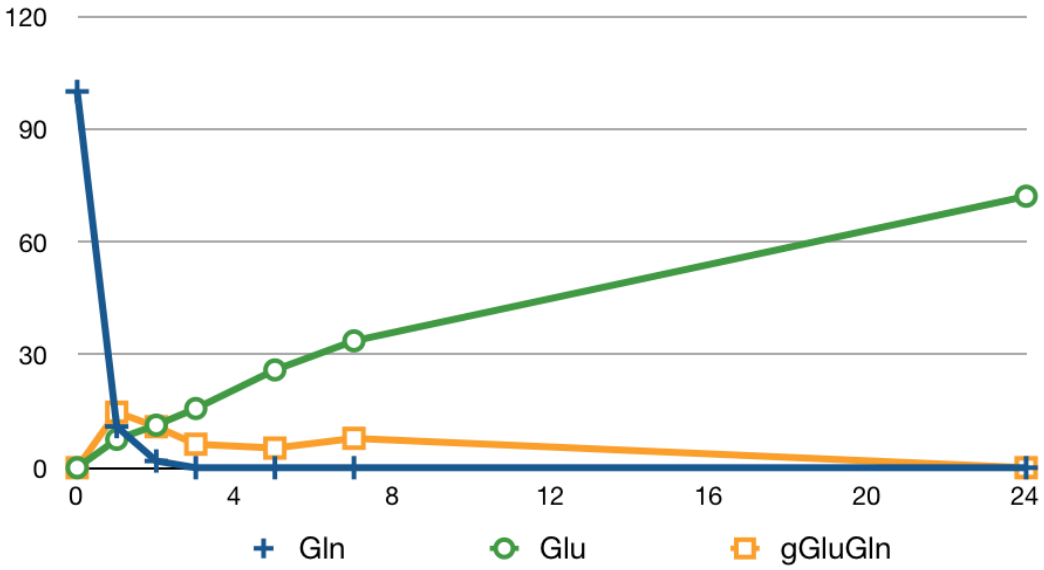
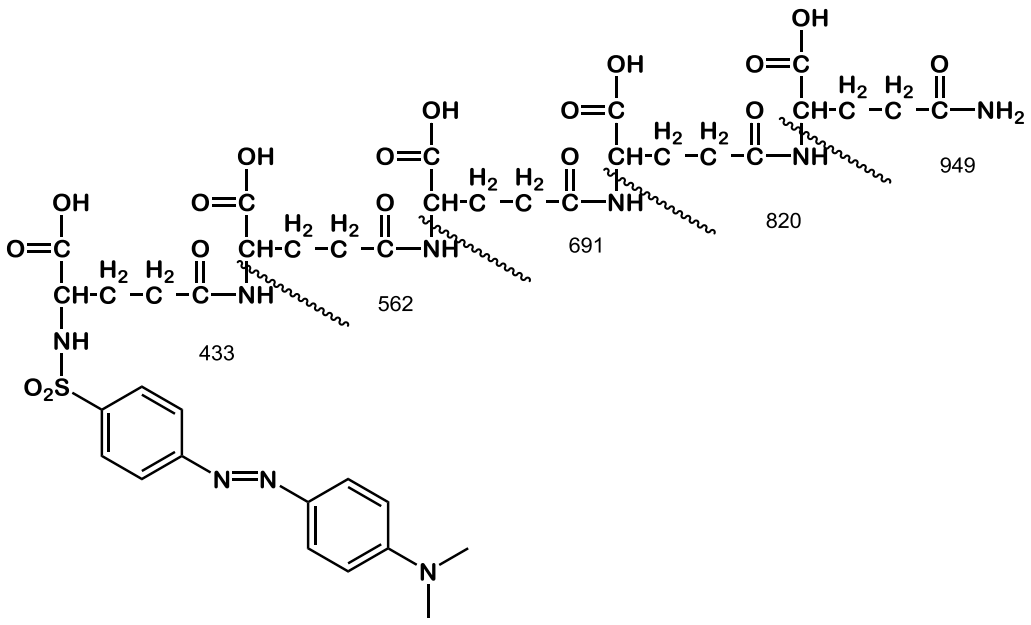


Figure 43: pH-dependence of Glutaminase activity of B. subtilis GGT- raising pH to 9,8, transpeptidation becomes significant

HPLC-ESI MS experiments showed that oligomeric species, containing up to four γ-glutamyl residues linked to a single glutamine molecule, were formed (Scheme 12).



Scheme 12: oligomeric species formed by transpeptidation

We were unfortunately unable to quantify these species, but the result is similar to that hypnotized using *B. subtilis* (*natto*) (Suzuki, 2002b).

The same experiment was repeated using D-glutamine, with identical outcomes.

The results obtained in these experiments are in our opinion noteworthy for at least three reasons. Firstly, while on one hand they confirmed the glutaminase activity of *B. subtilis* GGT, they showed also the ability of the enzyme to accept glutamine as a substrate in a transpeptidation reaction. It is in apparent contrast with results previously reported, in which the transpeptidase activity towards glutamine was referred to be very low (Suzuki, 2002b). Those results were however obtained at a lower pH value, below the pK_a of the glutamine amino group. It was indeed proposed that an alkaline pH is beneficial for the GGT-catalyzed transpeptidation reaction in that it increases the concentration of unprotonated nucleophilic amino groups (Proveen, 1983). At more acidic pH, the amino group of the acceptor molecule is protonated to a more extent, so the hydrolysis reaction is favored. In our case, the autotranspeptidation reaction was mainly observed at pH 9,8, slightly above 9,1, the pK_a of the amino group of glutamine.

Secondly, the enzyme can accept as substrates not only discrete molecules, but also oligomeric poly-γ-glutamyl derivatives, as showed by the HPLC-ESI MS experiments. This is a peculiar characteristic, not shared with mammalian or other bacterial GGTs, such as for example E. coli GGT. When *E. coli* GGT was used for synthetic purposes in the presence of a high concentration of glutamine as donor at elevated pH, only the formation of γ-glutamylglutamine was reported, but not the formation of oligomeric compounds (Suzuki, 2002b). A similar behaviour was noted for rat kidney GGT (Proeen, 1983). The ability of processing also oligomeric γ-glutamyl derivatives is ascribable to the possible role of *B. subtilis* GGT in the homeostasis of γ-PGA, which constitutes the cellular envelope of some strains of *B. subtilis* (Tate, 1974; Suzuki, 1986).

Thirdly, the ability of *B. subtilis* GGT to accept D-glutamine as an acceptor substrate

marks another difference from mammalian and *E. coli* GGT. The latter can indeed accept γ-glutamyl compounds with D-configuration only as donors, but amino acid acceptors have to be in the L-configuration (Tate, 1974; Proveen, 1983; Sukuzi, 2002b). The similar results obtained subjecting L- and D-glutamine to the action of *B. subtilis* GGT can be once again related to the involvement of the enzyme in the homeostasis of extracellular γ-PGA, in that this polymeric material is usually formed by L- and D-glutamic acid units in different proportion, depending on *Bacillus* strain and environmental conditions (Suzuki, 1986).

EXPERIMENTAL PART

Materials and Methods

Glutamic acid, N-Fmoc-OtBut-glutamic acid, 2-chloro-trytil-chloride resin and all other reagents 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.

Analytical TLC was performed on silica gel F254 precoated aluminum sheets (0.2 mm layer; Merck, Darmstadt, Germany); components were detected under an UV lamp (λ 254 nm) and by spraying with a ninhydrin 5% (w/v) in ethanol solution, followed by heating to ca. 150 °C.

^{1}H and ^{13}C NMR spectra were recorded at 400,13 and 100,61 MHz, respectively, on a Bruker AVANCE 400 spectrometer equipped with a XWIN-NMR software package (Bruker, Karlsruhe, Germany) at 300 K. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million and were referenced to the solvent signals. ^{13}C NMR signal multiplicities were based on APT (attached proton test) spectra.

Matrix assisted laser desorption/ionization spectra (MALDI TOF) were acquired on a Bruker Microflex LT Spectrometer.

UV measurements were carried out with a Jasco UV-vis/NIR V 630 instrument.

For HPLC runs, the instruments used was a HPLC Merck Hitachi: D-7000 interface, UV detector L-7400, column oven L-7350, L-7200 programmable autosampler, L-7150 pump.

For solid phase synthesis a Milligen 9050 Peptide synthesizer and a Biotage Initiator+ were used

SpectraPor3 membranes, MWCO 3500 Da, were used for dialysis.

Ultrafiltration was performed by means of Amicon device (250 and 10 ml), equipped with Millipore filtration membranes (regenerated cellulose, diameter 76-25 mm; NMWL 30 000 Da).

Abbreviations used

- Fmoc: fluorenylmethyloxycarbonyl
- Boc: tert-butoxycarbonyl
- BCC: benzylchlorocarbonate
- Cbz: carboxybenzyl
- DMF: dimethylformamide
- HOBt: hydroxybenzotriazole
- HBTU: O-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate
- DIPEA: N,N-diisopropylamine
- DCHA: dicyclohexylamine
- EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
- TEA: triethylamine
- TFFH: N,N,N',N'-tetramethyl-fluoro-formamidinium-exafluorophosphate
- $TMSCH_2N_2$: trimethylsilyldiazomethane

Bioproduction

A typical procedure is reported.

Cells growth and sporulation

B. subtilis PB5249 and PB5509 strains, grown overnight in 20 mL of LB medium, were diluted to optical density 0.2 at 600nm (OD_{600}) in rich Difco Sporulation Medium (DSM) and in Spizizen's Minimal Salt Medium (SMS) (Harwood and Cutting, 1990) and incubated at 37 °C in an orbital shaker. After 90 min, aliquots were withdrawn every 30 min for OD_{600} readings. After 24 h-incubation, cells were taken from cultures grown in DSM medium and serially diluted. An equal volume was plated on LB plates in duplicate, before and after incubation at 80 °C for 10 min (to kill vegetative cells). After incubation for 16 h at 37 °C, CFUs (colony forming units) were counted and sporulation efficiency was calculated as the ratio between heat resistant and total CFUs. Experiments were independently repeated at least three times.

γ-PGA culture

Cells were grown overnight in LB medium at 37 °C in a shaking incubator; cultures were diluted to OD_{600} 0.2 in E medium adapted from Leonard et al. (1958) (L-glutamic acid, 40.0 g/L; citric acid, 12.0 g/L; glucose, 80.0 g/L; NH_4Cl, 7.0 g/L; K_2HPO_4, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L, $FeCl_3 \cdot 6H_2O$, 0.04 g/L; $CaCl_2 \cdot 2H_2O$, 0.15 g/L; $MnSO_4 \cdot H_2O$, 0.104 g/L, pH 6.5) and incubated at 37 °C with shaking. Aliquots (500 µL in duplicate) were collected every 12 hours for OD_{600} readings and for γ-PGA extraction. After OD_{600} readings, each sample was centrifuged at 16000 rcf for 10 min at 4°C.

γ-PGA Separation on SDS-PAGE

To 10 μL γ-PGA samples, 2.5 μL loading buffer (Bromophenol Blue 5 mg/mL, Glycerol 50%, running buffer 50%) was added before separation on 8% SDS-PAGE. The gel was stained with 0.5% methylene blue in 3% acetic acid for 30 min and destained in H_2O.

Isolation

A typical procedure is given thereafter.

γ-PGA was precipitated from culture surnatant by addition of MeOH (3 volumes) at -20 °C overnight; the resulting crude product was suspended in milliQ water, and then dialyzed (SpectraPor3 membranes, MWCO 3500 Da) against milliQ water for 12 hours; the resulting solution was acidified to pH 2 by addition of concentrated HCl, reprecipitated with 1-Propanol (3 volumes) and rinsed with acetone/diethyl ether.

^{1}H NMR (δ ppm D_2O): 1.35 [b, 1H, $C^{\beta}H_2$], 1.50 [b, 1H, $C^{\beta}H_2$], 1.78 [m, 2H, $C^{\gamma}H_2$], 3,71 [b, 1H, CH], 8.00 [d, 1H, -CONH].

Stereochemical L/D composition determination

Derivatisation method

A 1% solution of FDAA in acetone (200 μL, 7.2 μL) and 40 μL of an aqueous 1% NaHCO3 solution were added to a 50 mM solution of D,L-glutamic acid (100 μL, 5 μmol) in a 1mL brown vial. The vial was closed with a screw cap, covered with aluminium sheet to protect it from light and put in an oven at 40 °C for 1 hour and 15 minutes, under magnetic stirring. After cooling at room temperature, a 2M HCl solution (20 μL, 40 μmol) was added. The solution was subsequently stirred for some minutes and then dried with a nitrogen flow in roughly 45 minutes.

The vial content was dissolved in 1mL of mobile phase A (see HPLC protocol) and diluted in 1:5 proportion with mobile phase B to get a final concentration around 1 mM which was injected in HPLC.

HPLC protocol

-column: RP Select B C_{18} (Merck) 5 μm (250×4.6 mm)

-wavelenght: 340 nm

-flow: 1.2 mL/min

-temperature: 27 °C

-eluent:

-mobile phase A=acetonitrile/water (10:90 v/v) and sodium acetate 0.04 M, final pH 5.3;

- mobile phase B=acetonitrile/water (50:50 v/v) and sodium acetate 0.04 M, final pH 5.3.

-gradient elution:

- 0-20% B from 0 to 15.0 min;

- 20-75% B from 15.1 to 30.0 min;

- 20-75% B from min 30.1 to the end of the analysis.

It was verified that the analyte, if protected by light, maintains its stability for up to 5 days. The order of elution of peaks was in accordance with that reported in literature (B'Hymer, 2001).

Commercial sample analysis

γ-PGA hydrolysis

A 0,3 molar solution of γ-PGA (sodium salt, molarity calculated on the monomer MW (169,12, g mol^{-1}51,5 mg weighted) in 1mL of 2N HCl was put, with a magnetic stirrer, in a 1,5 mL vial equipped with a sealed cap. The vial was put in a sand bath at 110 °C and kept under stirring for 3,5 hours. It was afterwards let cool to room temperature; solution was dried with a nitrogen flow (this operation took roughly 2 hours. A white solid was obtained, which was stored at 4 °C.

Derivatisation

Reaction with Marfey's reagent was carried out as previously described: 200 μL of Marfey's reagent mother solution (3,6 mg in 355 μL of acetone) were added to 100 μL of the sample's mother solution (7,9 mg of acid hydrolisate, MW 147.13, in 1,1 mL).

HPLC run

Analysis was carried out the previously described, conditions but for the temperature, which was raised from 27 °C to 35 °C. Dried reaction residue was dissolved with 1 mL of mobile phase B, containing 50% of acetonitrile, differently from the D-L in house prepared mixture, that was easily dissolved in mobile phase A, composed at 90% by acetonitrile. Analysis was repeated twice.

Results are summarized in table 9.

Table 9

Sample 1 (dil. 1:5)	**Rt (min)**	**Area (mV)**	**%**	**% L/D**
	17,95	1878426	14,20 (L)	47,97
	24,90	2037731	15,40 (D)	52,03
	35,21	8802742	66,53 (Marfey)	
			3,86 (other)	
Sample 2 (dil. 1:4)	**Rt (min)**	**Area (mV)**	**%**	**% L/D**
	16,60	2590954	14,68 (L)	48,24
	22,66	2779724	15,75 (D)	51,76
	35,04	12095685	68,54 (Marfey)	
			1,02 (other)	

Non commercial sample analysis

γ-PGA hydrolysis

Procedure was the same used in the previous case. A 0,3 M solution of γ-PGA (sodium salt) was prepared weighting 11,6 mg in 0,230 μL of 2 M HCl. A brown, vitreous solid was obtained and kept at 4 °C.

The product was quite rubbery and was tediously dissolved in 2M HCl, under stirring. Solubility was complete after 10 minutes at 110 °C.

Derivatisation

Reaction was carried out as described above: 100 μL of the mother solution were added to 200 μL of Marfey's reagent mother solution (2,8 mg in 281 μL of acetone). Since it was not possible to take an exactly weighted aliquot of hydrolisate solution, the initial

weight of the polymer (11,6 mg in 1,6 mL) in its acidic form (MW 147,13) was taken into account.

HPLC run

Analysis was carried out in the previously described, conditions but for the temperature, which was raised from 27 °C to 35 °C. Dried reaction residue was dissolved with 1 mL of mobile phase B, containing 50% of acetonitrile, as was done for the commercial sample.

Results are summarized in table 10.

Table 10

Sample 1 (dil. 1:5)	**Rt (min)**	**Area (mV)**	**%**	**% L/D**
	17,04	300053	4,010 (L)	14,33
	22,81	1794414	23,98 (D)	85,67
	35,15	4928788	65,87 (Marfey)	
			6,14 (other)	
Sample 2 (dil. 1:1)	**Rt (min)**	**Area (mV)**	**%**	**% L/D**
	15,99	1257696	4,21 (L)	14,33
	22,11	7518484	25,16 (D)	85,67
	35,03	19979016	66,87 (Marfey)	
			3,76 (other)	

Determination of molecular weight

Chromatographic method

Chromatographic system

A modular Waters SEC System was used: Alliance 2695 (degassing unit, pump, injector) column oven. Differential Refractometer (DRI) as concentration detector. To it a MALS detector was connected in line, in the serial disposition: Alliance-MALS-DRI.

Since DRI detector suffers a slight delay with respect to the MALS one, signals were re-aligned. DRI detector delay with respect to the MALS one was measured to be 0,270 mL.

Multi-Angle Laser Light Scattering (MALS)

A MALS photometer Dawn DSP-F (Wyatt) with K5 flow cell was used. The device employs a He-Ne laser, λ=632,8 nm, with vertically polarized light. It measures the intensity of scattered light in 18 angular positions at the same time.

0,1 M phosphate buffer (plus 0,1 M NaCl) at pH 7,0 at 35 °C was used as a solvent.

With the K5 cell used 16 angular positions (with values varying from 14,5° to 158,3°) were available in this aqueous solvent.

MALS photometer was calibrated with Toluene, assuming a Rayleigh Factor $Rq=1{,}406\cdot10^{-5}$ cm^{-1}.

Photodiodes were angularly normalized using a globular protein BSA (M=66.4 kg/mole, Rg=2.9 nm) assumed to isotropic.

dn/dc

The value of the dn/dc ratio (refraction index excess value of the macromolecule with respect to the solvent) was obtained from the literature (Wyatt, 1993). For γ-PGA in the mobile phase used for SEC at 25 °C it is equal to 0,140 mL/g.

Analysis conditions

Γ-PGA samples

–Columns: 2 Aquagel OH60 Polymer Laboratories (for sample PGA 12); 2 Ultrahydrogel (2000-500 Å) Waters (for Acid e Na Salt samples)

–Mobile phase: 0.1M NaCl + 0.1M Phosphate Buffer pH 7,0

– Temperature: 35 °C

– Flow: 0.8 mL/min

– Injection Volume: 150 μL

– Concentration: 1 or 5 mg/mL

Γ-PGA t-butyl ester

–Columns: 1 Mesopore, 1 Oligopore Polymer Laboratories

–Mobile phase: THF, stabilized with 0.025% BHT

–Temperature: 35 °C

–Flow: 0.8 mL/min

–Injection volume: 150 μL

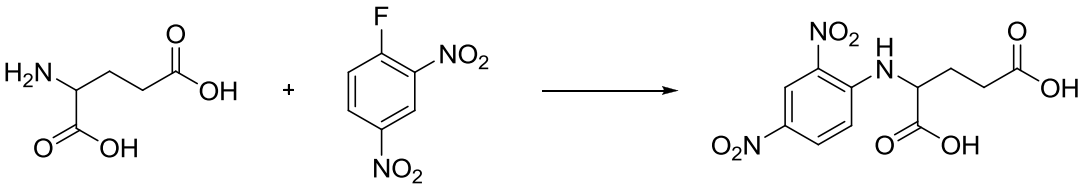
–Concentration: 3 mg/mL

All samples were dissolved in the mobile phase chosen for SEC at the reported concentration. Before injection all solutions were passed through 0,20 μm filters.

γ-PGA molecular weight assay by derivatisation with Sanger's reagent and hydrolysis

The following procedures were adapted from Park (Park, 2005) and Paraskevas (Paraskevas, 2002).

Preparation of DNP-Glu

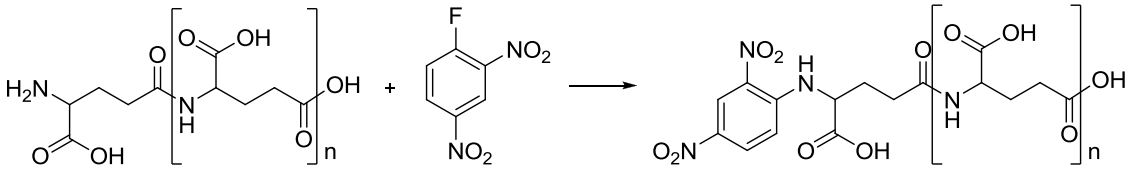


Glutamic acid (3,398 mmol, 0,500 g) was dissolved in an aqueous $NaHCO_3$ solution (13,60 mmol, 1,142 g in 20 ml). The solution was heated to 40 °C and then 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) was added (3,504 mmol 0,44 ml) under stirring.

The resulting solution was protected from light with an aluminium foil. After 18 hours, TLC control ($PrOH/H_2O$, 7/3) showed the complete consumption of the product. The solution was then washed with diethyl ether (three times with 10 ml aliquots), and acidified with concentrated HCl up to pH 2. The raw product was obtained as a yellow oil (1,042 g, 98%) after extraction with AcOEt (three times with 15 ml aliquots) and concentration to dryness at the rotary evaporator. After dissolution in 5 ml of CH_2Cl_2, it was converted in the corresponding dicyclohexylammonium salt by addition of a 10% excess of dicyclohexylammine (3,660 mmol, 0,73 ml). The resulting yellow precipitate was filtered and dried in desiccators (2,994 mmol, 1,481 g).

^{1}H NMR (δ ppm $CDCl_3$): 2.16 [m, 1H, $C^{\beta}H_2$], 2.24 [m, 1H, $C^{\beta}H_2$], 2.40 [m, 2H, $C^{\gamma}H_2$], 4.37 [m, 1H, CH], 7.07 [d, 1H, NH], 8.2 [dd, *J* = 12Hz, 1H], 9.15 [d, *J* =4 Hz, 1H], 9.52 [d, J = 8 Hz, 1H].

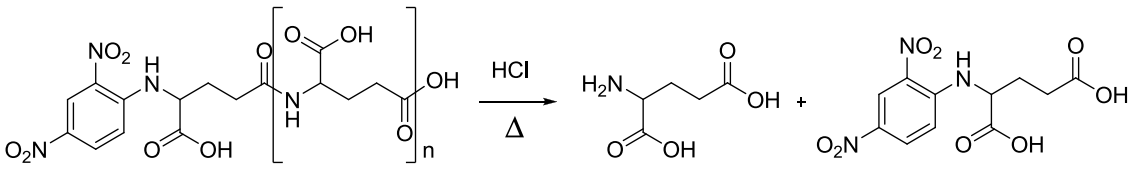
Preparation of DNP-γ-PGA



Γ-PGA (0,505 g) was dissolved in 35 ml of a saturated solution of $NaHCO_3$. The solution was heated to 60 °C, under stirring. As the temperature got stable, 60 µl of 1-fluoro-2, 4-dinitrobenzene (Sanger's reagent) were added. The round bottom flask was protected from light with an aluminium foil and left at 60 °C, under stirring, for 18 hours. The solution was then cooled, and brought to pH 2-3 with some drops of HCl 37%.

Methanol (25 ml) was added to the solution, and after a night at 4 °C, a precipitate (0,31 g) was obtained.

Hydrolysis of DNP-γ-PGA



Γ-PGA (0,128 g) was dissolved in 6 ml of a 6 M HCl solution in a screw cap test tube; the solution was put under stirring, heated to 110 °C and maintained at this temperature for 12 hours. The solution was afterwards put in a round bottom flask and brought to pH 7-8 with saturated $NaHCO_3$ solution (10 ml) and $NaHCO_3$. Finally, the solution was frozen and lyophilized.

UV measurements

For the evaluation of DNP-Glu contained in hydrolyzed samples, a calibration curve was constructed, taking points at 10, 20, 50, 100 μM concentration of DNP-Glu, prepared by dilution of a DNP-Glu mother solution in water (500 μM); measurements were performed at the wavelength of 356 nm.

The resulting calibration curve is expressed by the following equation:

$$y = 0{,}0095x + 0{,}0209, with\ R^2 = 0{,}996$$

Results for the low molecular weight commercial sample of γ-PGA purchased by Natto Bioscence, Co., are listed below.

Every sample was prepared dissolving the entire amount of hydrolyzed product in distilled water, and then diluting the resulting solution until Abs fit in the calibration curve.

All measurements were repeated three times.

Representative results are reported thereafter.

Table 11

Abs (corrected for blank absorption)	Molecular weight (g/mol)
0,248	30508
0,260	29742
0,249	28958

Average molecular weight estimated by this method results to be 29 736 g/mol.

Rheological measurements

All measurements were carried out using a rotational rheometer produced by Haake (Karlsruke, Germany) model Rheostress RS600.

Working conditions are reported for each sample. Strumental parameters are listed as follows.

- ✓ Cone and plate:

  these two parts of the sensor system are identified by their diameter and by the angle occurring between them. For instance, the notation "60/1" indicates a cone and plate system with a diameter of 60 mm, and an angle occurring between the two of 1°.
- ✓ Gap: distance between cone and plate, expressed in micrometers (μm).
- ✓ Waiting time: delay between measurements.
- ✓ Sample quantity deposed on plate.
- ✓ Temperature.

Samples were prepared dissolving 25 mg of γ-PGA in 2,5 ml of milliQ water. All measurements were repeated three times. Results are reported as the average.

Natto Bioscience sample

Working conditions:

- ✓ Cone and plate: 60/1.
- ✓ Gap: 52 μm.
- ✓ Waiting time: 180 s.
- ✓ Sample quantity: 1 ml.
- ✓ Temperature: 25 °C.

Results

Table 12

Average			ds	
τ (Pa)	**η (mPa s)**	**shear rate (1/s)**	**τ (Pa)**	**η (mPa s)**
0,0262	2,619	10,0	0,0015	0,1485
0,1025	2,049	50,0	0,0007	0,0113
0,1545	2,061	75,0	0,0021	0,0361
0,2095	2,094	100	0,0021	0,0247
0,2580	2,062	125	0	0
0,3095	2,063	150	0,0078	0,0509
0,3600	2,058	175	0,0014	0,0099
0,4100	2,048	200	0,0057	0,0283
0,5140	2,057	250	0,0042	0,0156
0,6170	2,057	300	0,0014	0,0071

Biological Tech sample

Working conditions:

- ✓ Cone and plate: 35/1.
- ✓ Gap: 52 μm.
- ✓ Waiting time: 180 s.
- ✓ Sample quantity: 200 μl.
- ✓ Temperature: 25 °C.

Results

Table 13

Average			ds	
τ (Pa)	**η (mPa s)**	**shear rate (1/s)**	**τ (Pa)**	**η (mPa s)**
1,616	161,5	9,999	0,0340	3,383
6,958	139,2	49,99	0,1344	2,676
9,641	128,5	75,00	0,1439	1,950
12,01	120,1	100,0	0,1305	1,305
14,17	113,4	125,0	0,1852	1,474
16,19	108,0	150,0	0,2254	1,531
18,05	103,1	175,0	0,2309	1,626
19,82	99,12	200,0	0,2627	1,323
22,99	91,95	250,0	0,2893	1,164
25,90	86,34	300,0	0,3304	1,104

University of Pavia sample

Working conditions:

- ✓ Cone and plate: 35/1.
- ✓ Gap: 52 μm.
- ✓ Waiting time: 180 s.
- ✓ Sample quantity: 200 μl.
- ✓ Temperature: 25 °C.

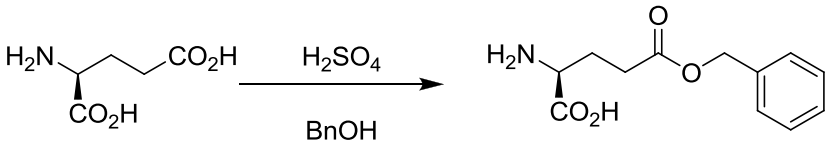
Results

Table 14

Average			ds	
τ (Pa)	**η (mPa s)**	**shear rate (1/s)**	**τ (Pa)**	**η (mPa s)**
1,320	132,0	9,999	0,0332	3,318
5,758	115,2	50,00	0,0943	1,922
8,097	107,9	75,00	0,1507	2,021
10,11	101,1	100,0	0,1723	1,724
11,99	95,95	125,0	0,2346	1,883
13,80	92,01	150,0	0,1833	1,207
15,45	88,28	175,0	0,1258	0,715
16,98	84,89	200,0	0,2138	1,067
19,82	79,27	250,0	0,1947	0,782
22,40	74,66	300,0	0,2146	0,721

Synthetic Procedures

Synthesis of γ-Benzyl Glutamic Acid (1)

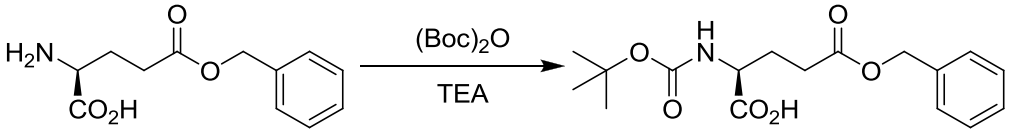


The procedure was adapted from Guttmann (1958). 4 ml of 98% H_2SO_4 and 40 ml (388 mmol) of benzyl alcohol were added to 40 ml of diethylether in a 250 ml flask. The mixture was stirred at room temperature for about 15 minutes and then the ether was removed *in vacuo*. 6 g (40 mmol) of glutamic acid were added in little aliquots to the obtained solution, waiting for it to completely dissolve each new addition.

The resulting solution was stirred at room temperature for about 24 hours and controlled by TLC (PrOH 7: H_2O 3: AcOH 0,5). Then 80 ml of absolute ethanol were added to the solution, under vigorous stirring, followed by 20 ml of Pyridine; the solution was kept at -18 °C for about 24 hours; after this time, it was possible to filter the product as a white powder, which washed with cold ethanol and recrystallised from a water (76 ml) and pyridine (8ml) solution to finally obtain 5,3 g of a translucent solid (56% yield).

^{1}H NMR (δ ppm, 9 D_2O/ 1 DCl): 2.17 [m, 2H, $C^{\beta}H_2$], 2.59 [m, 2H, $C^{\gamma}H_2$], 4.15 [t, 1H, $C^{\alpha}H$], 5.10 [s, 2H, -CH_2-Ph], 7.36 [m, 5H, Ph].

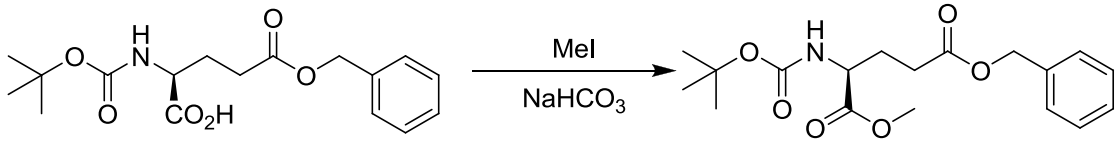
Synthesis of N-Boc γ-Benzyl Glutamic Acid (2)



500 mg of γ-benzyl-glutamic acid (2,1 mmol) were suspended in 6 ml of a 1:1 solution of dioxane and water in a 50 ml flask; the suspension was cooled to 0 °C and then 0,53 ml of Boc-anhydride and 0,43 ml of triethylamine were added. The resulting clear solution was stirred at room temperature for 12 h, controlled by TLC (PrOH 7: H_2O 3: AcOH 0,5) and then diluted with 5 ml of water; the solution was brought to pH 2 adding 5M HCl and then extracted with EtOAc (3 times with 6 ml for each extraction). The organic phase was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator to obtain 700 mg of a sticky oil (95% yield) that was directly used for the next reaction.

^{1}H NMR (δ ppm $CDCl_3$): 1.46 [s, 9H, $(CH_3)_3C$-], 2.05 [m, 1H, $C^{\beta}H_2$], 2.27 [m, 1H, $C^{\beta}H_2$], 2.55 [m, 2H, $C^{\gamma}H_2$], 4.37 [broad, 1H, $C^{\alpha}H$], 5.19 [s, 2H, -CH_2-Ph], 6.39 [broad, 1H, -OCONH-], 7.36 [m, 5H, Ph].

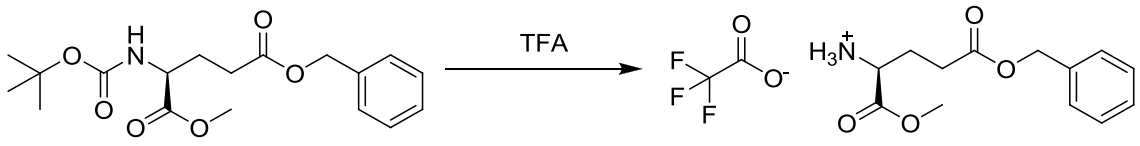
Synthesis of N-Boc-α-Methyl-γ-Benzyl Glutamic Acid (3)



700 mg of N-Boc-γ-benzyl-glutamic acid (2 mmol), previously dissolved in 6 ml of DMF were loaded under N_2 atmosphere in a 10 ml flask. 336 mg of NaHCO3 and then 0,2 µl of MeI were added under magnetic stirring to the resulting mixture. The solution was stirred at room temperature for about 24 hours and controlled by TLC (CH_2Cl_2 9: MeOH 1). Then it was diluted with 5 ml of water and extracted with diethyl ether (3 times with 6 ml for each extraction). The organic phase was washed with water, with a saturated $Na_2S_2O_3$ solution and finally with a saturated NaCl solution; it was then dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator. The obtained oil was dissolved in diethyl ether and subsequently treated with *n*-hexane to obtain, after 24 hours at -18 °C, a white crystalline solid that was filtered and washed with cold *n*-hexane (470 mg, 67% yield).

^{1}H NMR (δ ppm $CDCl_3$): 1.46 [s, 9H, $(CH_3)_3C$-], 1.99 [m, 1H, $C^{\beta}H_2$], 2.20 [m, 1H, $C^{\beta}H_2$], 2.45 [m, 2H, $C^{\gamma}H_2$], 3.75 [s, 3H, -$COOCH_3$], 4.37 [broad, 1H, CH], 5.14 [s, 2H, -CH_2-Ph], 7.38 [m, 5H, Ph].

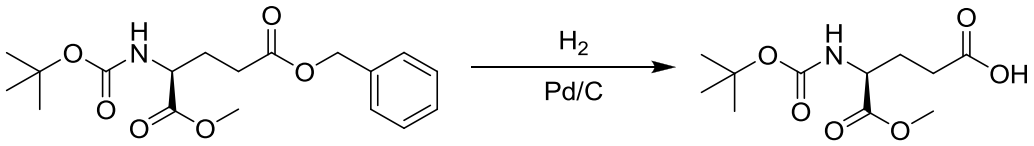
Synthesis of α-Methyl-γ-Benzyl Glutamate·CF_3CO_2H (4)



400 mg of N-Boc-α-methyl-γ-benzyl glutamic acid (1,14 mmol) were dissolved in a 10 ml flask, in 3,5 ml of CH_2Cl_2 containing 1,75 ml of CF_3CO_2H at 0 °C. The mixture was stirred at room temperature for about 1 hour and controlled by TLC (CH_2Cl_2 9: MeOH 1). After that the solution was concentrated at rotary evaporator and then at the pump to completely remove the CF_3CO_2H. The residue was then dissolved in diethyl ether and then treated with *n*-hexane to afford a precipitate after 24 hours at -18 °C. The white solid was filtered and washed with cold *n*-hexane. (365 mg, 87% yield).

^{1}H NMR (δ ppm $CDCl_3$): 2.27 [m, 2H, $C^{\beta}H_2$], 2.60 [m, 2H, $C^{\gamma}H_2$], 3.76 [s, 3H, -$COOCH_3$], 4.16 [broad, 1H, CH], 5.12 [s, 2H, -CH_2-Ph], 7.35 [m, 5H, Ph].

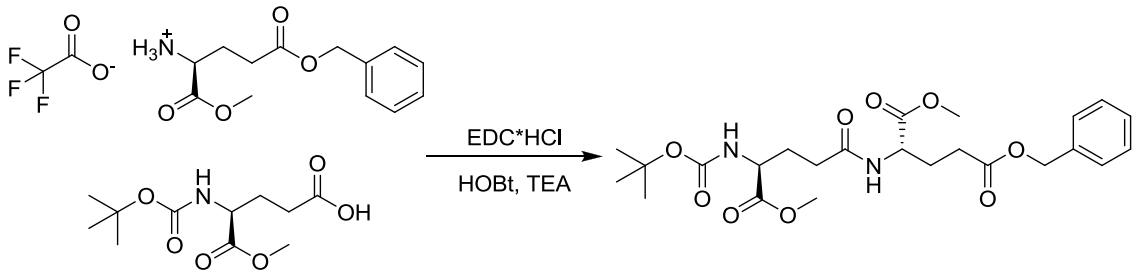
Synthesis of N-Boc-α-Methyl-Glutamic acid (5)



400 mg of N-Boc-α-methyl-γ-benzyl-glutamic acid (1,14 mmol) were dissolved in 10 ml of EtOH and 10 ml of *i*-PrOH in a 50 ml flask; 20 mg of Pd/C were then added and the reaction mixture was stirred under H_2 atmosphere for 18 hours and controlled by TLC (PrOH 9: H_2O 1: AcOH 0,5). The suspension was filtered on celite and the residue was washed with ethanol. The resulting clear solution was concentrated with a rotary evaporator and then to the pump to obtain an oil that was dissolved in diethyl ether and then treated with *n*-hexane to afford a white powder that was filtered and washed with cold *n*-hexane (265 mg, 89% yield).

^{1}H NMR (δ ppm $CDCl_3$): 1.45 [s, 9H, $(CH_3)_3C$-], 1.98 [m, 1H, $C^{\beta}H_2$], 2.19 [m, 1H, $C^{\beta}H_2$], 2.42 [m, 2H, $C^{\gamma}H_2$], 3.74 [s, 3H, -$COOCH_3$], 4.28 [broad, 1H, CH].

Synthesis of N-Boc-α-α'–Dimethyl-γ-Benzyl-Glutamic Acid Dimer (6)

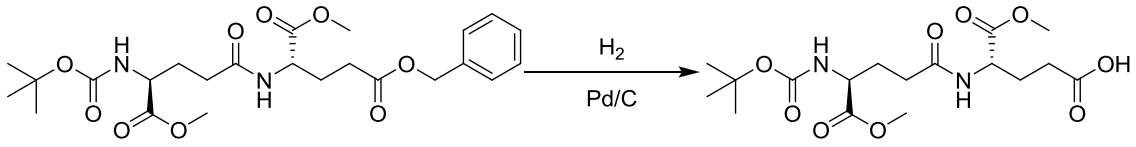


261 mg (1,0 mmol) of N-Boc-α-methyl-glutamic acid were dissolved under N_2 atmosphere in 5 ml of anhydrous CH_2Cl_2 in a 10 ml flask. The solution was cooled at 0 °C, then 230 mg (1,2 mmol) of EDC HCl were added under stirring; after 10 minutes 135 mg (1,0 mmol) of HOBt were added, and again after ten minutes 365 mg of α-Methyl-γ-Benzyl-glutamic acid (1,0 mmol) and 0,16 ml of triethylamine were added. The reaction mixture was stirred for about 20 hours and controlled by TLC (PrOH 9: H_2O 1: AcOH 0,5). Afterwards, the solution was diluted with 5 ml of CH_2Cl_2, washed with a saturated $NaHCO_3$ solution (3 times with 5 ml for each washing), with a 5% citric acid solution (3 times with 5 ml for each washing), water, and finally with a saturated NaCl solution. The organic phase was dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator to afford a colourless oil that was poured into ether/*n*-hexane and kept at -18 °C for about 24 hours. A white powder precipitated that could be filtered and washed with *n*-hexane (336 mg, 68% yield).

^{1}H NMR (δ ppm $CDCl_3$): 1.42 [s, 9H, $(CH_3)_3C$-], 1.85-2.55 [m, 8H, $C^{\beta}H_2$, $C^{\gamma}H_2$], 3.73 [s, 6H, 2*-$COOCH_3$], 4.14 [broad, 1H, CH], 4.6 [broad, 1H, CH], 5.11 [s, 4H, 2*-CH_2-Ph], 5.31 [broad d, *J* = 8.1 Hz, 1H, -CONH-], 6.57 [broad d, *J* = 7,5 Hz, 1H, -CONH-], 7.35 [m, 5H, Ph].

^{13}C NMR (δ ppm $CDCl_3$): 27.12, 28.60 [$C^{\beta}H_2$], 28.21 [$(CH_3)_3C$-], 30.21, 32.14 [$C^{\gamma}H_2$], 51.64, 52.35, 52.42, 52.84 [CH, -$COOCH_3$], 66.51 [-CH_2-Ph], 80.12 [$(CH_3)_3C$-], 128.21, 128.52, 135.71 [Ph], 155.67 [-OCONH], 171.72, 172.24, 172.60, 172.67 [carbonyl].

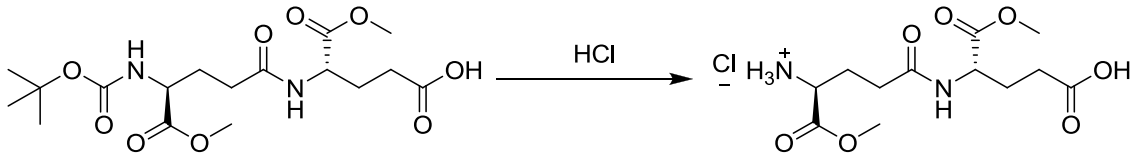
Synthesis of N-Boc-α-α'–Dimethyl-Glutamic Acid Dimer (7)



232 mg of N-Boc-α-α'-dimethyl-γ-benzyl-glutamic acid dimer (0,5 mmol) were dissolved in10 ml of ethanol and 5 ml of *i*-PrOH in a 50 ml flask. Then, 13 mg of Pd/C were added and the reaction mixture was stirred under H_2 atmosphere for 15 hours (TLC: PrOH 9: H_2O 1: AcOH 0,5). The suspension was filtered on celite and the residue was washed with ethanol. The resulting clear solution was concentrated with a rotary evaporator and then to the pump to obtain 182 mg of a colourless oil (90% yield) that was directly used for the next reaction.

^{1}H NMR (δ ppm $CDCl_3$): 1.45 [s, 9H, $(CH_3)_3C$-], 1.86-2.50 [m, 8H, $C^{\beta}H_2$, $C^{\gamma}H_2$], 3.78 [s, 6H, 2*-$COOCH_3$], 4.20 [m, 1H, CH], 4.63 [m, 1H, CH].

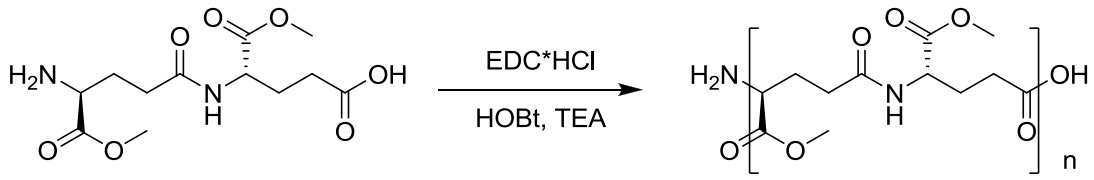
Synthesis of α-α'–Dimethyl-Glutamic Acid Dimer·HCl (8)



182 mg of N-Boc-α-α'–dimethyl-glutamic acid dimer (0,45 mmol) were dissolved in 1,5 ml of CH_3Cl in a 10 ml flask. 0,6 ml of a 5M HCl solution in dioxane were added at 0°C and the the resulting mixture was stirred at 0° until the precipitation of a white solid. Residual solvent was removed *in vacuo;* the product was washed with ether and ethyl acetate and then filtered (101 mg, 74% yield).

^{1}H NMR (δ ppm DMSO): 1.78 [m, 4H, $C^{\beta}H_2$], 2.34 [m, 4H, $C^{\gamma}H_2$], 3.61 [s, 3H, -$COOCH_3$], 3.71 [s, 3H, -$COOCH_3$], 3.99 [m, 1H, CH], 4.25 [m, 1H, CH].

Polycondensation (9)

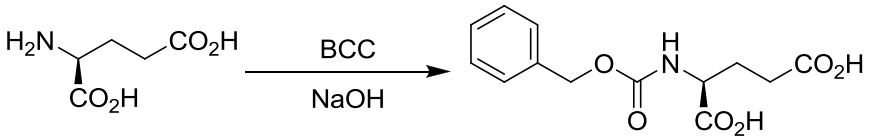


160 mg (0,526 mmol) of α-α'-methyl glutamic acid dimer were dissolved in 1 ml of DMF and loaded under N_2 atmosphere in a 5 ml flask. The solution was cooled to 0 °C and then, one after the other, 120 mg (0,631 mmol) of EDC·HCl, 71 mg (0,526 mmol) of HOBt and 88 µl of triethylamine were added. The reaction mixture was stirred for 24 hours, controlled by TLC (PrOH 9: H_2O 1: AcOH 0,5) and then concentrated with a rotary evaporator. The residue was dissolved in 5 ml of water and extracted with CH_2Cl_2 (3 times with 2 ml for each extraction); the organic phase was dried over anhydrous Na_2SO_4 and concentrated to obtain a sticky oil; this was triturated in diethyl ether until the formation of a pale yellow powder that was filtered, swiftly washed with *n*-Hexane and put in a drier (60 mg, 38% yield).

^{1}H NMR (δ ppm $CDCl_3$): 1.68-2.38 [broad, 4H, $C^{\beta}H_2$, $C^{\gamma}H_2$], 3.72-3.79 [broad, 3H, -$COOCH_3$], 4.59-4.78 [broad, 1H, CH].

Mass spectrum (MALDI-TOF): molecular weight distribution centered on *m/z* 3909.

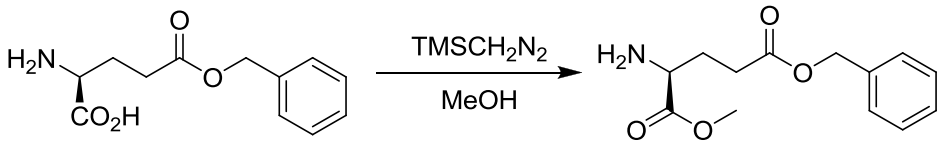
Synthesis of N-Cbz-Glutamic Acid (10)



14 g of glutamic acid (95 mmol) were dissolved in 20 ml of water in a 50 ml flask. The solution was basified at 0°C with 30 ml of 5M NaOH. 18 ml of benzylchlorocarbonate (BCC) (125 mmol) were added in little aliquots keeping the pH high during the process with 2M NaOH solution. The reaction mixture was stirred at 0 °C for 1,5 hours and then let to room temperature; it was basified to pH 10 and washed with ethyl acetate. The aqueous phase was acidified to pH 2 with a 5M HCl solution and left at -18 °C for 24 hours to afford a white solid that was filtered and washed with *n*-hexane (21 g, 80% yield).

^{1}H NMR (δ ppm DMSO): 1.75 [m, 1H, $C^{\beta}H_2$], 1.97 [m, 1H, $C^{\beta}H_2$], 2.29 [m, 2H, $C^{\gamma}H_2$], 3.99 [m, 1H, CH], 5.03 [s, 2H, -CH_2-Ph], 7.35 [m, 5H, Ph], 7.59 [d, 1H, -CONH-], 12.40 [broad, 2H, -COOH].

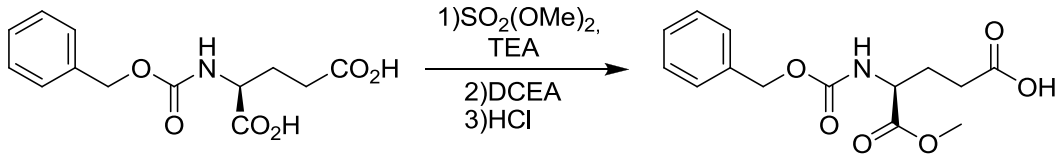
Synthesis of α-Methyl-γ-Benzyl Glutamic Acid (11)



γ-benzyl-glutamic acid (148 mg, 0,6 mmol) were dissolved in 7,2 ml of a mixture of CH_2Cl_2 (6 ml) and MeOH (1,2 ml), under N_2 flow. $TMSCH_2N_2$ (0,78 mmol, 0,375 ml of a 2N solution in diethyl ether) was added; the solution immediately got an intense yellow colour, tending to vanish with reaction's progress, which was controlled by TLC (PrOH 9: H_2O 1: AcOH 0,5); loss of colour along some 6 hours indicated the consumption of the reagent; solvent was afterwards evaporated, and the crude product was dissolved in AcOEt and washed with saturated $NaHCO_3$ solution (twice with 8 ml each time); the aqueous phase was then extracted with AcOEt (twice with 4 ml each time). Organic phases were pooled together, dried with saturated NaCl solution and Na_2SO_4, and concentrated at the rotary evaporator to yield 105 mg (70%) of a white solid product. This procedure was adapted from Kühnel, 2007.

^{1}H NMR (δ ppm $CDCl_3$): 1.99 [m, 1H, $C^{\beta}H_2$], 2.20 [m, 1H, $C^{\beta}H_2$], 2.45 [m, 2H, $C^{\gamma}H_2$], 3.75 [s, 3H, -$COOCH_3$], 4.37 [broad, 1H, CH], 5.14 [s, 2H, -CH_2-Ph], 7.38 [m, 5H, Ph].

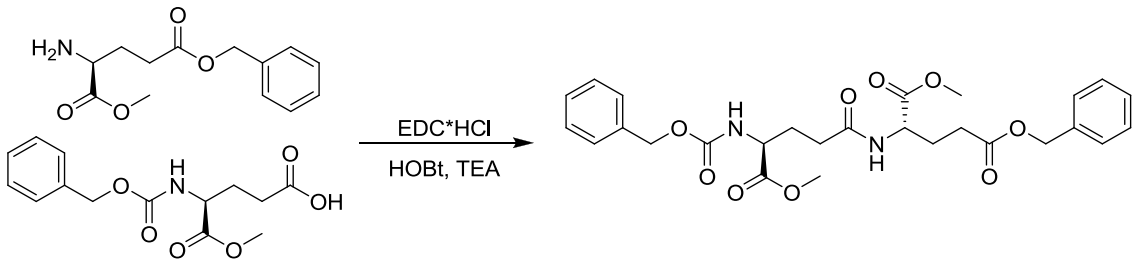
Synthesis of N-Cbz-α-Methyl-Glutamic Acid (12)



562 mg of N-Cbz glutamic acid (2,0 mmol) were suspended under N_2 atmosphere in 1 ml of distilled CH_3Cl in a 25 ml flask; 0,28 ml of triethylamine (2,0 mmol) were added to the suspension and the mixture was stirred at room temperature until the complete dissolution of the solid, then 0,22 ml (2,2 mmol) of dimethylsulphate were added and the reaction mixture was stirred at Room temperature for 4,5 hours and followed by TLC (PrOH 9: H_2O 1: AcOH 0,5). The solvent was removed with a rotary evaporator and the residue was dissolved in 5 ml of water and extracted with ethyl acetate (3 times with 3 ml for each extraction). The organic phase was then washed with water and a saturated NaCl solution, dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator. The resulting oil was dissolved in 20 ml of ethyl acetate and treated with 0,44 ml of diciclohexylamine to afford, after 24 hours at -18 °C, a white solid that was filtered and washed with cold ethyl acetate (572 mg, 60% yield). The salt was then dissolved in 10 ml of 0,5M HCl and the resulting solution was stirred at room temperature for half an hour; then it was extracted with CH_2Cl_2 (3 times with 6 ml for each extraction). The organic phase was washed with water and a saturated NaCl solution, dried over Na_2SO_4 and concentrated with a rotary evaporator to give 333 mg of a colourless oil (94% yield) that was directly used for the next reaction.

^{1}H NMR (δ ppm $CDCl_3$): 2.28 [m, 2H, $C^{\beta}H_2$], 2.94 [m, 2H, $C^{\gamma}H_2$], 3.74 [s, 3H, -$COOCH_3$], 4.32 [m, 1H, CH], 5.10 [s, 2H, -CH_2-Ph], 6.85 [broad, 1H, -CONH-], 3.23 [m, 5H, Ph], 8.5[broad, CO_2H].

Synthesis of N-Cbz-α-α'–Dimethyl-γ-Benzyl-Glutamic Acid Dimer (13)

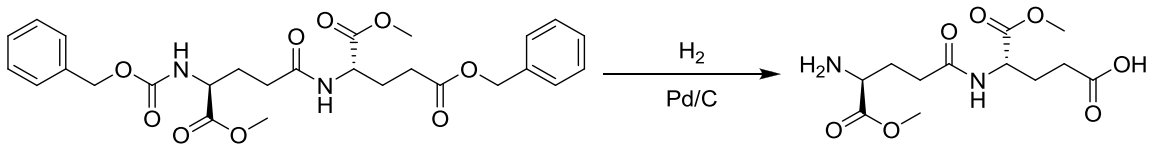


295 mg of N-Cbz α-methyl glutamic acid (1 mmol) were dissolved under N_2 atmosphere in 5 ml of anhydrous CH_2Cl_2 in a 10 ml flask. The solution was cooled at 0 °C then 230 mg (1.2 mmol) of EDC·HCl were added under stirring; after 10 minutes 135 mg (1 mmol) of HOBt were added and again after ten minutes 365 mg of α-Methyl-γ-Benzyl Glutamic Acid (1 mmol) and 0.16 ml of triethylamine. The reaction mixture was stirred for about 16 hours and controlled by TLC (PrOH 9: H2O 1: AcOH 0,5). Then the solution was diluted with 5 ml of CH_2Cl_2, washed with a saturated $NaHCO_3$ solution (3 times with 6 ml each), with a 5% citric acid solution (3 times with 6 ml each) water and finally with a saturated NaCl solution. The organic phase was dried over anhydrous Na_2SO_4 and concentrated with a rotary evaporator to obtain a colourless oil that was cooled at -18 °C to precipitate a white powder that was filtered and washed with n-hexane (350 mg, 65% yield).

^{1}H NMR (δ ppm $CDCl_3$): 2.03 [m, 2H, $C^{\beta}H_2$], 2.25 [m, 4H, $C^{\beta}H_2$, $C^{\gamma}H_2$], 2.49 [m, 2H, $C^{\gamma}H_2$], 3.73 [s, 3H, -$COOCH_3$], 3.74 [s, 3H, -$COOCH_3$], 4.38 [broad, 1H, CH], 4.62 [broad, 1H, CH], 5.11 [s, 2H, -CH_2-Ph], 5.13 [s, 2H, -CH_2-Ph], 5.64 [broad, 1H, -CONH-], 6.44 [broad, 1H, - CONH-], 7.33 [m, 10H, Ph].

13C NMR (δ ppm CDCl3): 27.19, 28.32 [$C^{\beta}H_2$], 30.09, 32.06 [$C^{\gamma}H_2$], 51.75 [CH], 52.53 [-$COOCH_3$], 53.39 [CH], 66.58, 67.11 [-CH_2-Ph], 129-127 [CH Ph], 135.74, 136.20 [C-Ph], 156.20 [CO-CH_2Ph], 171.69, 172.24, 172. 34, 172. 65 [carbonyl].

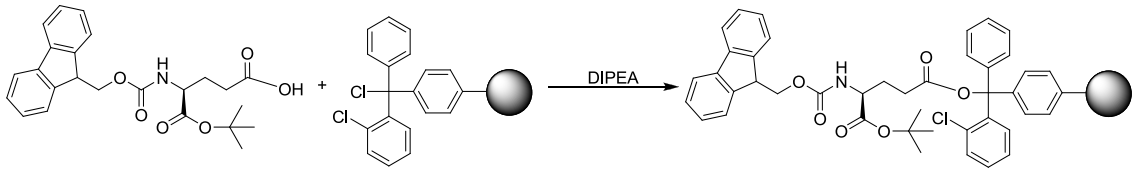
Synthesis of α-α'–Dimethyl-Glutamic Acid Dimer (14)



300 mg (0.57 mmol) of N-Cbz α-α' –methyl-γ-benzyl glutamic acid dimer were dissolved in 10 ml of EtOH and 10 ml of *i*-PrOH in a 50 ml flask, then 15 mg of Pd/C were added and the reaction mixture was stirred under H_2 atmosphere for 18 hours and controlled by TLC (PrOH 9: H_2O 1: AcOH 0,5). The suspension was filtered on celite and the residue was washed with ethanol. The resulting clear solution was concentrated with a rotary evaporator and then to the pump to obtain 160 mg of a colourless oil (94% yield) that was directly used for the polycondensation.

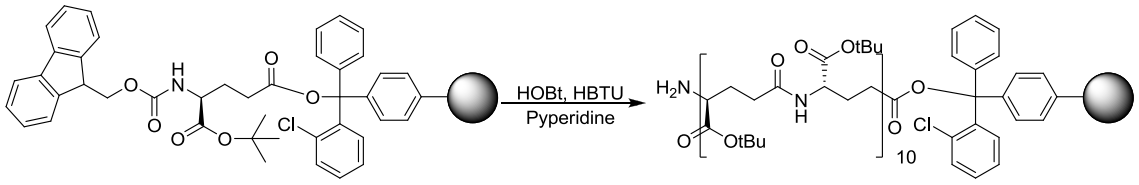
^{1}H NMR (δ ppm DMSO): 1.77 [m, 4H, $C^{\beta}H_2$], 2.34 [m, 4H, $C^{\gamma}H_2$], 3.60 [s, 3H, -$COOCH_3$], 3.72 [s, 3H, -$COOCH_3$], 3.98 [m, 1H, CH], 4.25 [m, 1H, CH].

Functionalisation of 2-Chloro-Trytil-Chloride Resin (15)



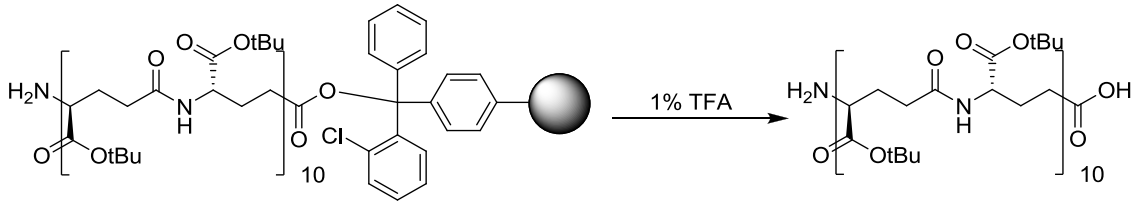
213 mg of N-Fmoc-O-tert-butyl-glutamic acid (0,5 mmol) and 0,33 ml of DIPEA (2 mmol) were dissolved in 3 ml of dry CH_2Cl_2 and loaded in a fritted plastic tube. Then 312,5 mg of 2-chlorotrityl chloride resin (loading 1,6 mmol/g) were added and the tube was kept under orbital stirring overnight. The resin was washed three times with 3 ml of a CH_2Cl_2/CH_3OH/DIPEA (17:2:1) solution for each washing, then three times with of CH_2Cl_2,with 4 ml for each washing, two times with DMF, with 3 ml for each washing, and finally two times of CH_2Cl_2, each time with 3 ml. The resin was dried overnight *in vacuo;* 488 mg of loaded resin were obtained (96% recover).

Synthesis of α-tert-butyl-glutamic acid γ-decamer (16)



Nine vials containing 212 mg of N-Fmoc-O-tertbutyl-glutamic acid (0,5 mmol), 67 mg of HOBt (0,5 mmol) and 189 mg of HBTU (0,5 mmol) were prepared. Then, 101 mg of loaded resin (0,1 mmol of amino acid) were suspended in DMF and put in the synthesizer reaction column. The couplings were performed in a 2M DIPEA in DMF solution and the deprotection of the amino terminus was carried out with a 20% piperidine in DMF solution. 175 mg of α-tert-butyl-glutamic acid γ-decamer linked to the resin were obtained (71% recover).

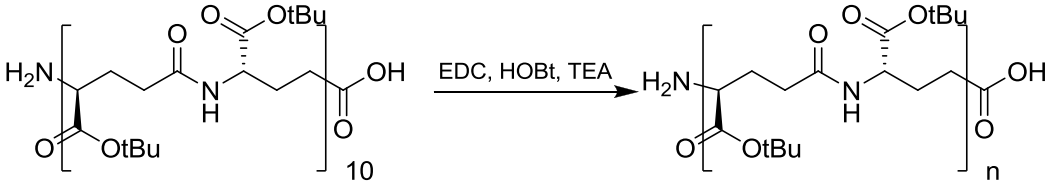
Cleavage of the Resin (17)



175 mg of loaded resin (0,07 mmol of α-tert-butyl-glutamic acid γ-decamer) were suspended in 4ml of CH_2Cl_2 in a fritted plastic tube; CH_2Cl_2 was filtered and then the resin was washed ten times with 3 ml of a 1% TFA in CH_2Cl_2 solution, each time collecting the filtered solvent in the same flask containing 20 ml of a 10% pyridine in CH_3OH solution. Eventually the resin was washed with CH_2Cl_2 (three times with 6 ml for each washing), CH_3OH (three times, each time with 6 ml), CH_2Cl_2 (twice, each time with 6 ml) and CH_3OH (with 6 ml, twice).The filtered fractions were combined with the pyridine solution that was then evaporated, under reduced pressure, to 5% of the staring volume. The residue was freeze-dried to give the decamer as a yellow powder (100 mg, 53% yield).

Mass spectrum (MALDI-TOF): *m/z* 1870 (MH_2^{2+}).

Coupling of the decamers (18)

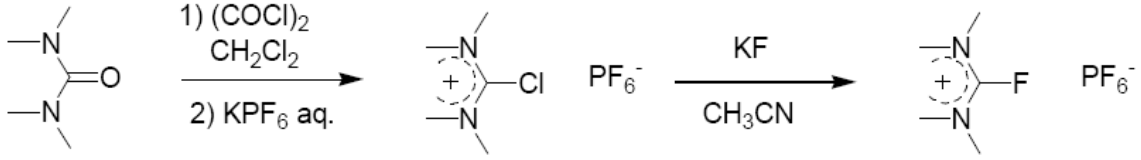


89 mg (0,0047 mmol) of γ-PGA butyl ester decamer and 32 µl (0,188 mmol) of DIPEA were dissolved in 4 ml of DMF; 12,5 mg (0,047 mmol) of TFFH were added to the reaction mixture just before putting the reactor in the microwave oven. The mixture was heated at 50 °C for 30 minutes (oven power: 200 Watt). Afterwards, it was diluted with 20 ml of distilled water, frozen and freeze dried. SEC-MALS analysis showed the product had M_p= 17,950.

Mass spectrum (MALDI-TOF): *m/z* 5993, 5480, 5188, 4647, 4355, 3772, 2740 (base peak)

M_p= 17,950 (SEC-MALS)

Sinthesis of N,N,N',N'-Tetramethyl-fluoro-formamidinium hexafluorophosphate (TFFH)



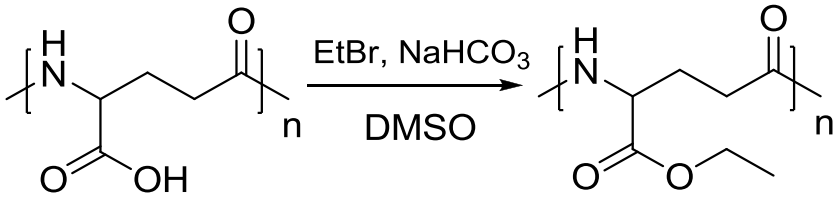
Step I: 0,480 ml (4,0 mmol) of tetramethylurea were diluted in 12 ml of CH_2Cl_2; the solution was cooled to 0 °C and then 0,343 ml (4,0 mmol) of oxalyl chloride were added, keeping the mixture under magnetic stirring at 0 °C for 10 minutes and at room temperature for 3 hours. After this time, solvent was removed under reduced pressure and the obtained solid was washed with anhydrous diethyl ether. The solid is then dissolved in CH_2Cl_2, and stirred for 30 minutes with a saturated aqueous solution of KPF_6. The organic phase is subsequently washed with saturated NaCl solution, dried on anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford 446 mg of a white solid (yield 40%).

Step II: 400 mg (1,4 mmol) of N,N,N',N'-tetramethyl-chloro-formamidinium-hexafluorophosphate were dissolved in 4 ml of anhydrous acetonitrile; 244 mg (4,2 mmol) of previously dried KF were then added. The mixture was left under stirring at 60 °C for three hours, and then cooled to room temperature. Excess KF was filtered, and the solid was washed with acetonitrile. Organic phases were concentrated under reduced pressure; the solid residue was dissolved in CH_2Cl_2 and filtered when hot. Filtered organic phase was concentrated under reduced pressure up to a third of its original volume, then diethyl ether was added under vigorous stirring. It was then observed the precipitation of a white solid, which could finally be filtered (228 mg, yield 62%).

^{1}H NMR (δ ppm $CDCl_3$): 3.33 [d, 12H].

^{19}F NMR (δ ppm $CDCl_3$): -76.32, -73.79 [d, 6F, J=757 Hz]; -44.32 [s, 1F].

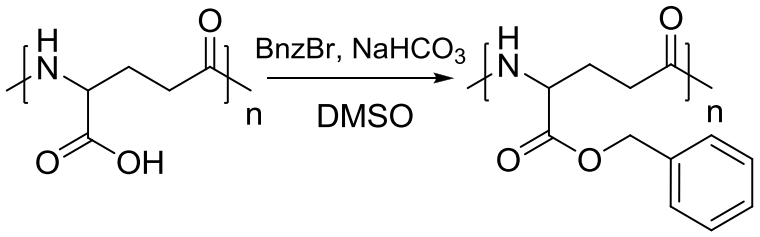
α-ethyl ester of γ-PGA



γ-PGA (2 g, 11, 83 mmol) was suspended in DMSO, under stirring; and was heated up to reach the temperature of 45 °C. As it got homogenous, $NaHCO_3$ (1,99 g, 23,66 mmol) was added, and after the salt got homogenously dispersed in the suspension, EtBr (4,38 ml, 59,15 mmol) was poured in. The reaction was left at 45 °C for 5 days; the solution was cooled to room temperature and then added dropwise in 750 ml of acid water which was kept in the pH interval 1-2 with conc HCl. Evolution of CO_2 was observed. A sticky, gluish compound precipitated from the solution; after filtration, ^{1}H-NMR showed it to be the α-ethyl ester of γ-PGA, with a low (44%) functionalisation degree. The material was again reacted in the same conditions for 3 more days; after precipitation, the material was put at the rotary evaporator, which was connected to an acetone/solid CO_2 trap to eliminate DMSO traces, for some three hours. The second generation product was finally obtained in the form of yellowish powder (525 mg). Recover 25%, functionalisation 67%.

^{1}H NMR (δ ppm DMSO): 1.8 [t, 3H, -$COOCH_2CH_3$], 1.77 [m, 1H, $C^{\beta}H_2$], 1.97 [m, 1H, $C^{\beta}H_2$], 2.18 [m, 2H, $C^{\gamma}H_2$], 4.05 [q, 2H, -$COOCH_2CH_3$], 4.20 [m, 1H, CH], 8.22 [d, 1H, -CONH].

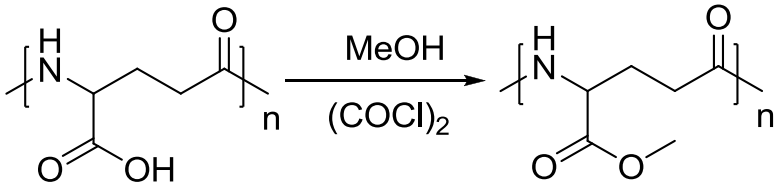
α-benzyl ester of γ-PGA



γ-PGA (2,00 g, 11, 83 mmol) was suspended in DMSO, under stirring; and was heated up to reach the temperature of 45 °C. Once homogenous, $NaHCO_3$ (1,99 g, 23,66 mmol) was added; BnBr (7,0 ml, 59,15 mmol) was poured in only when the suspension got homogenous again. The reaction was left at 45 °C for 5 days, afterewards the solution was cooled to room temperature and subsequently added, dropwise, in 750 ml of acid water which was kept in the pH interval 1-2 by addition of concentrated HCl. Evolution of CO_2 was observed. A yellow, translucent powder precipitated from the solution; ^{1}H-NMR revealed it to be α-benzyl ester of γ-PGA, with 50% functionalisation degree. The material was again reacted in the same conditions for 3 more days; after precipitation, it was put at the rotary evaporator, which was connected to an acetone/solid CO_2 trap to eliminate DMSO traces, for a couple of hours. The second generation product was obtained in the form of brownish powder (1,71 g). Recover 61%, functionalisation degree 70%.

^{1}H NMR (δ ppm DMSO): 1.76 [m, 1H, $C^{\beta}H_2$], 1.92 [m, 1H, $C^{\beta}H_2$], 2.21 [m, 2H, $C^{\gamma}H_2$], 4.21 [m, 1H, CH], 5.11 [s, 2H, -CH_2Ph], 7.37 [s, 5H Ph] 8.12 [d, 1H, -CONH].

α-methyl ester of γ-PGA

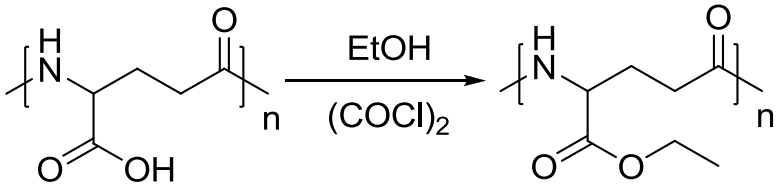


γ-PGA (500 mg, 3,40 mmol) was suspended in anhydrous methanol (8 ml), and cooled to 0 °C in an ice bath. A catalytic amount of anhydrous DMF (0,1 ml) and oxalyl chloride (1,75 ml, 20,33 mmol) were then cautiously added dropwise under nitrogen flow, paying attention not to let the temperature increase above 5 °C. Evolution of gas was observed. Once the additions were completed, the ice bath was removed and the solution temperature was let raise to ambient value. After 24 hours, the solution was diluted with methanol (16 ml) and neutralized with saturated $NaHCO_3$ solution (20 ml). After removal of methanol at the rotary evaporator, the formation of a white precipitate was observed. The product, a white, sticky powder (101 mg) was filtered and washed with little portions of distilled water and diethyl ether. Recover 25 %, functionalisation degree 90%.

The general procedure used for the preparation of the next products follows the previous one, used for the preparation of the methyl ester.

^{1}H NMR (δ ppm DMSO): 1.75 [m, 1H, $C^{\beta}H_2$], 1.95 [m, 1H, $C^{\beta}H_2$], 2.21 [m, 2H, $C^{\gamma}H_2$], 3.62 [s, 3H, -$COOCH_3$], 4.22 [m, 1H, CH], 8.27 [d, 1H, -CONH].b

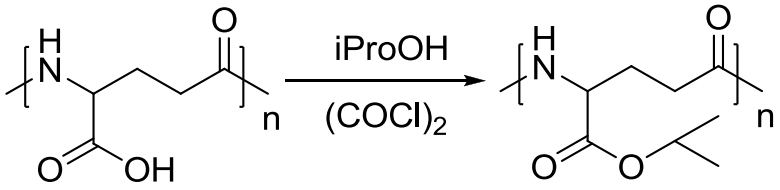
α-ethyl ester of γ-PGA



γ-PGA (250 mg, 1,70 mmol) was suspended in anhydrous ethanol (4 ml), and cooled to 0 °C in an ice bath. A catalytic amount of anhydrous DMF (0,1 ml) and oxalyl chloride (0,87 ml, 10,20 mmol) were then cautiously added dropwise under nitrogen flow, paying attention not to let the temperature increase above 5 °C. Evolution of gas was observed. Once the additions were completed, the ice bath was removed and the solution temperature was let raise to ambient value. After 48 hours, the solution was diluted with ethanol (14 ml) and neutralized with saturated $NaHCO_3$ solution (20 ml). After removal of methanol at the rotary evaporator, the formation of a white precipitate was observed. The product, a white powder (101 mg) was filtered and washed with little portions of distilled water, ethanol and diethyl ether. Recover 95 %, functionalisation degree 25%.

^{1}H NMR (δ ppm DMSO): 1.18 [t, 3H, -$COOCH_2CH_3$], 1.77 [m, 1H, $C^{\beta}H_2$], 1.97 [m, 1H, $C^{\beta}H_2$], 2.18 [m, 2H, $C^{\gamma}H_2$], 4.05 [q, 2H, -$COOCH_2CH_3$], 4.20 [m, 1H, CH], 8.22 [d, 1H, -CONH].

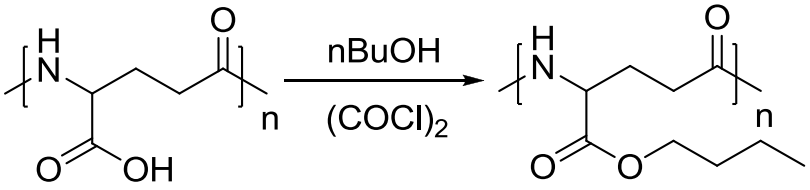
α-isopropyl ester of γ-PGA



γ-PGA (250 mg, 1,70 mmol) was suspended in anhydrous isopropanol (4 ml), and cooled to 0 °C in an ice bath. A catalytic amount of anhydrous DMF (0,1 ml) and oxalyl chloride (0,87 ml, 10,20 mmol) were then cautiously added dropwise under nitrogen flow, paying attention not to let the temperature increase above 5 °C. Evolution of gas was observed. Once the additions were completed, the ice bath was removed and the solution temperature was let raise to ambient value. After 48 hours, the solution was diluted with isopropanol (8 ml) and neutralized with saturated $NaHCO_3$ solution (20 ml). After removal of methanol at the rotary evaporator, the formation of a white precipitate was observed. The product, in the form of a white powder (227 mg) was filtered and washed with little portions of distilled water, ethanol and diethyl ether. Recover 90 %, functionalisation degree 70%.

^{1}H NMR (δ ppm DMSO): 1.27 [d, 6H, -OCH(CH_3)$_2$] 1.77 [m, 1H, $C^{\beta}H_2$], 1.97 [m, 1H, $C^{\beta}H_2$], 2.21 [m, 2H, $C^{\gamma}H_2$], 4.04 [m, 1H, CH], 4.17 [m, 1H, -OCH(CH_3)$_2$] 8.13 [d, 1H, -CONH].

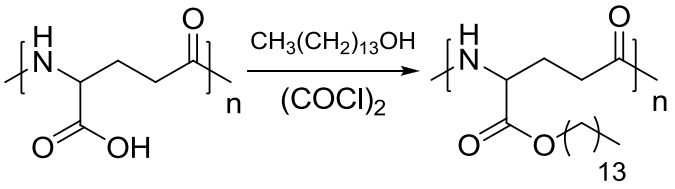
α-*n*-butyl ester of γ-PGA



γ-PGA (250 mg, 1,70 mmol) was suspended in anhydrous *n*-butanol (4 ml), and cooled to 0 °C in an ice bath. A catalytic amount of anhydrous DMF (0,1 ml) and oxalyl chloride (0,87 ml, 10,20 mmol) were then cautiously added dropwise under nitrogen flow, paying attention not to let the temperature increase above 5 °C. Evolution of gas was observed. Once the additions were completed, the ice bath was removed and the solution temperature was let raise to ambient value. After 48 hours, the solution was diluted with methanol (12 ml) and neutralized with saturated $NaHCO_3$ solution (20 ml). After removal of methanol at the rotary evaporator, the formation of a white precipitate was observed. The product, a white powder (126 mg) was filtered and washed with little portions of methanol. Recover 50 %, functionalisation degree 25-70%, depending on preparation.

^{1}H NMR (δ ppm DMSO): 0,91 [t, 3H, -$COOCH_2CH_2CH_2CH_3$], 1.62 [m, 2 H, -$OCH_2CH_2CH_2CH_3$] 1.39 [m, 2H, -$OCH_2CH_2CH_2CH_3$], 1.74 [m, 1H, $C^{\beta}H_2$], 1.97 [m, 1H, $C^{\beta}H_2$], 2.21 [b, 2H, $C^{\gamma}H_2$], 4.05 [m, 2H, -$COOCH_2CH_2CH_2CH_3$], 4.17 [m, 1H, CH], 8,13 [d, 1H, -CONH].

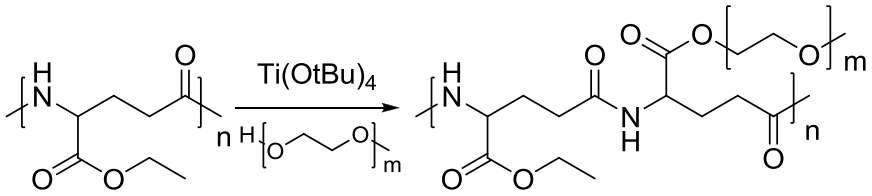
α-tetradecanoyl ester of γ-PGA



γ-PGA (500 mg, 3,40 mmol) were suspended in anhydrous THF (100 ml), and cooled to 0 °C in an ice bath. A catalytic amount of anhydrous DMF (0,3 ml) and oxalyl chloride (5,2 ml, 61,2 mmol) were then cautiously added dropwise under nitrogen flow, paying attention not to let the temperature increase above 5 °C. Evolution of gas was observed. Once the additions were completed, the ice bath was removed and the solution temperature got to ambient value, and then tetradecanol (14,6 g, 68,0 mmol) was added. After 72 hours, the solution was neutralized with saturated $NaHCO_3$ solution; after removal of THF at the rotary evaporator, the formation of a white precipitate was observed. The product, a white powder (133 mg) was filtered and washed with portions of methanol, and then diethyl ether. Recover 53 %, functionalisation degree 4-25% depending on preparation.

^{1}H NMR (δ ppm DMSO): 0,85 [t, 3H, -COO$(CH_2)_{13}CH_3$], 1.23 [s, 24H, -O$CH_2(CH_2)_{12}CH_3$] 1.74 [m, 1H, $C^{\beta}H_2$], 1.95 [m, 1H, $C^{\beta}H_2$], 2.19 [b, 2H, $C^{\gamma}H_2$], 4.06 [b, 2H, -COO$CH_2(CH_2)_{12}CH_3$], 4.16 [b, 1H, CH], 8,14 [d, 1H, -CONH].

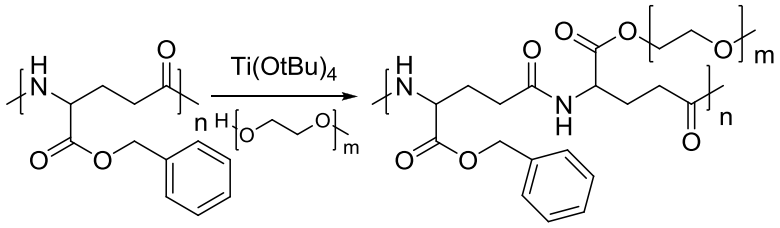
α-ethyl-α'-poly(ethylene glycol)-yl ester of γ-PGA



α-ethyl ester of γ-PGA (175 mg, 1 mmol, 67% functionalisation) was suspended in poly (ethylenglycol) (average molecular weight 550, 11 g, 20 mmol); Ti (O*t*-Bu)$_4$ was added (68 mg, 0,2 mmol) under nitrogen flow and the reaction mixture was heated to 100 °C and maintained at the same temperature for 3 hours, under stirring. It was then cooled to room temperature and diluted, drop by drop, in methanol (25 ml). The formation of a precipitate (47 mg) was observed, which was filtered off and washed with little aliquots of methanol and diethyl ether. Recover 27 %, functionalisation degree 59% ethyl ester, 27% poly (ethylenglycol).

^{1}H NMR (δ ppm DMSO): 1.21 [t, 3H, -COOCH$_2$CH$_3$], 1.74 [m, 1H, C$^\beta$H$_2$], 1.95 [m, 1H, C$^\beta$H$_2$], 2.21 [m, 2H, C$^\gamma$H$_2$], 3.45 [s, (OCH$_2$CH$_2$)$_n$], 3.53 [s, (OCH$_2$CH$_2$)$_n$], 4.01 [q, 2H, -COOCH$_2$CH$_3$], 4.21 [m, 1H, CH], 8.26 [d, 1H, -CONH].

α-benzyl-α'-poly(ethylene glycol)-yl ester of γ-PGA



α-benzyl ester of γ-PGA (237 mg, 1 mmol, 50% functionalisation) was suspended in poly (ethylenglycol) (average molecular weight 550, 11 g, 20 mmol); Ti (O*t*-Bu)$_4$ was added (68 mg, 0,2 mmol) under nitrogen flow and the reaction mixture was heated to 100 °C and maintained at the same temperature for 3 hours, under stirring. It was then cooled to room temperature and diluted, drop by drop, in methanol (25 ml). The formation of a precipitate (107 mg) was observed, which was filtered off and washed with little aliquots of methanol and diethyl ether. Recover 45 %, functionalisation degree 42 % benzyl ester, 21% poly (ethylenglycol).

^{1}H NMR (δ ppm DMSO): 1.77 [b, 1H, C$^\beta$H$_2$], 1.99 [b, 1H, C$^\beta$H$_2$], 2.51 [b, 2H, C$^\gamma$H$_2$], 4.21 [m, 1H, CH], 3.46 [s, (OCH$_2$CH$_2$)$_n$], 3.52 [s, (OCH$_2$CH$_2$)$_n$], 5.10 [s, 2H, -CH$_2$Ph], 7.33 [s, 5H Ph] 8.31 [b, 1H, -CONH].

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