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Review

Analytical approaches to poly-γ-glutamate: Quantification, molecular size determination, and stereochemistry investigation☆

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ABSTRACT

Poly-γ-glutamate, a nylon-like polyamide material typically consisting of both enantiomers of glutamate, exhibits reasonable biodegradability and its multi-functionality is attracting particular attention. Thus, its industrial application as a versatile and chiral polymer is in increasing demands. Poly-γ-glutamate is presently synthesized using several biocatalysts (*e.g.*, bacterial cells), but the uncontrollable structural diversity of such biosynthesized materials is an area of concern. This review highlights analytical approaches of interest to assure the functional and structural reproducibility of poly-γ-glutamate.

Contents

1. Introduction

Poly-γ-glutamate is a protease-resistant isopolypeptide with a nylon-like backbone (Fig. 1), and some experiments with esterification of its α-carboxyl side chains indicate that it exhibits substantially the nylon-like properties [1]. Unlike synthetic nylons, poly-γ-glutamate with multiple chirotopic carbons possesses a fair biodegradability (presumably due to naturally occurring depolymerases [2], and its multi-functionality is attracting particular attention (Table 1). It is not presently possible to chemically synthesize high-molecular-size poly-γ-glutamate; however, it can be easily produced from glutamate monomers by biocatalysts (Fig. 2, step *a*), such as viable cells of *Bacillus subtilis* [2,3] or an *Escherichia coli* clone that bears a poly-γ-glutamate synthetase-gene cluster [4,5] and the membrane-associated enzymes from *B. subtilis* subsp. *chungkookjang* [6]. Of particular concern here, poly-γ-glutamate, especially when produced by *B. subtilis* cells, is quite changeable in molecular structure [2,7], depending on the strains used, broth components, culture conditions, and other factors. In fact, many attempts have demonstrated that it is difficult to control both the polymer quality and functionality. At the same time, analytical approaches to poly-γ-glutamate have advanced significantly, and are now poised to provide insights into the correlation(s) between the multi-functionalities and structural diversity of poly-γ-glutamate.

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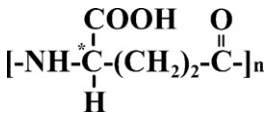

Fig. 1. Probable molecular structure of PGA. The backbone structure is virtually the same as that of a chemically synthesized (and achiral), high-performance polyamide materials, such as nylon-4. The chiral carbon(s) of PGA are indicated with asterisk(s).

This review article highlights some valuable methods for analyzing the yield, the molecular sizes, and the stereochemistry of poly-γ-glutamate.

2. Isolation and purification of poly-γ-glutamate (PGA)

Crude PGA samples can be prepared by water extraction from the surfaces of traditional Japanese fermented soybeans (*natto*), or by alcohol precipitation of the culture filtrates of *B. subtilis* [2]. Treatment with acid and proteases are then required for the removal of polysaccharides and proteins from the samples, respectively. Further purification is indispensable prior to structural analyses of PGA to remove low-molecular-size compounds, such as glutamate monomers (as remaining substrates) and culture-medium constituents, and affords pure high-molecular-size PGA (Fig. 2, step *b*). As the acidity of PGA increases with increasing molecular size, corresponding to the number of γ-glutamyl residues, anion-exchange chromatography is the preferred method for purification and molecular-size fractionation of PGA. For instance, recent literature shows that pure PGA samples with average molecular masses of 13, 52, 130, 390, and 1300 kDa are obtained from fractions eluted with 0.3, 0.4, 0.5, 0.6, and 0.7-M NaCl solutions, respectively, from a silica-based hydrophilic strong anion-exchanger with large pore-size (*e.g.*, QMA) [8].

3. Rapid quantification of PGA

To date, PGA yields can be estimated by various methods, including high-performance liquid chromatography (HPLC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [2] and a colorimetric assay based on safranin-induced precipitation of PGA, a well-known and rapid quantification method, but one which suffers from comparatively low accuracy and limited applicability. Hence, this article presents another rapid, more accurate quantification of PGA: the cetyltrimethylammonium bromide (CTAB)-dependent spectrophotometric assay. CTAB binds very specifically to PGA to form a water-insoluble, highly dispersed micelle-like complex, resulting in an increase in turbidity. As shown in Fig. 3, the turbidity-based calibration curve ($y = 0.5(\pm 0.025)x$, where x and y represent the concentration of PGA and the turbidity at 400 nm of the mixtures) gives a good linearity over at least the range of 0.2–2 μg ml^{-1} PGA, regardless of the polymer stereochemistry. The CTAB assay thus has the potential for rapid quantification of PGA.

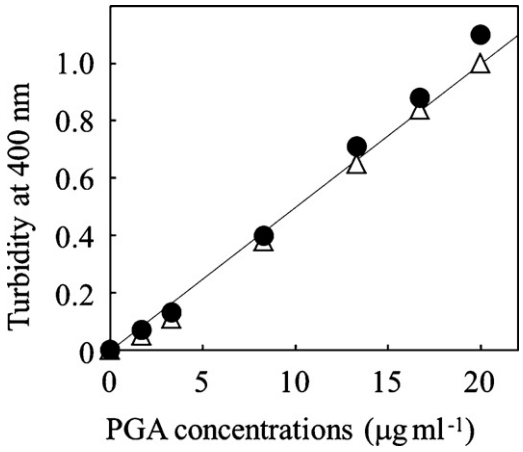

Fig. 3. CTAB-dependent spectrophotometric assay for rapid quantification of PGA. The calibration curve was made using both the 1000-kDa polymers of archaeal L-*homo*-PGA (*closed circles*) [23] and bacterial DL-*co*-PGA (*open triangles*) [8]. Data represent the means of three independent tests, and standard errors were below 3%.

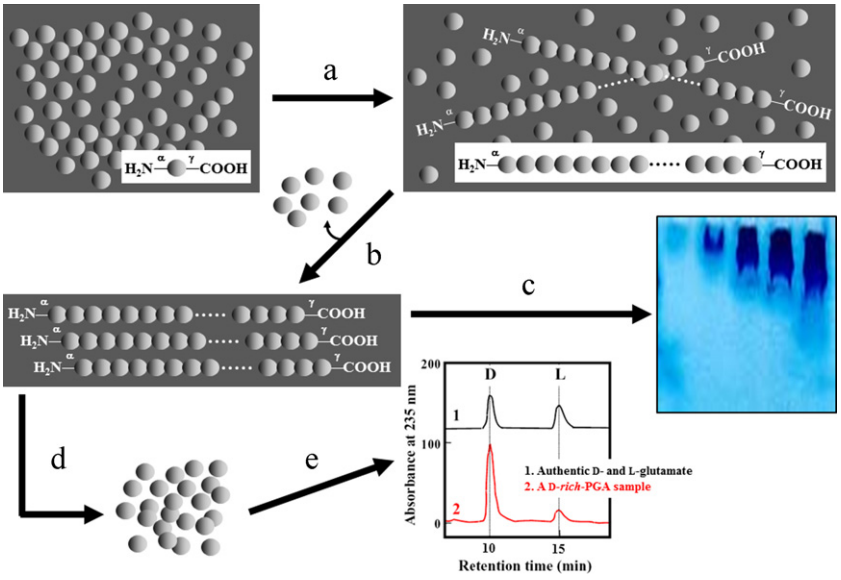

Fig. 2. Analytical processes for the determination of PGA structure. (a) Initial PGA biosynthesis. In the glutamate monomers, the α-amino and γ-carboxyl groups, and the core constituents are indicated by NH_2, COOH, and gray bowls, respectively. For each molecule of PGA polymers, a huge number of the constituents (bowls) align from a sole α-amino (NH_2) group to the γ-carboxyl (COOH) residue at the end. (b) Purification of PGA polymers. (c) SDS-PAGE and visualization of PGA polymers, helping to understand its molecular-size distribution. (d) Hydrolysis of PGA polymers and (e) chiral HPLC to estimate the stereo-chemical composition of hydrolysates, resulting in the characterization of PGA.

Table 1
Potential applications of PGA and its derivatives [2].

Categories	Applications	Details
Biodegradable materials	Thermoplastics, fibers, films	Substitution for chemically synthesized, non-biodegradable plastics as industrial and daily necessity
	Hydrogels	Substitution for non-biodegradable water-absorbents such as polyacrylate in diaper; potential application for desert greening
Bioremediation	Flocculants	Substitution for non-biodegradable flocculants such as polyacrylamide
	Metal absorbents	Removal of heavy metals and radionuclides
Others	Cryoprotectants	Preservation of cryolabile nutrients
	Bitterness-relieving agents	Relief of bitter taste by amino acids, peptides, quinine, caffeine, minerals, etc.
	Thickeners	Viscosity enhancement for drinks; prevention of aging of foods such as bakery products and noodles; improvement of textures
	Mineral absorbents	Promotion of absorption of bioavailable minerals such as Ca^{2+}: live stocks, increase in egg-shells strength, decrease in body fat, etc.; human, prevention of osteoporosis
	Humectants	Use for skin-care in cosmetics
	Drug delivers	Improvement of anticancer drugs
	Gene vectors	Use for gene therapy
	Curable biological adhesives	Substitution of fibrin
	Membranes	Separation of heavy metals; enantioselection of amino acids
	Dispersants	Uses in detergents, cosmetics, paper making, etc.
	Biomacromolecules-immobilizing materials	

4. Molecular-size determination of PGA

Generally, the molecular-size distribution of *B. subtilis* PGA ranges widely from 10 kDa to over 1000 kDa [2,9]. Recently, Sung and colleagues found that super-high-molecular-size PGA (*e.g.*, ~2000 kDa) is a potential antitumor agent, modulating the immune system against tumors [9], suggesting that the precise analysis of molecular size of PGA is indispensable to designing and improving polymer function. In this section, a selection of methods to determine the molecular size of PGA is presented.

4.1. Assay by gel permeation chromatography (GPC)

GPC is a commonly used method for assaying the size of biomacromolecules, including PGA. Using GPC reveals specific size parameters of the polymer of interest, such as number-averaged molecular mass (M_n), weight-averaged molecular mass (M_w) and polydispersity (M_w/M_n) [10,11]. Experimentally, dried PGA is suspended in $NaNO_3$ solution, dissolved with an alkaline solution, and subjected to GPC at a constant flow rate [8,12]. PGA will be detected with a refractometer [8,12] and give a typical chromatogram for molecular-size determination [12]. Its apparent molecular size can be estimated using polyethylene oxide as an approximate standard marker.

4.2. Electrophoretic assay

PGA is visualized as smeared bands on an SDS-PAGE gel by staining with basic dyes, such as methylene blue and alcian blue [2] (Fig. 2, step *c*), helping to deepen the understanding of the molecular-size distribution of PGA [2,6,8,13–15]. Size-distribution profiles can be obtained using a densitometry system [8]. Alternatively, a simple autoradiography assay has been established using ^{14}C-labeled *B. subtilis* PGA [16]. Due to its simplicity, the SDS-PAGE assay is significantly more convenient than the GPC assay, though the former has an important weakness in that it is notably less precise in the analysis of PGA with a super-high molecular size of over 2000 kDa [8].

4.3. Chemical assay

Based on the fact that every molecule of PGA has one terminal free amino group irrespective of the linkage number and the ratio of numbers between the amino group and γ-glutamyl residues of PGA is parallel to average molecular mass (or weight), a new strategy for chemical determination of the polymer size of PGA has been proposed [6,8]. As shown in Fig. 4, 1-fluoro-2,4-dinitrobenzene (FDNB) is a key reagent in the chemical assay. Experimentally, PGA is converted into *N*-dinitrophenyl (DNP)-PGA by incubation in an FDNB solution, followed by polyamide hydrolysis under acidic conditions at high temperature. The resulting DNP-glutamate and free glutamate monomers are determined by a colorimetry [6,8] and an HPLC assay [2,5,6,8], respectively. Average molecular mass (or weight) of PGA is estimated using Eq. (1), where the factor 129 corresponds to the molecular mass (or weight) of one γ-glutamyl residue.

$$\text{Average molecular mass} = 129 \times \frac{\text{Number of γ-glutamyl residues}}{\text{Number of amino groups}} \quad (1)$$

This chemical assay is simple, but quite efficient. For instance, even in the case of a high-molecular-size PGA (*e.g.*, a 1000-kDa polymer), a small amount of the polymer (*e.g.*, ~0.15 mg) is enough for the size analysis.

5. Investigation of PGA stereochemistry

Various PGA-producing microorganisms, other than *B. subtilis*, have been discovered and characterized (Table 2), revealing that PGA is a remarkably divergent biopolymer in terms of chirality. To date, two distinct determinants of PGA stereochemistry are predicted: the stereo-chemical composition of intracellular glutamate pools and the stereoselectivity of PGA synthetase for glutamate substrate. In the first category, glutamate racemase (GLR, catalyzing the racemization of intracellular L-glutamate to DL-glutamate) and D-amino acid aminotransferase (DAT, catalyzing the *trans*-conversion of 2-oxoglutarate and various D-amino acids (*e.g.*, intracellularly abundant D-alanine) into D-glutamate and the corresponding oxo-acids) are L-glutamate-dependent and -independent D-glutamate-supplying enzymes *in vivo*. In fact, *B. subtilis* cells with a high GLR activity produce DL-*co*-PGA [2,14],

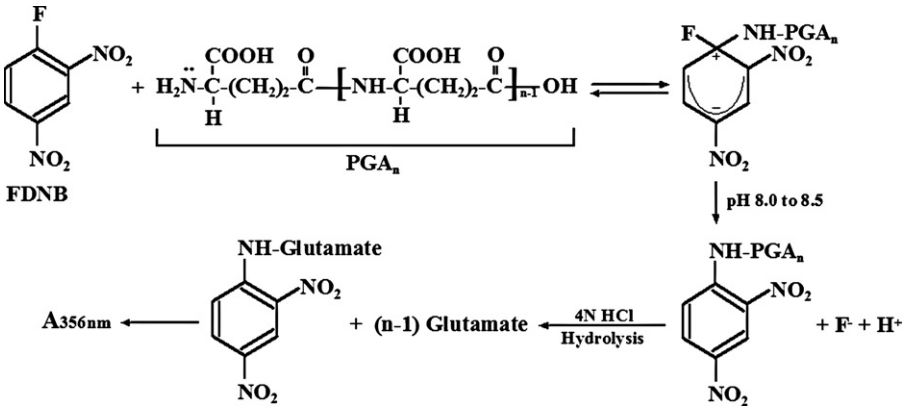

Fig. 4. FDNB-dependent spectrophotometric assay for estimation of the average molecular size of PGA [6,8].

while D-*homo*-PGA-producing *Bacillus anthracis* possesses DAT in abundance [2]. *Bacillus megaterium* and *Natrialba aegyptiaca* (an extremely halophilic archaeon; Ref. [17]), which show little or no D-glutamate-supplying enzyme activity [18], synthesize L-*rich* and *homo*-PGA, respectively. In the second category, the performance of two operon architectures for PGA synthesis, *i.e.*, *pgs* and *cap* (Table 2), is the focus. The *pgs* and *cap* operons operate in the production of PGA as an abundant *exo*-polymer and an important capsular component, respectively. To our knowledge, the B, C, and A components are highly conserved. In contrast, the E component, which may be a potent stimulator in *B. subtilis* PGA synthesis [19], is only found in Gram-positive bacteria, including bacilli and staphylococci. At least, *B. subtilis* Pgs system indiscriminately utilizes both enantiomers of glutamate as substrates [6]. The Cap systems specifically encompass the CapD enzyme, which is required for the anchoring of PGA to peptidoglycans [20]. Many of the *Staphylococcus epidermidis* group, such as *Staphylococcus haemolyticus* [21], possess the Cap system and produce DL-*co*-PGA [22]. Interestingly, the CapBC complex (*viz.*, the catalytic component of staphylococcal PGA synthetase) is more similar in primary structure to *B. subtilis* PgsBC complex than *B. anthracis* CapBC complex. On the other hand, L-*rich*-PGA-producing *B. megaterium*, though it is taxonomically close to *B. subtilis*, possesses the Cap-like system (DDBJ/EMBL/GenBank accession number, AB571872), suggesting that typical Cap systems serve to produce stereo-regular polymers, such as L-*rich*- and D-*homo*-PGAs. Genetic research on L-*homo*-PGA synthesis in *N. aegyptiaca* is now in progress.

The peculiar stereochemistry of PGA is also attractive in biochemical applications [2,23]. Here, the article introduces traditional and advanced methods for the investigation of PGA stereochemistry.

5.1. Immunochemical assay

Half a century has passed since immunochemical strategies for the assay of PGA stereochemistry were established [2]. During the course of these studies, antibodies recognizing the L-isomer-containing regions of PGA were developed [24,25], though until recently there was little progress on the development of high-performance antibody tools for the precise analysis (or detection) of non-immunogenic D-*homo*-PGA [2,26–28]. On the basis of traditional immunochemical analyses of the stereochemistry of *B. megaterium* PGA [24,25], it has been assumed that *B. megaterium* produces DL-*co*-PGA (*e.g.*, DL ratio, 50:50), as does *B. subtilis* [2].

5.2. Chiral HPLC assay

Currently, various chiral carriers are developed and chiral HPLC can be available even for the investigation on the stereochemistry of chiral-polymers including PGA. In the case of PGA, the stereochemistry is usually determined by measuring the stereochemical compositions of hydrolysates from PGA samples of interest. Experimentally, PGA samples are first hydrolyzed with a high concentration of HCl at a high temperature *in vacuo* (Fig. 2,

Table 2
Biochemical comparisons of some PGA-producing microorganisms: stereo-chemical features (DL-ratios) of the polymer product, major enzymatic activities for the *in vivo* D-glutamate supply, and differences in the organization of PGA-production operons.

Producers	PGA stereochemistry (D-ratios; %)	Major D-glutamate-supplying enzyme activities[a]	Organization of PGA-production operons	References
Gram-positive bacteria				
Bacillus subtilis	20–80	GLR	*pgsB-C-A-E-pgdS*	[2,32]
Bacillus subtilis subsp. *chungkookjang*	60–70	GLR, DAT	*pgsB-C-A-E-pgdS*	[4,14,15]
Bacillus anthracis	100	DAT	*capB-C-A-D-E*	[2,19,33]
Bacillus megaterium	5–10	*n.m.a.*[c]	*capB-C-A-D-E-F*	[18]
Staphylococcus epidermidis	40–50	*n.d.*	*capB-C-A-D-E*	[22]
Staphylococcus haemolyticus	*n.d.*[b]	GLR, DAT	*cap*-like operon	[22]
Gram-negative bacteria				
Fusobacterium nucleatum	*n.d.*	*n.d.*	*pgsB-C-A*	[34]
Archaea				
Natrialba aegyptiaca	0	*n.m.a.*	*n.d.*	[2,17]

[a] GLR, glutamate racemase activity; DAT, D-amino acid aminotransferase activity.
[b] *n.d.*, not determined.
[c] *n.m.a.*, no measurable activity.

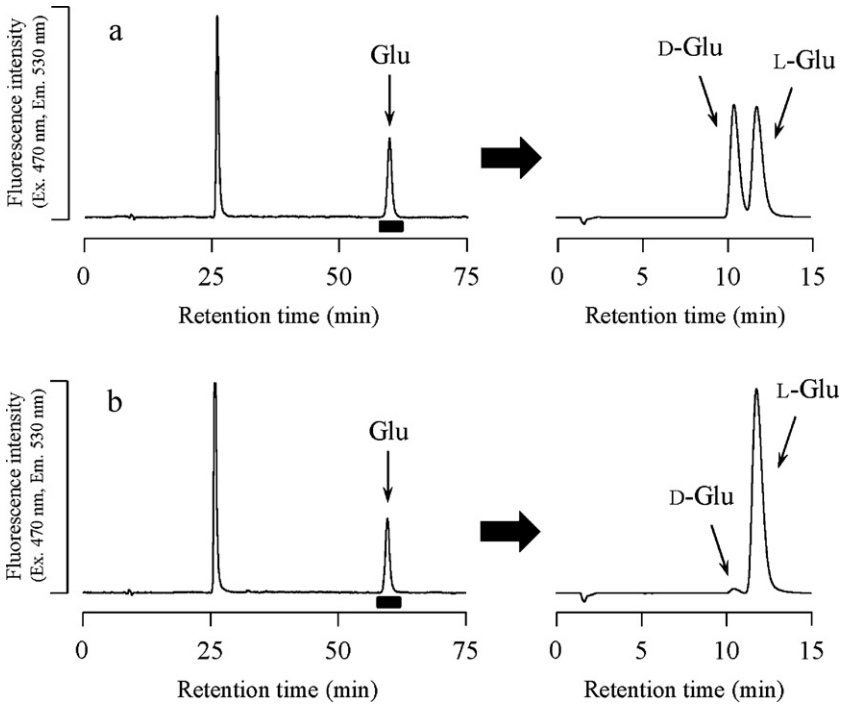

Fig. 5. Determination of PGA stereochemistry. PGA was hydrolyzed using 6 M HCl at 108 °C for 24 h, then derivatized with a fluorescence reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and subjected to 2D-HPLC analysis using a series of NANOSPACE SI-2 (Shiseido, Tokyo, Japan) [29]. Subjects: *a*, *B. subtilis* PGA (D:L = 5:5); *b*, *N. aegyptiaca* PGA (D:L = 0:10). In the first dimension (*left panels* in *a* and *b*), a monolithic ODS column (1000 by 0.53 mm; Shiseido) was developed with a mobile phase, consisted of 6% (v/v) acetonitrile and 0.06% (v/v) trifluoroacetic acid in H_2O, at a flow rate of 25 μl/min to isolate NBD-derivatized glutamate (NBD-Glu) from the hydrolysates. The NBD-Glu was detected using a 3013 fluorescence detector (Shiseido) and automatically transferred to the second dimension. In the second dimension (*right panels* in *a* and *b*), NBD-Glu was enantioselectively separated with a Sumichiral OA-2500S column (250 by 1.5 mm; self-packed, material was from Sumika Chemical Analysis Service, Osaka, Japan) and detected by the second fluorescence detector.

step *d*) and the resulting hydrolysates are subjected to chiral HPLC with a CHIRALPAK MA(+) column (4.6 by 50 mm; DAICEL, Tokyo, Japan) (Fig. 2, step *e*), which is eluted isocratically with 2 mM $CuSO_4$ solution [5,6,8,13]. For data standardization, it is important to assay reactant blanks and negative controls. Typically, standard curves for D- and L-glutamate give a good linearity over a range of 0.5–100 nmol of glutamate, and the yield of PGA (usually as a μg order) can be calculated according to a definition in which the value 129 corresponds to the mole number of one glutamate unit of PGA. Table 2 includes selected recent data on the stereo-chemical compositions of PGA from some representative producers. Most recently, *B. subtilis* DL-*co*- and *N. aegyptiaca* l-*homo*-PGAs were investigated by micro-two-dimensional (2D) HPLC [29]. Fig. 5 reveals these typical chromatograms, suggesting that *B. subtilis* DL-PGA contains a large number of D-glutamyl residues, whereas such D-units coexist at only a trace ratio (<3%) in *N. aegyptiaca* L-PGA.

6. Conclusion

Current development of analytical techniques for PGA has highlighted the structural diversity of PGA. In fact, *B. megaterium* can over-produce L-*rich*-PGA, in which the L-isomer makes up over 90%of the polymer (Table 2), yet it is likely that eubacteria, including bacilli and staphylococci, produce polymers in which D-glutamate residues occur at a considerably high ratio (*e.g.*, DL-*co*- or D-*homo*-PGA) [2]. Regulation of PGA stereochemistry has become an important issue in the practical application of PGA [2,23,30] as well as being of interest in fundamental research on the precise synthesis of molecularly diverse chiral biopolymers [2,31]. To date, many techniques have been developed to estimate the molecular size of biopolymers, which has been found to be critical to function. For instance, *via* an SDS-PAGE assay, it was demonstrated that high-performance PGA from *B. subtilis* subsp. *chungkookjang* [9] is significantly higher than that from *B. subtilis* (*natto*) in average molecular size [8]. Industrial applications of PGA as a versatile chiral-polymer are in increasing demands. In future, the analytical approaches to PGA outlined in this article, and further improved analytic technology, will be required to assure the functional and structural reproducibility of this useful polymer.

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