Biological Synthesis of Monodisperse Derivatives of Poly(α,L-glutamic acid): Model Rodlike Polymers

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Poly(α,L-glutamic acid) (PLGA) and its derivatives have played a central role in the physical chemistry and materials science of polymers. PLGA itself has been used in fundamental studies of the helix–coil transition[1] and polyelectrolyte chemistry, and rodlike esters of PLGA—most notably poly(γ-benzyl α,L-glutamate) (PBLG)—have been shown to form liquid crystalline solutions[2] and beautifully ordered monolayer films.[3] The traditional synthetic route to PLGA[4] and its esters involves the ring-opening polymerization of *N*-carboxy-α-amino acid anhydrides. This technique affords heterogeneous populations of chains characterized by relatively broad distributions of molecular weights, and subsequent conversion of PLGA esters to the parent acid can be accompanied by substantial racemization.[5] The heterogeneity of the resulting products complicates the interpretation of their hydrodynamic,[6] spectroscopic,[7,8] and liquid crystalline[9] behavior and limits the utility of these polymers in the design and synthesis of complex macromolecular architectures. We report herein a general biosynthetic route to monodisperse derivatives of PLGA, unique rodlike polymers. The method is illustrated by the synthesis of polymer **1**.

$$\underset{\mathbf{1}}{\text{HGluAsp (Glu}_{17}\text{Asp)}_4\text{GluGluOH}}$$

The oligonucleotide duplex **2**, which encodes 17 glutamic acid residues followed by a single aspartic acid unit, was synthesized on a Milligen/Biosearch Model 8700 DNA synthesizer employing β-cyanoethyl phosphoramidite chemistry.[10] The design of the coding sequence reflects the fact that glutamic acid is encoded by two codons, GAA and GAG, with the former more abundant in native *Escherichia coli* genes.[11] The periodic aspartic acid residues provide recognition and cleavage sites for the restriction enzyme *Bbs*I, which is used to liberate the coding sequence after amplification and sequence verification (vide infra). Incorporation of *any* restriction site into an uninterrupted poly(glutamic acid) coding sequence would require that the remainder of the sequence consist of only a single codon, a situation we preferred to avoid because of concern about genetic stability. Aspartic acid was chosen as the second residue because of its structural similarity to glutamic acid, which is expected to reduce to a minimum any perturbation of the chemical and physical properties of the chain. The oligonucleotides were purified by electrophoresis on a 10% polyacrylamide denaturing gel, annealed, enzymatically phosphorylated at the 5′ termini, and ligated with *Bam*HI-digested, phosphatase-treated

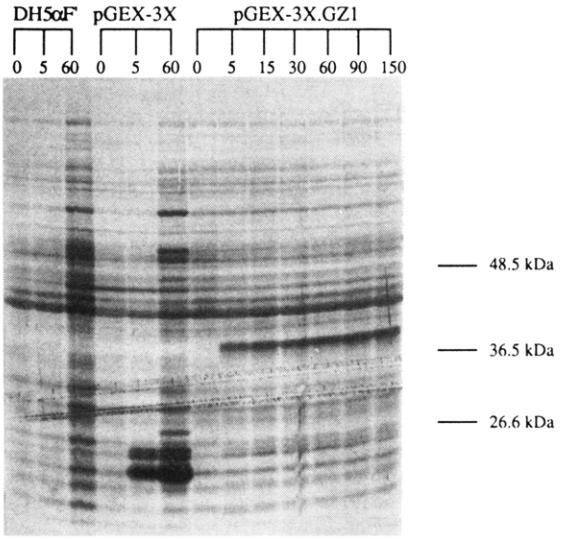

Figure 1. SDS–PAGE (12%) analysis of [^{35}S]-labeled proteins in cell lysates. Lanes 1–6 are negative controls. The nascent polypeptide derived from cells transformed with pGEX-3X.GZ1 migrates at the anticipated position (~36 kDa) in lanes 8–13. *E. coli* strain DH5αF′, DH5αF′ transformed with pGEX-3X, and DH5αF′ transformed with pGEX-3X.GZ1 were grown overnight at 37 °C in M9AA media[14] supplemented with vitamin B_1 and appropriate antibiotics. When OD_{600} reached 0.6–0.8, IPTG was added to a final concentration of 0.4 mM. [^{35}S]Methionine (Amersham International) was added 5 min before induction. Immediately before (t = 0) and at certain times (in minutes) after induction, a 1-mL cell culture was removed and cells were collected by centrifugation at 13 500*g* for 1 min and washed with 1 mL of YT to remove unincorporated radioactive methionine. Cells were lysed by adding a suitable amount (so that the cell concentration was 1.0 OD_{600}/mL) of the sample buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.01% bromophenol blue). Lysates were analyzed by discontinuous SDS–polyacrylamide gel electrophoresis using the method of Laemmli:[17] lanes 1–3, 20 μL of DH5αF′ (no plasmid control) cell lysates at time 0, 5, and 60 min; lanes 4–6, 20 μL of lysates from transformants carrying pGEX-3X (no insert control); lanes 7–13, 40 μL of lysates of cells transformed with pGEX-3X.GZ1. Lysates were heated at 100 °C for 3 min before being loaded. The gel was run at 10 mA for 12 h and then fixed in 50% methanol, 40% distilled deionized H_2O, and 10% acetic acid for 2 h. The gel was dried at 80 °C for 2 h and exposed to an X-ray film for 36 h. The molecular weight markers are prestained (Sigma Chemical Co.).

pUC18.[12,13] *E. coli* strain DH5αF′ cells were transformed with the recombinant plasmid and screened by insertional inactivation of the β-galactosidase gene, and the sequence of the insert was verified by the Sanger dideoxy sequencing strategy.[14] *Bbs*I digestion of the recombinant plasmid afforded a 54 base-pair fragment encoding $Glu_{17}Asp$ and bearing nonpalindromic termini. This DNA "monomer" was purified on a 10% polyacrylamide gel and self-ligated in head-to-tail fashion with T4 DNA ligase to yield a population of multimers. A portion of the ligation mixture was analyzed on a 1.5% agarose gel; multimers consisting of up to 20 monomer units were observed. The ligation mixture was cloned into the unique *Bbs*I site of pUC803, which was constructed by inserting adaptor sequence **3** at the *Bam*HI site of pUC18. The adaptor encodes a single methionine residue upstream of the *Bbs*I site to allow cyanogen bromide digestion of fragments fused to the N-terminus of the sequence of interest and a stop codon at the 3′-end of the coding region. DH5αF′ cells were transformed with the population of recombinant pUC803 plasmids.

A plasmid containing four repeats of the DNA monomer was isolated, and the nucleotide sequence of the tetramer

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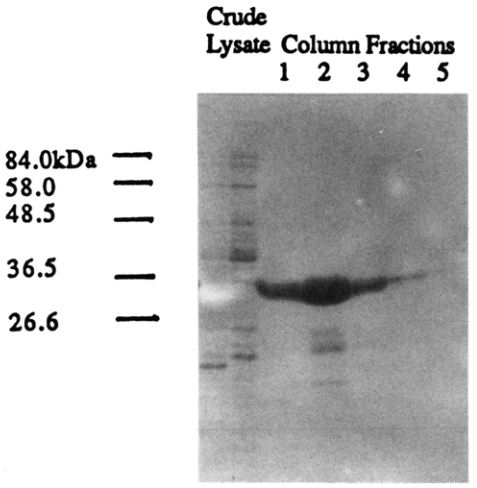


Figure 2. Purification of the fusion protein by affinity chromatography on glutathione-linked Sepharose 4B. Lanes 1 and 2, lysates of cells transformed with pGEX-3X and pGEX-3X.GZ1, respectively; lanes 3–7, column fractions eluted with 5 mM glutathione. A 1-L culture of *E. coli* DH5αF′ pGEX-3X.GZ1 was grown overnight in 2xYT medium at 37 °C. When OD_{600} reached 0.8, IPTG was added to a final concentration of 0.4 mM. Cells were harvested 2 h after induction by centrifugation at 5000*g* in 250-mL bottles. Pellets were resuspended in 20 mL of ice-cold PBS (150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, pH 7.3) and transferred to an SS-34 centrifuge tube. Cells were lysed by sonication, and Triton X-100 (10%) was added to a final surfactant concentration of 1%. Lysates were centrifuged at 13000*g* for 20 min at 4 °C, and the cleared supernatant was passed through a Sepharose 4B glutathione affinity column (2 mL, Pharmacia LKB) prewashed with 20 mL of PBS and equilibrated with 6 mL of PBS containing 1% Triton X-100. The column was washed twice with 10 mL of PBS, and the fusion protein was eluted with 10 mL of elution buffer (5 mM glutathione in 50 mM Tris-HCl, pH 8.0); the eluent was collected as five 2-mL fractions. Each fraction was lyophilized and redissolved in 500 μL of H_2O. A 10-μL portion of each fraction was mixed with 2x sample buffer and separated on a 12% SDS–polyacrylamide gel at 10-mA constant current for 12 h. The gel was stained with Coomassie Brillant Blue R-250 for 2 h and destained overnight.

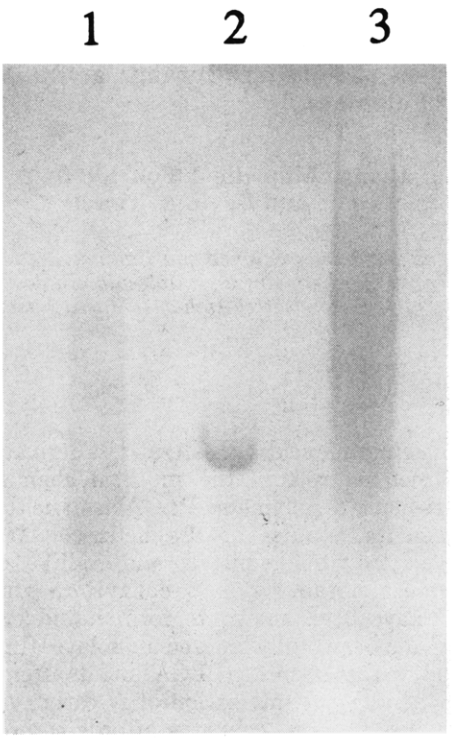
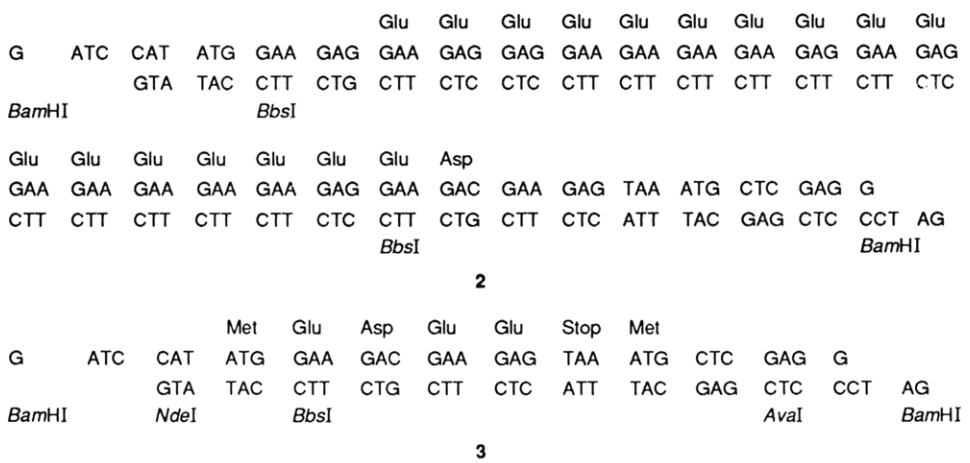


Figure 3. Comparison of molecular weight distribution of purified target polymer 1 with those of two commercial samples of PLGA on a 12% polyacrylamide gel. Lane 1, PLGA of molecular weight 9050 and polydispersity index 1.20 (Sigma Chemical Co.); lane 2, 1 (expected molecular weight 9760); lane 3, PLGA of molecular weight 21 000 and polydispersity index 1.38 (Sigma Chemical Co.). All molecular weights are based on the protonated side-chain form. Fusion protein GST$(E_{17}D)_4$ was digested for 24 h with cyanogen bromide in 70% formic acid.[18] Solvent was removed under vacuum. The powder was dissolved in 100 mM Tris-HCl, pH 8.0, and a portion of this solution was mixed with glycerol to a final glycerol concentration of 10%. The mixture was loaded on a 12% nondenaturing polyacrylamide gel using 0.01 M Na_2HPO_4 as the electrophoresis buffer. The gel was run at 25 mA for 2.5 h, stained in 0.01% methylene blue (pH 6.5) for 15 min, and destained in H_2O for 8 h with frequent changes of water. The bands corresponding to polymer 1 were excised from the gel and dialyzed first against 1 M NaCl to displace methylene blue and then against H_2O to remove salt. The sample was collected, lyophilized, and then dissolved in 100 mM Tris-HCl, pH 8.0. About 15 μg of the commercial polymers and 10 μg of polmer 1 were loaded on a 12% nondenaturing polyacrylamide gel. The electrophoresis, staining, and destaining procedures were the same as described above.

was confirmed by sequencing of the double-stranded DNA. The *Bam*HI segment was recovered, purified, and inserted into the expression vector pGEX-3X.[10] The ligation mix was used to transform DH5αF′ cells, and the presence and orientation of the insert were checked by digestion with *Bam*HI and *Ava*I, respectively.

Cells containing recombinant plasmid of the correct orientation (pGEX-3X.GZ1) were used to inoculate 2xYT media. Expression of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) during midlog growth (OD_{600} = 0.6–0.8) and monitored through in vivo incorporation of [^{35}S]methionine. The rate of cell growth was found to be normal prior to induction but then declined as a prominent new protein product of anticipated molecular weight (36 000) appeared in whole cell lysates analyzed on a 12% SDS–polyacrylamide gel



(PAGE; Figure 1). No such product was found in the crude lysates of cells lacking the artificial coding sequence.

Fermentation on a 12-L scale was done in a New Brunswick Scientific Microferm Fermenter. Cells were grown with vigorous aeration in a YT medium to $OD_{600} \sim 1.0$ at 600 nm, and protein synthesis was induced by adding IPTG to a concentration of 0.4 mM. Cells were harvested 2 h after induction by centrifugation at 4000*g* for 20 min at 4 °C.

After cell lysis, the fusion protein was purified by affinity chromatography on glutathione-linked Sepharose 4B.[16] Figure 2 shows the results of SDS–PAGE analysis of column fractions eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0; ca. 4 mg of purified protein was obtained per liter of fermentation medium. Chemical cleavage with CNBr in 70% formic acid followed by electrophoretic purification on a nondenaturing polyacrylamide gel afforded a product which migrates as a single band at the expected molecular weight and which yields amino acid analyses consistent with sequence 1. Figure 3 compares the electrophoretic behavior of 1 with that of the best commercial (i.e., chemically synthesized) samples of PLGA and illustrates strikingly the power of the biosynthetic strategy in controlling macromolecular architecture. We are pursuing studies of the solution and solid-state behavior of this new polymer, its chain-length variants, and its side-chain esters.

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