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POLY(ALPHA,L-GLUTAMIC ACID): SYNTHESIS OF ITS MONODISPERSE DERIVATIVES AND INTERACTION OF ITS ALKYLATED DERIVATIVES WITH PHOSPHOLIPID BILAYER MEMBRANES

A Dissertation Presented
by
GUANGHUI ZHANG

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

September 1994

Department of Polymer Science and Engineering
POLY(ALPHA,L-GLUTAMIC ACID): SYNTHESIS OF ITS MONODISPERSE DERIVATIVES AND INTERACTION OF ITS ALKYLATED DERIVATIVES WITH PHOSPHOLIPID BILAYER MEMBRANES

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ABSTRACT

POLY(ALPHA,L-GLUTAMIC ACID): SYNTHESIS OF ITS MONODISPERSE DERIVATIVES AND INTERACTION OF ITS ALKYLATED DERIVATIVES WITH PHOSPHOLIPID BILAYER MEMBRANES

SEPTEMBER 1994

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Directed by: Professor David A. Tirrell

A general strategy has been developed to synthesize biologically monodisperse polypeptides with the major repeat unit 1. These polymers are derivatives of poly(\(\alpha\),L-glutamic acid) (PLGA).

- Glu\(_{17}\)Asp - 1

Such polymers should adopt an \(\alpha\)-helical structure when the side chain carboxylate groups are protonated, since Glu has the highest helix forming propensity (\(P_\alpha=1.59\)) of all twenty natural amino acids. Polymer 2 was synthesized as a fusion protein with glutathione S-transferase (GST) in a bacterial host, and was liberated from the GST fragment by CNBr cleavage.

\[ \text{GluAsp(Glu}_{17}\text{Asp})_4\text{GluGlu} \quad 2 \]

DNA sequencing and amino acid analysis confirmed the composition of 2. Polymer 2 undergoes a conformational transition in aqueous solution from a random coil to an \(\alpha\)-helix as indicated by circular dichroism measurements. The \(\alpha\)-helical structure persists when the solvent is removed as demonstrated by Fourier transform infrared (FTIR) spectroscopy. Electrophoresis shows that polymer 2 is much more
homogeneous in terms of molecular weight than chemically synthesized PLGAs of comparable molecular weight.

A method was adopted to make the monodisperse rodlike molecule 3, a derivative of poly(\(\gamma\)-benzyl \(\alpha\),L-glutamate) (PBLG), by reacting 2 with phenyl diazomethane. Quantitative conversion was indicated by nuclear magnetic resonance spectroscopy. In helicogenic solvents for PBLG, 3 also assumes an \(\alpha\)-helical structure and transforms into a random coil when trifluoroacetic acid is added to the solution. Polymer 3 self-assembles into \(\alpha\)-helical structure when cast as a film from tetrahydrofuran solution. Gel permeation chromatography shows that 3 has a much narrower molecular weight distribution than chemically synthesized PBLG of comparable molecular weight.

\[
\text{Glu(OBzl)Asp(OBzl)}\{[(\text{Glu(OBzl)})_{17}\text{Asp(OBzl)}]\text{4Glu(OBzl)Glu(OBzl)} \quad 3
\]

where OBzl denotes a benzyl ester.

High molecular weight PLGA was chemically synthesized from \(\gamma\)-benzyl \(\alpha\),L-glutamate \(N\)-carboxy anhydride. This polymer was modified with 8 mol % or 15 mol % hexylamine. The modified polymers can disrupt dilauroylphosphatidylcholine multilamellar vesicles and egg yolk phosphatidylcholine small unilamellar vesicles in a manner which is dependent on the solution pH. Fluorescent probes, specifically pyrene and 8-anilino-naphthalene-1-sulfonic acid, ammonium salt, indicate that the modified polymers associate in a pH-dependent fashion and provide hydrophobic domains to solubilize lipid membrane vesicles.
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CHAPTER 1

SYNTHESIS OF MONODISPERSE DERIVATIVES OF POLY(α,L-GLUTAMIC ACID)

1.1 Introduction

1.1.1 Chemical Synthesis of Polymers

Macromolecular engineering,\(^1\) including protein engineering,\(^2\) is based on but transcends the chemical and/or biological synthetic methods of polymers. Designing a polymer that might demonstrate desired performance requires a thorough understanding of structure-property relationships. An evaluation of the behavior of designer polymer may, in turn, enrich our knowledge of molecular structural correspondence with mechanical, thermal, electrical, optical, or medical properties.

Chemical synthetic strategies are able to produce materials to meet a variety of needs. High performance polymers can be obtained with relative ease due to the tremendous advances of synthetic techniques. For example, to increase the temperature resistance of polymers, a high percentage of aromatic content can be incorporated into macromolecular structures.

Chemically synthesized polymers are characterized by heterogeneity of macromolecular species in terms of chain length, stereochemistry, comonomer sequence and overall compositions. A brief review of current status of polymer synthetic strategies proves that none of the available techniques can provide absolute control over molecular architecture.\(^3\) Step-growth and radical polymerization can neither yield polymers with narrow molecular weight distributions nor control
sequences and compositions if more than one monomer is involved. Living polymerization provides good, although not absolute, control over chain length, but can not dictate comonomer sequence and is useful only within a limited monomer group. Pure stereochemistry can be achieved by employing Ziegler-Natta strategies, but products from such reactions are very polydisperse. If coreactants are used, the resulting products are again mixtures of polymers with different sequences, compositions and molecular lengths. Polydispersity is intrinsically linked to synthetic polymers.

In many practical applications, molecular nonuniformity is itself advantageous and can help to achieve desired properties. For example, processability and transparency are often due to the presence of molecules of different lengths and configuration. But to accurately and precisely understand how molecular structure influences the bulk or surface characteristics, polymers of well-defined length, stereochemistry, sequence and composition should be synthesized.

1.1.2. α-Amino Acid-Based Polymers

1.1.2.1 Secondary Structures of Proteins

Our interest in choosing α-amino acids as building blocks arises from their wide array of chemical functional groups which are amenable to further modification, and from the fact that the resulting polymers can adopt secondary structures composed of helices, sheets and turns. The right handed α-helix is among the most common type of helices which include the 310-helix, the collagen-helix and more recently the β-helix. The stable α-helix (Figure 1.1) is characterized by intrachain hydrogen bonding between the C = O group of the n th residue and the N - H group of the n+4 th residue along the protein backbone. These hydrogen bonds are 2.86 Å from the
Figure 1.1 Structure of an α-helix. From Ref. 4.
oxygen atom to the nitrogen atom. The direction of this interaction is nearly parallel to the helix axis. It requires 18 residues to complete 5 turns with a pitch of 5.41 Å and a helix diameter of 4.6 Å. The torsion angles are favorable for most amino acid residues with \( \phi = -57^\circ \) and \( \psi = -47^\circ \). The amide group in the protein backbone has a partial negative charge on the O atom and a partial positive charge on the N-H group, making an effective amide dipole moment of 3.5 Debye. Since the peptide groups point in the same direction, the resulting α-helix is in fact a macrodipole. The dipolar character of the α-helix is believed to play a very important role in protein function.

In Figure 1.2, another regular conformation, the β-sheet structure, is shown. The β-sheet is made up of β-strands, with the protein backbone almost fully extended. Each residue is rotated 180° with respect to its adjacent amino acid, forming an equivalent of a 2\( \frac{1}{2} \) helix with a translation of 3.4 Å per residue. The β-strand is usually stabilized through interchain hydrogen bonding in parallel or antiparallel β-sheets.

Other common features of polypeptide conformation are turns and random coils. Reversal of the polypeptide backbone is often achieved by forming tight turns at the surfaces of molecules. β-hairpins serve as connectors of adjacent strands in an antiparallel β-sheet. More commonly observed β-turns are defined by four residues at positions identified as \( n \) to \( n+3 \). In these secondary structures, two residues at the turn position are not involved in hydrogen bonding (Figure 1.3). Random coils are just the opposite of the aforementioned nonrandom conformations. Depending on environment, polypeptides can assume different secondary structures, some of which are interconvertible upon changing temperature, solvent, ionic strength, or solution pH. In most proteins, 90% of the residues are implicated in either α-helices (38%), β-strands (20%), or reverse turns (32%).

Amino acids, when incorporated into peptide backbones, have different propensities for secondary structure formation. These probabilities are shown in Table
Figure 1.2 Structure of a β-sheet. From Ref. 4.
Classical γ turn

Inverse γ turn

Type I

Type I'

Type II

Type II'

Figure 1.3 Structures of turns. From Ref. 7.
1.1. Some residues are likely to be found in the α-helix conformation, while others prefer to stay in β-sheets, at turn positions or in random coils. These data can provide a guide to rational protein design, although the propensity of a specific amino acid for secondary structure changes depending on its location along the peptide chain.

1.1.2.2 Chemical Synthesis

Because of the biological importance\textsuperscript{12} and materials potential of amino acid-based molecules,\textsuperscript{13} controlled synthesis of peptides, proteins and polypeptides has been a subject of intensive study. The principal reaction of synthesis is the amide bond formation, or acylation of the amino group of the second amino acid (AA\textsubscript{2}) by the carboxyl group of the first amino acid (AA\textsubscript{1}). Solution synthesis\textsuperscript{14,15} of peptides of defined sequence usually requires protection of the amino group of AA\textsubscript{1} and the carboxyl group of AA\textsubscript{2}. If an amino acid with a side chain functional group is involved, this potentially reactive group must be blocked before carrying out the coupling reaction. Since direct amide bond formation is a slow reaction, the carboxyl group must be activated to become a strong acylating species. Reaction temperature should not be high in order to avoid racemization or other side reactions. If more amino acids are to be coupled, one of the protecting groups must be removed to react with incoming protected amino acids. The desired peptide is obtained by deblocking all protective groups.

Solid-phase peptide synthesis (SPPS) developed by Merrifield\textsuperscript{16,17} makes use of a polymeric support and provides ease of peptidyl polymer purification. One amino acid is attached to the polymer resin, and other amino acids are coupled to it. After the coupling reaction, the peptide is removed from the polymer resin and further purified.

As in solution synthesis, SPPS also necessitates extensive protecting and deprotecting steps, in addition to the limitation of resin functionality and accumulated error from
Table 1.1 Conformational preferences of amino acid residues. $^7$ $P_\alpha$, $P_\beta$ and $P_t$ are the preferences for an amino acid to reside in $\alpha$-helices, $\beta$-strands or reverse turns, respectively. Highlighted values indicate the highest possibility that an amino acid can be located at that particular conformation.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>$\alpha$-helix ($P_\alpha$)</th>
<th>$\beta$-sheet ($P_\beta$)</th>
<th>turn ($P_t$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>1.59</td>
<td>0.52</td>
<td>1.01</td>
</tr>
<tr>
<td>Ala</td>
<td>1.41</td>
<td>0.72</td>
<td>0.82</td>
</tr>
<tr>
<td>Leu</td>
<td>1.34</td>
<td>1.22</td>
<td>0.57</td>
</tr>
<tr>
<td>Met</td>
<td>1.30</td>
<td>1.14</td>
<td>0.52</td>
</tr>
<tr>
<td>Gln</td>
<td>1.27</td>
<td>0.98</td>
<td>0.84</td>
</tr>
<tr>
<td>Lys</td>
<td>1.23</td>
<td>0.69</td>
<td>1.07</td>
</tr>
<tr>
<td>Arg</td>
<td>1.21</td>
<td>0.84</td>
<td>0.90</td>
</tr>
<tr>
<td>His</td>
<td>1.05</td>
<td>0.80</td>
<td>0.81</td>
</tr>
<tr>
<td>Val</td>
<td>0.90</td>
<td>1.87</td>
<td>0.41</td>
</tr>
<tr>
<td>Ile</td>
<td>1.09</td>
<td>1.67</td>
<td>0.47</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.74</td>
<td>1.45</td>
<td>0.76</td>
</tr>
<tr>
<td>Cys</td>
<td>0.66</td>
<td>1.40</td>
<td>0.54</td>
</tr>
<tr>
<td>Typ</td>
<td>1.02</td>
<td>1.35</td>
<td>0.65</td>
</tr>
<tr>
<td>Phe</td>
<td>1.16</td>
<td>1.33</td>
<td>0.59</td>
</tr>
<tr>
<td>Thr</td>
<td>0.76</td>
<td>1.17</td>
<td>0.90</td>
</tr>
<tr>
<td>Gly</td>
<td>0.43</td>
<td>0.58</td>
<td>1.77</td>
</tr>
<tr>
<td>Asn</td>
<td>0.76</td>
<td>0.48</td>
<td>1.34</td>
</tr>
<tr>
<td>Pro</td>
<td>0.34</td>
<td>0.31</td>
<td>1.32</td>
</tr>
<tr>
<td>Ser</td>
<td>0.57</td>
<td>0.96</td>
<td>1.22</td>
</tr>
<tr>
<td>Asp</td>
<td>0.99</td>
<td>0.39</td>
<td>1.24</td>
</tr>
</tbody>
</table>
incomplete reactions, although SPPS is being used for widespread synthesis through automated instrumentation.

Despite tremendous advances in discovering and selecting protective groups,18 activating agents,19 and coupling additives,20,21 it remains impractical to make large peptides or proteins in good yield. Although the so-called segment condensation can produce large sequential polypeptides (of the structure AA1AA2AA3AA1AA2AA3AA1AA2AA3......),22 the molecular weight distribution of the resulting polymer is problematic.

Polypeptides of simple compositions can be synthesized within a short time by ring-opening polymerization of N-carboxy α-amino acid anhydrides (NCAs).23 Numerous homopolymers and copolymers have been made as model compounds to study solution and solid-state behavior of polypeptides. Polymers obtained through this strategy are characterized by relatively broad molecular weight distributions, and it is hard to control comonomer sequences and compositions.

Direct polycondensation of amino acids pioneered by Higashi et al.24 offers another pathway to synthesizing polypeptides of low molecular weight in the presence of N-phosphonium salts of pyridine. Direct coupling reactions can also be carried out by using diphenyl phosphoryl azide as the condensation agent.25 These types of reactions can be performed with ease, but the resulting polymers are heterogeneous in terms of molecular weight, monomer sequence and composition.

1.1.2.3 Recombinant DNA Technique

Template-directed polymerization under the catalysis of enzymes yields biopolymers of specified primary structure. DNA can be transcribed into mRNA with remarkable accuracy and mRNA can be translated into polypeptide with very high fidelity.4 Polymers based on α-amino acid building blocks should provide a prototype
for absolute control of molecular structural variables. Amino acids can be natural ones which have a wide array of chemical functional groups, and unnatural ones which are likely to afford unique bulk and surface properties. The resulting polypeptides can assume well-characterized higher order structures.

The widespread use of recombinant DNA technology is the result of three major scientific developments: the ability to introduce DNA into *E. coli* and to select infected bacteria, the capacity to obtain DNA in high yield and purity, and the discovery and purification of restriction enzymes. Other advances, such as automation of oligonucleotide synthesis, double-stranded DNA sequencing, construction of powerful expression plasmids, and polymerase chain reaction, speed up the application of recombinant DNA techniques. This method has also been applied to other expression systems including yeast and mammalian cells.

Figure 1.4 shows a general strategy to make polypeptides using this biological pathway. A complementary DNA (cDNA) corresponding to the desired polypeptide is first constructed. A plasmid vector with the necessary elements for high-level expression in *E. coli* (e.g., strong promoter, signals permitting efficient translation by ribosomes) is opened up at its polylinker region and recombines with the cDNA to make a hybrid plasmid. This newly formed plasmid is then introduced into bacterial cells. Once the inducing factor (either chemical or temperature) turns on mRNA synthesis, ribosomes will translate the message into a protein product. After lysing the cells, target protein can be separated from other compounds produced by the cells.

Steps from DNA to proteins are shown in Figure 1.5. The protein sequence is specified by triplets of nucleotides that comprise the genetic information in DNA. This code is transcribed in the form of a messenger RNA. The information is perpetuated as expressed protein using mRNA as the blueprint. Numerous peptides and proteins of biological and pharmacological importance have been produced by
Figure 1.4 General strategy to make polypeptides through biological pathways in E. coli. From Ref. 28.
Protein synthesis within cells is an elaborate process.

Figure 1.5 From DNA to proteins. From Ref. 29.
employing recombinant DNA methods. Expression of corresponding chemically
synthesized DNA has produced human insulin, human growth hormone and other
proteins.

Rational design of proteins has generated “Felix”, a four-helix bundle
protein. DeGrado et al. have designed and expressed the “α4” helix
bundle using a minimalist approach. A designer protein that has catalytic activity has
also been reported.

Polypeptides of materials potential have been designed and expressed in
bacteria. Noticing that repeats of hexapeptide GAGAGS are dominant in silk fibroin,
Cappello and coworkers successfully produced silk-like proteins based on
this repeat sequence and were able to show β-sheet formation through hydrogen
bonding. A commercial product, Pronectin™, is a copolymer that incorporates both
β-sheet forming sequence GAGAGS and the RGD cell binding domain of human
fibronectin. This material has been proven to adopt a conformation where the sheet
forming structures provide a rugged skeleton and the periodic cell attachment domains
demonstrate biological activities.

Extending their work on protein-based polymers synthesized via segmental
condensation, Urry et al. obtained elastomeric sequential polypeptides, G-(VPGVG)19-VPGV, through fermenting E. coli cells harboring the corresponding
gene cassettes. A structural component of the mussel adhesive protein has also been
expressed.

Incorporation of unnatural amino acids or amino acid analogs has been
demonstrated by Fournier and coworkers and Schultz and colleagues. The
former group was able to replace methionine residues quantitatively by
selenomethionine in a periodic polypeptide. The latter group devised a strategy to
incorporate dozens of analogs (including an α-hydroxy acid) into peptides and the
detailed methods were described in a recent review.
Tirrell et al. have been systematically investigating the solid state behavior of a series of repetitive polypeptides represented by the general formulae [(AG)mPEG]n49,50 and [(AG)pXG]q51,52 where X=E, D, V, N, etc. In the sequences, the alanylglucose diads are expected to form antiparallel β-sheets, and PEG and XG should constitute reverse turns as periodic β-sheet breakers. All of these polymers were expressed at high levels in bacteria. A wealth of information from structural analysis was obtained from such studies.

Difficulties in expression of desired polypeptides was reported.53,54 In spite of these failures, recombinant DNA technique has become an indispensable route to well engineered polypeptides.

1.1.3 Overview of Chapter 1

Chapter 1 of this thesis addresses the synthesis of monodisperse derivatives of poly(α,L-glutamic acid) (PLGA). Historically, PLGA and its derivatives have played a central role in polymer physical chemistry, molecular biology, and materials science. PLGA itself has been used in fundamental studies of the helix-coil transition and polyelectrolyte chemistry. The most notable γ-ester of PLGA - poly(γ-benzyl L-glutamate) (PBLG) has been subjected to even more extensive studies55 because of the ease of its chemical synthesis and its α-helical nature in many organic solvents and the solid state. To make monodisperse PBLG is a major focus of this project. Modifying PLGA is the subject of Chapter 2.

Theoretically, PLGA can be synthesized by any of the chemical methods mentioned above. In practice, PLGA is most readily synthesized by the NCA method and subsequent debenzylation. This topic will be discussed in more detail in Chapter 3.
The helix-coil transition of PLGA induced by changing solution pH is accompanied by changes of degree of ionization, specific rotation, and intrinsic viscosity.\textsuperscript{56} These experiments demonstrate that the helix-coil transition takes place through intermediate species containing both helical and coil states. Since polydisperse samples have always been used, ambiguity arises over this intermediate explanation or an all-or-none mechanism.

In studies of polyelectrolytes, poly(acrylic acid) and poly(styrene sulfonic acid) are mainly used as weak and strong acids, respectively. Even the latter with narrow molecular weight distribution shows broad distributions by electrophoresis. Monodisperse PLGA can be used as a model compound in studies of physical chemistry of polyelectrolytes, either in solution or in restricted geometries.\textsuperscript{57} Crystallization and monolayer studies can also benefit from this chemically pure macromolecule.

The potential application of PLGA as an ion sensing and ion channeling component in multiphase materials has been reported.\textsuperscript{58} Block copolymers of poly(styrene-co-acrylonitrile) and PLGA enable the latter to be immobilized onto electrodes. The modified electrode can respond to very small Ca\textsuperscript{2+} changes (10\textsuperscript{-6} M) due to PLGA and exhibits good stability due to the vinyl polymer chains.

All studies of PLGA to date have been based on polydisperse samples. This inherent character complicates the interpretation of the hydrodynamic, spectroscopic and crystalline behavior of the polymer. To fully understand the fundamental aspects of solution and solid state properties of PLGA, monodisperse materials are needed.

The conventional polymer synthetic methods can not provide PLGA of uniform chain length. A potential route to such materials is to apply the recombinant DNA technique described above. In bacteria, proteins with long stretches of glutamate residues have not been found, although segments of tandem glu residues have been reported in higher organisms. (Poly(γ-glutamic acid) obtained from
culturing bacteria *Bacillus subtilis* should not be confused with poly(α,L-glutamic acid)). Sugimoto et al. found a glutamic acid-rich protein from bovine retina, where 68 of 109 amino acids are glutamic acid and the longest run of glu is 4. Ambler listed some unusual sequences including Glu30 as the C-terminal domain of *Drosophila* troponin-T, Glu30 in a *Plasmodium* glutamic acid-rich protein, and runs of glu at the C-terminus of several vertebrate developmental proteins.

*In vivo* synthesis of longer runs or homopolypeptides of glutamic acid has not been reported. This chapter of the dissertation outlines the rationale for and difficulty in designing the target polypeptide with GluAsp as the major repeat unit and explains why we need to incorporate one foreign amino acid in a repetitive 18-amino-acid element. The Experimental Section details the procedures to obtain necessary oligonucleotides, plasmids, and finally proteins. Procedures are described for obtaining I.1 starting with frozen stock of cells transformed with the plasmid containing the corresponding artificial gene. Major results are given in the Results and Discussion section. Several conclusions are drawn from the experimental results.

\[
\text{GluAsp(Glu17Asp)4GluGlu} \\
\text{(or ED(E17D)4E2)}
\]

1.1

1.2 Experimental Section

1.2.1 Materials and Methods

Materials used and procedures performed in the experiment described in this chapter are listed and detailed in the following pages.
1.2.1.1 Materials

This part lists all the chemical reagents and their solutions used in the experiment. DNA and protein molecular weight standards, plasmids, and competent *E. coli* cells employed are also included in this section.

1.2.1.1.1 Reagents

All reagents and solvents used in the experiment described in this chapter are listed below. All materials were used as received unless otherwise indicated. Sources are given for all chemicals.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid, glacial, ACS grade</td>
<td>Fisher</td>
</tr>
<tr>
<td>Acetonitrile, anhydrous</td>
<td>Fisher</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Sigma</td>
</tr>
<tr>
<td>Adenosine 5'-triphosphate, disodium salt (ATP)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Agarose</td>
<td>Gibco BRL</td>
</tr>
</tbody>
</table>

Amino acids

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L</em>-Alanine</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Arginine</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Asparagine</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Aspartic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Cysteine</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Glutamic acid</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Glutamine</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>L</em>-Histidine</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
L-Isoleucine, Sigma
L-Leucine, Sigma
L-Lysine, Sigma
L-Methionine, Sigma
L-[35S]Methionine, DuPont NEN
L-Phenylalanine, Sigma
L-Proline, Sigma
L-Serine, Sigma
L-Threonine, Sigma
L-Tryptophan, Sigma
L-Tyrosine, Sigma
L-Valine, Sigma
Ammonium chloride, ACS grade, Fisher
Ammonium acetate, ACS grade, Fisher
Ammonium persulfate, Gibco BRL
Ammonium hydroxide, ACS grade, Fisher
Ammonium sulfate, ACS grade, Fisher
Ampicillin, sodium salt, Sigma
Bacto-agar, Difco
Bacto-tryptone, Difco
Bacto-yeast extract, Difco
Boric acid, ACS grade, Sigma
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), Gibco BRL
Bromophenol blue, Sigma
n-Butanol, Fisher
Calcium chloride, reagent grade, Fisher
Chloramphenicol, 99%, Sigma
Coomassie brilliant blue R-250
Cyanogen bromide
Deoxyadenosine 5'-α-[35S]thiotriphosphate, triethylammonium salt
Dimethylformamide, reagent grade
Dimethyldichlorosilane
Dithiothreitol (DTT)
Ethanol, 95% and 100%
Ethidium bromide, 10 mg/mL in H2O
Ethylendediaminetetraacetic acid, disodium salt (EDTA)
Formamide, ACS grade
Formic acid, 88%
Formic acid, 90%
Glucose
Glutathione, reduced form
Glycerol
Hydrogen chloride, reagent grade
Isopropanol
Isopropyl β-D-thiogalactopyranoside (IPTG)
Lithium chloride, ACS grade
M13/pUC sequencing primer (-40), 17 mer
Magnesium chloride, ACS grade
Magnesium sulfate, ACS grade
Methanol
β-Mercaptoethanol
N,N'-Methylene-bis-acrylamide
Methylene blue
Morpholino ethane sulfonic acid (MES)
3-(N-Morpholino) propane sulfonic acid (MOPS) Sigma
Oyster glycogen Sigma
Phenol, 99+% Sigma
Poly(ethylene glycol) (PEG), MW 8000 Sigma
Potassium acetate, reagent grade Fisher
Potassium hydroxide, reagent grade Fisher
Sodium acetate, reagent grade Fisher
Sodium chloride, reagent grade Fisher
Sodium dodecylsulfate (SDS), 99% Sigma
Sodium hydroxide Fisher
Sodium phosphate, dibasic Fisher
Sodium phosphate, monobasic Fisher
Spermidine, free base Sigma
\(N,N,N',N'\)-Tetramethylethylenediamine (TEMED) Sigma
Trizma base, (Tris[hydroxymethyl]aminomethane, Tris) Sigma
Triton X-100 Sigma
Urea Sigma
Vitamin B1 Sigma
Xylene cyanol Sigma
Zinc chloride Fisher

1.2.1.2 Enzymes

Restriction enzymes, phosphatase, ligase, kinase and protease were purchased from commercial sources. Concentrated buffers (10 X) that came with restriction enzymes were used directly. Values of Units are specific to the suppliers.
Avai, 10 U/μL
BamHI, 20 U/μL
BbsI, 5 U/μL
Calf intestinal alkaline phosphatase (CIP), 16 U/μL
Factor Xa
NdeI, 20 U/μL
T4 DNA ligase, 400 U/μL
T4 polynucleotide kinase, 10 U/μL
New England Biolabs
New England Biolabs
New England Biolabs
United States Biochemical
Boehringer Mannheim or New England Biolabs
New England Biolabs
New England Biolabs
New England Biolabs

1.2.1.1.3 Molecular Weight Standards for DNA and Protein

123 bp DNA ladder
1 Kb DNA ladder
Lambda DNA-BstEII digest
ϕX174 DNA-HaeIII digest
pBR322 DNA-MspI digest
Prestained kits for protein molecular weights (27 - 180 kDa)
Protein molecular weight standards, high range (15 - 200 kDa)
Protein molecular weight standards, low range (3 - 43 kDa)
Gibco BRL
Gibco BRL
New England Biolabs
New England Biolabs
New England Biolabs
Sigma
Gibco BRL
Gibco BRL

1.2.1.4 Stock Solutions

Most of the stock solutions were prepared in distilled, deionized water. Some of them were prepared in other solvents as indicated. Aqueous solutions were sterilized either by autoclaving at 120°C and 15 lb/sq. in. on liquid cycle for 20 minutes or by passing through a 0.2 μm filter (Nalgene).
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Concentration/Condition</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate</td>
<td>10 mM</td>
<td>Filter</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>10% (wt/v)</td>
<td>Use as made</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>200 mg/mL</td>
<td>Filter</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>25 mg/mL in 95% ethanol</td>
<td>Use as made</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>1 M</td>
<td>Filter</td>
</tr>
<tr>
<td>DTT</td>
<td>1 M</td>
<td>Filter</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>0.5 M, pH to 8.0 with NaOH</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Glucose</td>
<td>20% (wt/v)</td>
<td>Filter</td>
</tr>
<tr>
<td>Glycerol</td>
<td>80% (v/v)</td>
<td>Autoclave</td>
</tr>
<tr>
<td>IPTG</td>
<td>0.2 M</td>
<td>Filter</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1 M</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>1 M</td>
<td>Autoclave</td>
</tr>
<tr>
<td>MES</td>
<td>1 M, pH to 6.2 with KOH</td>
<td>Filter</td>
</tr>
<tr>
<td>MOPS</td>
<td>1 M</td>
<td>Filter</td>
</tr>
<tr>
<td>Poly(ethylene glycol) (PEG), MW 8000</td>
<td>40% (wt/v)</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Potassium acetate</td>
<td>3 M, pH to 4.8 with acetic acid</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>3 M, pH to 4.8 with acetic acid</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 M</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Sodium dodecylsulfate, SDS</td>
<td>10% (wt/v)</td>
<td>Filter</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>2 M</td>
<td>Use as made</td>
</tr>
<tr>
<td>Tris-HCl pH 8.8</td>
<td>1.5 M, pH to 8.8 with HCl</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>1 M, pH to 8.0 with HCl</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Tris-HCl pH 7.6</td>
<td>1 M, pH to 7.6 with HCl</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Tris-HCl pH 7.5</td>
<td>1 M, pH to 7.5 with HCl</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Tris-HCl pH 6.8</td>
<td>1 M, pH to 6.8 with HCl</td>
<td>Autoclave</td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>1.0% (wt/v)</td>
<td>Filter</td>
</tr>
</tbody>
</table>
Zinc chloride 0.1 M  Filter
X-gal 40 mg/mL in DMF Use as made

1.2.1.1.5 Other Solutions

Solutions needed for the separation and detection of DNA and proteins are listed below. They were usually various combinations of stock solutions. Concentrations were for working conditions.

1.2.1.1.5.1 GTE Solution
This solution was used for alkaline lysis miniprep of plasmid DNA.

- 50 mM Glucose
- 25 mM Tris-HCl, pH 8.0
- 10 mM EDTA, pH 8.0

Sterilize by filtration.

1.2.1.1.5.2 NaOH/SDS Solution
This solution was used for alkaline lysis miniprep of plasmid DNA.

- 200 mM NaOH
- 1 % SDS

Prepare fresh solution for each use.

1.2.1.1.5.3 TELT Solution
This solution was used for lithium miniprep of plasmid DNA.

- 50 mM Tris-HCl, pH 8.0
- 62.5 mM EDTA, pH 8.0
- 2.5 M LiCl
4% (v/v) Triton X-100
Sterilize by filtration.

1.2.1.1.5.4 Elution Buffer

This solution was used for recovering DNA fragments from polyacrylamide gel.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 mM</td>
<td>Ammonium acetate</td>
</tr>
<tr>
<td>10 mM</td>
<td>Magnesium acetate</td>
</tr>
<tr>
<td>1 mM</td>
<td>EDTA, pH 8.0</td>
</tr>
<tr>
<td>0.1%</td>
<td>SDS</td>
</tr>
</tbody>
</table>

Sterilize by filtration.

1.2.1.1.5.5 TE Solution

This solution was used for dissolving DNA and resuspending pelleted cells.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>Tris-HCl, pH 7.6</td>
</tr>
<tr>
<td>1 mM</td>
<td>EDTA, pH 8.0</td>
</tr>
</tbody>
</table>

Sterilize by filtration.

1.2.1.1.5.6 TFB 1 Buffer

This solution was used for preparing competent cells.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM</td>
<td>MES</td>
</tr>
<tr>
<td>100 mM</td>
<td>RbCl₂</td>
</tr>
<tr>
<td>10 mM</td>
<td>CaCl₂</td>
</tr>
<tr>
<td>10 mM</td>
<td>MnCl₂</td>
</tr>
</tbody>
</table>

All salts in solid form were added to distilled, deionized water. Acetic acid to adjust pH to 5.8 and sterilize by filtration. TFB 1 was stored at 4°C.
1.2.1.5.7 **TFB 2 Buffer**

This solution was used for preparing competent cells.

- 10 mM MOPS
- 75 mM CaCl₂
- 10 mM RbCl₂
- 15% Glycerol

All salts in solid form were added to distilled, deionized water. Adjust pH to 6.5 with acetic acid. Filter sterilize and store at 4°C.

1.2.1.5.8 **Phenol Solution**

This solution was used for alkaline lysis miniprep of plasmid DNA.

Redistilled phenol was warmed in a water bath at 60°C until molten. An equal volume of Tris-HCl (1 M, pH 7.5) was used to extract the melted phenol. The procedure was repeated until the pH of the aqueous phase was 7.5. 8-Hydroxyquinoline (10 mg/100 mL phenol) was added to stabilize the buffered phenol which then was divided into small aliquots and kept at 4°C in the dark.

1.2.1.5.9 **Acrylamide Solution**

This solution was used for DNA and protein gel electrophoresis.

- 190 g Acrylamide
- 10 g Bisacrylamide

Add distilled, deionized water to 500 mL. The solution was sterilized by filtration and stored in the dark at 4°C.

1.2.1.5.10 **10 X TBE Buffer**

This solution was used for DNA gel electrophoresis.
216 g  Tris base
110 g  Boric acid
18.6 g  EDTA

Add distilled, deionized water to 2 L.

1.2.1.5.11 5X Running Buffer

This solution was used for protein electrophoresis.

15.1 g  Tris base
94 g  Glycine
1 g  SDS

Add distilled, deionized water to 1 L.

1.2.1.5.12 2X Formamide Loading Buffer

This solution was used for gel electrophoresis of oligonucleotides.

10 mL  Formamide
10 mg  Xylene cyanol
10 mg  Bromophenol blue
0.2 mL  0.5 M EDTA pH 8.0

Thoroughly mix and store at room temperature.

1.2.1.5.13 10X Gel Loading Buffer

This solution was used for gel electrophoresis of double-stranded DNA.

25 mg  Xylene cyanol
25 mg  Bromophenol blue
5 mL  Glycerol
5 mL  Water

Thoroughly mix and store at room temperature.
1.2.1.5.14 2 X SDS-Gel Loading Buffer

This solution was used for gel electrophoresis of proteins.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl, pH 7.5</td>
<td>1 % SDS</td>
</tr>
<tr>
<td>1 % SDS</td>
<td>1 % β-mercaptoethanol</td>
</tr>
<tr>
<td>0.01 % Bromophenol blue</td>
<td>10 % Glycerol</td>
</tr>
</tbody>
</table>

Prepare fresh solution for each use.

1.2.1.5.15 Staining Solution

This solution was used for visualizing proteins on gel.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 g Coomassie brilliant blue R-250</td>
<td>45 mL Methanol</td>
</tr>
<tr>
<td>45 mL Methanol</td>
<td>10 mL Acetic acid</td>
</tr>
<tr>
<td>45 mL Water</td>
<td></td>
</tr>
</tbody>
</table>

Thoroughly mix and store at room temperature.

1.2.1.5.16 Destaining Solution

This solution was used for washing away unbound dye molecules.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 mL Methanol</td>
<td>10 mL Acetic acid</td>
</tr>
<tr>
<td>45 mL Water</td>
<td></td>
</tr>
</tbody>
</table>

Thoroughly mix and store at room temperature.

1.2.1.5.17 25 X Amino Acid Solution

This solution was used for preparing minimal media.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg Each of the 20 L-amino acids</td>
<td></td>
</tr>
</tbody>
</table>
Add distilled, deionized water and 2 M NaOH to adjust pH to 7, and final volume to 100 mL. Sterilize by filtration and store at 4°C in dark.

1.2.1.1.5.18 5 X M9 Salts

This solution was used for preparing minimal culture media.

- 64 g Na2HPO4•7H2O
- 15 g KH2PO4
- 2.5 g NaCl
- 5.0 g NH4Cl

Add distilled, deionized water to 1 L. Sterilize by autoclaving.

1.2.1.1.6 Plasmids

Plasmid DNA was usually first transformed into suitable cells which were stored as frozen stock, and then purified from overnight culture. On occasion, commercial plasmid DNAs were used directly.

- pUC18 (2686 bp), 500 μg/mL Pharmacia
- pGEX-3X (4952 bp), 500 μg/mL Pharmacia

1.2.1.1.7 Competent Cells

Commercial competent cells have transformation efficiency at least one order of magnitude higher than house-made competent cells for pUC and pGEX related vectors. When transforming house-made competent cells failed, 50 μL commercial cells were used to do transformation.
DH10B™ competent cells (MAX EFFICIENCY)

DH5αF™ competent cells (LIBRARY EFFICIENCY)

1.2.1.1.8 Culture Media

Nutrients were mixed in different ways to make up media for cell growth.

1.2.1.1.8.1 2X YT Medium

This was used as a complex medium for cell growth.

- 10 g Bacto-yeast extract
- 16 g Bacto-tryptone
- 5 g NaCl

Add distilled water to 1 L. Mix well and sterilize by autoclaving.

1.2.1.1.8.2 YT Medium

This was used as a complex medium for cell growth.

- 5 g Bacto-yeast extract
- 8 g Bacto-tryptone
- 5 g NaCl

Add distilled water to 1 L. Mix well and sterilize by autoclaving.

1.2.1.1.8.3 M9AA Medium

This was used as a minimal medium for cell growth.

- 40 mL 5 X M9 salts (see above)
- 8 mL 25 X amino acid solution (see above)
- 4 mL 20 % Glucose
- 400 μL 1 M MgSO₄
20 μL  1 M CaCl2
40 μL  1 % vitamin B1
160 mL  Sterilized H2O

This solution was mixed in a pre-sterilized container and made fresh for each experiment.

1.2.1.8.4 Media Containing Agar

Agar (15 g) was added to a liter of liquid medium and sterilized by autoclaving. When the mixture was cooled to ~ 50°C, suitable antibiotics were put into it. About 30 mL mix was poured into a petri dish. After the media solidified, the plates were stored in an inverted position at 4°C. Before plating, the plates were incubated at 37°C for two hr to remove moisture. Liquid culture was spread on the surface to allow the growth of colonies.

1.2.1.2 Routine Procedures and Measurements

The procedures of manipulating DNA, competent cells, and proteins were adopted from published literature62,63,64 and instructions supplied by manufacturers.

1.2.1.2.1 Preparation of Competent Cells

A single colony was used to inoculate 5 mL 2 X YT medium. The culture was then allowed to grow to saturation at 37°C. A portion (400 μL) of this saturated culture was again used to inoculate 20 mL fresh 2 X YT medium. This culture was incubated at 37°C on a shaker until OD600 reached 0.5 - 0.6 (about 3 hr). Cells were spun down in an SS-34 centrifuge tube for 5 min at 10 krpm and 4°C. After the supernatant was poured off, the cells were gently suspended in 6 mL cold TFB 1
buffer. This mixture was incubated on ice for 20 min and centrifuged as before. The collected cells were resuspended gently in 2 mL cold TFB 2 buffer. Aliquots (100 μL) were flash frozen in a dry ice-ethanol bath and then stored at -80°C.

1.2.1.2.2 Transformation of Competent Cells with Plasmid DNA

An aliquot (100 μL) of competent cells was thawed on ice and mixed with 10 μL ligation mixture (~10 ng DNA, 10 μL ddH2O). The incubation was performed on ice for 2 hr. The cells were heat-shocked at 43°C for 1.5 min, and placed back on ice for 10 min. Fresh 2 X YT medium (900 μL) was added to the transformation mixture and cells incubated at 37°C for 45 min to express antibiotic resistance. The cell mix (300 μL) was plated on sterile agar plates containing appropriate antibiotics. Plates were incubated at 37°C overnight.

1.2.1.2.3 Preparation of Frozen Cell Stock

A single colony from a transformation plate was used to inoculate 5 mL 2 X YT medium with appropriate antibiotics. This culture was incubated at 37°C overnight. A small portion (850 μL) of the cell culture was mixed thoroughly with 150 μL glycerol. The mixture was frozen in a dry ice-ethanol bath and stored in a -80°C freezer.

1.2.1.2.4 Isolation of Plasmid DNA from Cell Culture

Three techniques were used to isolate plasmid DNA from cell growth media. An alkaline lysis miniprep procedure followed by PEG treatment yielded the purest DNA, most suitable for sequencing and transformation. The lithium miniprep method
excelled at rapid screening for presence and orientation of insert but suffered severe RNA contamination. The commercial Maxi prep kits (Qiagen, Inc.) had the advantage of obtaining hundreds of μg pure plasmid DNA in short times.

1.2.1.2.4.1 Alkaline Lysis Miniprep65,66

A portion of saturated overnight culture (1.5 mL) was centrifuged at 14,000 rpm for 1 min at room temperature, and the supernatant decanted. The harvested cells were resuspended in 100 μL GTE buffer with vigorous vortexing. After incubation on ice for 5 min, the cells were lysed by gently mixing with 200 μL cold NaOH/SDS solution. The mixture was put on ice for 5 min. NaOAc (150 μL, 3 M, pH 4.8) was used to precipitate chromosomal DNA. The suspension was put on ice for another 5 min. The chromosomal DNA was removed by centrifugation at 14,000 rpm for 10 min and the supernatant transferred to a new tube. DNase-free RNase A (1 μL, 10 μg/μL) was added to the cleared supernatant and the reaction was incubated at 37°C for 40 min. Protein impurities were removed by extraction with 450 μL 1:1 phenol/chloroform mixture. The aqueous layer was collected in a new tube and the plasmid DNA was precipitated by adding 1 mL 100 % ethanol and subsequent incubation at -10°C overnight. The plasmid DNA was pelleted by centrifugation at 4°C (14,000 rpm), washed twice with 200 μL 70 % cold ethanol, dried in vacuo and redissolved in 30 μL ddH2O. PEG (10 μL, 25 %) containing 2.5 M NaCl was added to the solution and the mixture was incubated on ice for 30 min. The pure DNA was isolated by centrifugation, dried in vacuo and resuspended in 30 μL ddH2O. Typical yields for high copy number plasmids were 1.5 - 2 μg.

1.2.1.2.4.2 Lithium Miniprep67

All steps were performed at room temperature. Cells from 1.5 mL saturated culture were pelleted by centrifugation at 14,000 rpm for 30 sec. The supernatant was
discarded and the last trace of it was removed by a drawn-out Pasteur pipet connected to an aspirator. The cells were resuspended in 100 μL TELT, followed by addition of 100 μL phenol/chloroform (1:1 v/v). This mixture was vortexed vigorously for 15 sec and centrifuged at 13,500 x g for 1 min. The upper phase was carefully transferred to a new tube and mixed with 200 μL cold ethanol (100%). DNA was pelleted by centrifugation for 10 min, and the precipitate was washed with 1 mL cold 100 % ethanol. The pellet was dried under vacuum and redissolved in 30 μL TE. Typical yields for high copy number plasmids were 2 - 3 μg.

1.2.1.2.4.3 Maxi Prep

The protocol provided with this commercial plasmid Maxi prep kit (Qiagen, Inc.) was followed. All buffers were supplied with the kit. Briefly, cells from saturated culture (typically 250 - 500 mL) were pelleted and resuspended in 10 mL buffer P1 (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, and 100 μg/mL RNase A). Cells were lysed by adding 10 mL buffer P2 (200 mM NaOH, and 1 % SDS). Chromosomal DNA was removed by adding 10 mL buffer P3 (3.0 M KOAc, pH 5.5) and centrifugation at ≥ 30,000 x g for 30 min (4°C). The supernatant was centrifuged again at ≥ 30,000 x g for 15 min (4°C). The supernatant was passed through (gravity flow) a prepacked anion exchange column which was preequilibrated with 10 mL buffer QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15 % Triton X-100). The column was washed twice with 30 mL buffer QC (1.0 NaCl, 50 mM MOPS, 15 % ethanol, pH 7.0). Plasmid DNA was eluted with 15 mL buffer QF (1.25 M NaCl, 50 mM Tris-HCl, 15 % ethanol, pH 8.5), and precipitated by 10.5 mL isopropanol. The suspension was stored at -20°C overnight (although this step was not recommended by the protocol accompanying the kit). The DNA was pelleted at ≥ 15,000 x g for 30 min (4°C), dried briefly, and redissolved in 1 mL TE, pH 8.0. From 250 mL culture, ~ 500 μg high copy number plasmid DNA can be obtained.
1.2.1.2.5 Double-Stranded DNA Sequencing

After recovery of recombinant plasmids, or when plasmids were reused, double-stranded DNA sequencing methods were used to verify the sequences of interest. The dideoxy method of Sanger was followed, although the exact steps were those suggested by suppliers.

Commercial kits (T7 Sequencing™ (Pharmacia) and Sequenase™ (United States Biochemical)) were employed and the accompanying protocols followed exactly to determine the sequences of inserts. Briefly, plasmid DNA was denatured and a complementary oligonucleotide (17 mer) was annealed to the plasmid 40 bases upstream from the insert site. The oligonucleotide was extended, using one strand of the plasmid as template, by incorporation of dATP (labeled with [35S] at α position), dCTP, dGTP and dTTP, under the catalysis of DNA polymerase. After a certain time, the reaction mixture was divided into separate tubes containing ddATP, ddCTP, ddGTP, or ddTTP, respectively, to terminate chain extension. The four mixtures were loaded on a denaturing urea-polyacrylamide gel at elevated temperature. The gel was dried and exposed to an X-ray film. The artificial gene sequences were read manually from the gel patterns.

1.2.1.2.6 Recovery of DNA Fragments from Enzyme Digest

Target DNA fragments from enzyme digestions or from the DNA synthesizer were isolated in pure form using two methods. For DNA monomer or small multimers, the “chop and soak” technique was used. Large DNA fragments, such as dephosphorylated plasmid, was recovered from agarose gel by the QIAEX extraction method (Qiagen, Inc.)
1.2.1.2.6.1 Chop and Soak Method

This is a modification of the so-called "crush and soak" method. (Because crushed gel pieces tend to swell too much, recovery of DNA-containing buffer is only minimal.) The enzyme reaction mix was loaded on a polyacrylamide gel which was then stained in distilled water containing ethidium bromide. After destaining the gel and locating the target DNA band, the gel slice was cut out, finely chopped, and soaked in elution buffer in a microcentrifuge tube. The slurry was incubated at 37°C overnight with shaking. The mixture was loaded on a microcentrifuge filter (Sigma) and the solution was collected in the accompanying tube. To this tube, 1/50 volume of oyster glycogen (10 mg/mL), and 2.5 volumes of absolute ethanol were added. Following incubation at -10°C overnight, the monomer was pelleted, washed, dried, and redissolved in TE.

1.2.1.2.6.2 QIAEX Agarose Gel Extraction Method

This commercial kit (Qiagen, Inc.) offers speed and high yield for recovery of large DNA fragments. The supplied protocol was followed exactly. Briefly, the enzyme reaction mix was loaded on a 1% agarose gel. The gel slice containing target DNA was cut out and put in buffer QX1 (3 M NaI, 4 M NaClO4, 5 mM Tris-HCl, pH 7.5, and 0.1 % Na2SO4) in a microcentrifuge tube. The gel was melted at 50°C in the presence of the proprietary "QIAEX suspension" containing DNA absorption beads. The DNA-bound beads were collected by centrifugation, washed twice with buffer QX2 (8 M NaClO4), and twice with buffer QX3 (70 % ethanol, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.5). DNA was separated from the beads simply by washing with ddH2O.
1.2.1.2.7 Quantification of DNA

Amounts of double-stranded DNA and oligonucleotides were measured by recording the absorption at 260 nm (path length 1 cm) on a Hitachi UV-Vis Spectrophotometer. A portion of DNA stock was diluted in 1 mL ddH2O, and OD260 was measured. The concentration of the stock was calculated using the following correspondence: 1.0 OD260 = 50 μg/mL for double-stranded DNA; 1.0 OD260 = 40 μg/mL for single-stranded DNA; and 1.0 OD260 = 20 μg/mL for single-stranded oligonucleotides.

A reading of OD280 was also taken if the purity of DNA was measured. A ratio OD260/OD280 of 1.8 ~ 2.0 corresponds to a protein-free DNA preparation.

DNA amounts were readily estimated by comparing the band intensity stained with ethidium bromide with that of approximately equally positioned DNA bands from molecular weight standards under UV light. The intensity ratio was estimated, and the amount of DNA standard was used to determine the amount of target DNA under the assumption that DNAs of equal mass give equal intensities.

1.2.1.2.8 Estimation of Cell Numbers

The number of cells in growth medium was estimated by measuring OD600 on a Hitachi UV-Vis Spectrophotometer. With 1 cm path length, 1.0 OD600 = 10^9 cells/mL for E. coli.

A linear relationship of OD600 with cell number was assumed regardless of growth phase or possible cell aggregation.
1.2.2 Construction of Artificial Genes

1.2.2.1 Synthesis and Purification of Oligonucleotides

Coding and anti-coding oligonucleotides were synthesized on a Milligen/Biosearch Model 8700 DNA Synthesizer employing the β-cyanoethylphosphoramidite chemistry developed by Caruthers et al.\(^6\) Syntheses were carried out on a 1.0 μmole scale using a controlled pore glass support functionalized with a guanine which is the 3' terminal base of the oligonucleotide sequences. The oligonucleotides were synthesized from 3' to 5'. At five positions, mixed bases were used on an equal weight basis. Crude oligonucleotides were cleaved from the supports by treating with concentrated NH\(_4\)OH at 65°C overnight. After removal of the glass support by centrifugation, the solutions were dried on a SpeedVac. The resulting pellet was suspended in 500 μL ddH\(_2\)O. The suspension was centrifuged for 2 min to remove particulate matter. The supernatant was transferred to a new tube and dried again. The pellet was dissolved in 400 μL ddH\(_2\)O. The quantification of the products was based on the optical absorbance measurements at 260 nm of diluted samples.

Coding strand (Oligo 1, 89 bases, crude yield = 7.84 mg, 34.3 %):


Anticoding strand (Oligo 2, 89 bases, crude yield = 4.71 mg, 16.3 %):

where \([A,G] = A\) and \(G\) mixed on an equal feed (weight) basis, \([T,C] = T\) and \(C\) mixed on an equal feed (weight) basis.

Preparative gel electrophoresis was used to purify the crude oligonucleotides. Portions (100 µL) of solutions containing Oligo 1 and Oligo 2 were mixed with 2 X formamide loading buffer. The mixtures were heated to 95°C for 5 min prior to loading on a 10 % polyacrylamide gel containing 50 % urea (8.3 M). The mixtures were electrophoresed at 350 constant volts for 3 hr, after which the product bands were visualized via ultraviolet shadowing and excised from the gel. The coding and anti-coding oligonucleotides were recovered by the chop and soak method and were redissolved in 50 µL ddH2O.

1.2.2.2 Annealing and Phosphorylation of Oligonucleotides

Purified oligonucleotides (6.6 µg each, 19.4 µL of Oligo 1, 30 µL of Oligo 2) were mixed in 100 µL of 100 mM NaCl and 20 mM MgCl2. The mixture was first put on a 95°C sand bath for 2 min, and transferred to an 80°C water bath which was allowed to cool gradually to room temperature over a 10 hr period, followed by incubation at 15°C for 1 hr. Oyster glycogen (20 µL, 10 µg/µL), NaOAc (12 µL, 3 M, pH 4.8) and ethanol (300 µL, 100 %) were added to the annealing reaction, and the mixture was incubated at -10°C overnight. The approximate yield of the duplex was 12 µg based on comparison of fluorescence intensities with those of DNA fragments from pBR322-MspI digestion.

The annealed oligonucleotides (200 pmoles) were dissolved in 10 µL 10 X kinase buffer (New England Biolabs), 10 µL 10 mM ATP and 75 µL ddH2O. T4 polynucleotide kinase (5 µL, 50 units) was added last and the reaction was incubated at 37°C for 1 hr. The reaction was heated to 70°C for 5 min, extracted with 100 µL of phenol/chloroform (1:1, v/v) and then 100 µL chloroform. The duplex was
precipitated by adding NaOAc (10 μL, 3 M, pH 4.8) and ethanol (250 μL, 100%). The pellet was washed with 70% ethanol and redissolved in 100 μL ddH2O.

1.2.2.3 Insertion of DNA Duplex into pUC18

pUC18 (10 μg, Figure 1.6) was digested with 5 μL BamHI restriction enzyme (50 Units) in a total volume of 50 μL at 37°C overnight. NaOAc (5 μL, 3 M, pH 4.8) and 2 volumes of 100% ethanol were added to precipitate the DNA. The collected pellet was washed, briefly dried and redissolved in 44 μL of buffer solution containing 50 mM Tris-HCl, pH 8.0, 1 mM ZnCl2, 1 mM MgCl2 and 1 mM spermidine. Calf intestine alkaline phosphatase (CIP, 1 μL, 16 Units) was added to the solution which was incubated at 37°C for 60 min. The dephosphorylation reaction was terminated by adding 10 μL 10X STE (100 mM Tris-HCl, pH 8.0, 1 mM NaCl, 10 mM EDTA), 35 μL ddH2O and 5 μL SDS (10%). After the reaction was heated to 70°C for 15 min, it was extracted with phenol/chloroform (100 μL, 1:1, v/v) twice and chloroform twice. The linearized vectors were further purified by electrophoresis on a 1% agarose gel, and recovered by the QIAEX extraction method.

Into a microcentrifuge tube were added dephosphorylated BamHI digested pUC18 (4 μL, 500 ng), oligonucleotide duplex solution (2 μL, 240 ng), 5 μL 10X ligase buffer, 5 μL 10 mM ATP, 33 μL ddH2O and lastly 1 μL T4 DNA ligase. The reaction was incubated on ice for 10 min and slowly brought to 14°C overnight. The resulting recombinant plasmids were designated as pUC18-89m.

1.2.2.4 Transformation of DH5αF' with pUC18-89m

An aliquot (100 μL) of competent cells was thawed on ice and mixed with 10 μL pUC18-89 ligation solution and 10 μL ddH2O. The incubation was performed on
Figure 1.6 General cloning vector pUC18.
ice for 2 hr. The cells were heat-shocked at 43°C for 1.5 min, and placed on ice for 5 min. Fresh 2 X YT medium (900 μL) was added to the transformation mixture and the cells were incubated at 37°C for 30 min to express antibiotic resistance. The cell mix (300 μL) was plated on sterile agar/YT plates containing ampicillin (200 μg/mL), isopropyl β-D-thiogalactoside (100 μg/mL) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (200 ng/mL). Plates were incubated at 37°C overnight for blue/white screening.

1.2.2.5 Isolation of pUC18-89m

Twenty white colonies were chosen and used to inoculate 5 mL aliquots of sterile 2 X YT media containing 200 μg/ml ampicillin. The cultures were incubated to saturation overnight at 37°C. Plasmid DNA was isolated by a modified alkaline lysis miniprep and resuspended in 30 μL ddH2O. The recombinant plasmid from a portion of this solution (17 μL) was digested with BamHI to check the presence of insert. The enzyme digestion showed that all twenty colonies contained inserts of anticipated size.

1.2.2.6 Double-Stranded Sequencing of pUC18-89m

Six colonies with recombinant plasmid were chosen for sequencing with M13mp18 as a control using the Sanger dideoxy sequencing strategy. The T7Sequencing™ Kit supplied by Pharmacia LKB Biotechnology was used and the accompanying protocol followed exactly. A -40 universal primer was annealed to the denatured template. The sequencing mixture were run on a 6% polyacrylamide gel containing 50% urea at 60 W constant power for 90 min. The gel was transferred onto a Whatman 3 MM filter paper, dried in vacuo and exposed to a sheet of X-ray film for 48 hr. Of the six plasmids chosen, only one gave unambiguous coding strand
sequence information as shown below, and the colony containing this insert was used to do the experiments described herein. The plasmid with this insert sequence was designated as pUC18-89.

5' - G ATC CAT ATG GAA GAC GAA GAG GAA GAA GAA GAG GAA GAA GAG GAA GAG GAA GAA GAG TAA ATG CTC GAG G -3'

1.2.2.7 Large Scale Preparation of DNA Monomer

The colony containing pUC18-89 was used to inoculate 5 mL 2 X YT + amp medium and the culture was allowed to grow overnight. This saturated culture was used to inoculate 1 L 2 X YT + amp. After 16 hr, the cells (from 500 mL culture) were harvested and plasmid was separated from the lysed cells following the Qiagen Maxi Plasmid Prep protocol. The pUC18-89 was dissolved in 1 mL ddH2O.

pUC18-89 (50 µL, 1 µg/µL) was mixed with 50 µL 10 X BbsI buffer and 350 µL ddH2O. BbsI enzyme (50 µL, 250 U) was added last and the reaction was incubated at 37°C overnight. The digest was directly loaded on an 8 % polyacrylamide gel which was run at 250 constant volts for 1.5 hr. The monomer bands were visualized by ethidium bromide staining and cut out from the gel, finely chopped and soaked in 2 mL elution buffer. The mixture was incubated at 37°C overnight with constant shaking. The slurry was passed through a microcentrifuge separation tube and washed twice with elution buffer. To the combined solution (2 mL) were added 40 µL oyster glycogen and 5 mL 100 % ethanol. Following incubation at -10°C overnight, the monomer was pelleted, washed, dried and redissolved in 20 µL TE. The same procedure was repeated to purify more monomer to give a combined volume of 40 µL.
1.2.2.8 Generation of Multimers

A portion of the monomer solution (32 μL) was mixed with 4 μL 10 X ligation buffer, and 4 μL 10 mM ATP. T4 DNA ligase (1 μL, 400 U) was added and the reaction was incubated at 14°C. After 4.5 hr, more T4 DNA ligase (1.5 μL, 600 U) was added to the reaction and the incubation continued for another 20 hr. A portion of the ligation mixture (27 μL) was run on a 1.5 % agarose gel. The multimers were divided into several fractions each of which contained ~40 ng DNA. The multimer band was recovered by the QIAEX agarose gel extraction method.

1.2.2.9 Insertion of Multimers into pUC803

pUC803 is equivalent to pUC18-89 minus the 54 bp BbsI fragment (803 was the room number of the laboratory where the vector was first constructed.) Linearized pUC803 was obtained by digesting pUC18-89 with BbsI, purifying by the QIAEX agarose gel extraction method, and dephosphorylating by CIP treatment as in the case of pUC18. The ligation between pUC803 and the multimer fraction was typically performed as follows: into a microcentrifuge tube containing a multimer fraction (~40 ng, in a volume of 14 μL) were added 1 μL BbsI digested, dephosphorylated pUC803 (20 ng), 2 μL 10 X ligation buffer, 2 μL 10 mM ATP, and 1.5 μL T4 DNA ligase. The reaction was incubated at 14°C overnight. The recombinant plasmid derived from pUC803 and a multimer is designated as pUC803-multimer.

Insertion of multimers into pUC803 was also performed by direct ligation of BbsI digested, phosphatase-treated pUC803 (30 ng) with multimer mix (4 μL) in a 20 μL reaction volume.
1.2.2.10 Transformation of DH5αF' with pUC803-multimer

Recombinant plasmid pUC803-multimer (in 15 μL out of 20 μL ligation mix from the last step) was introduced into competent E. coli DH5αF' cells the same way as described before. Nine colonies appeared on the transformation plate.

1.2.2.11 Restriction Analysis and Sequencing of pUC803-multimer

Plasmids from the 9 colonies were digested with BamHI to check the presence and the size of the insert. The largest multimer found was a tetramer. The double-stranded sequencing method was used to verify the nucleotide sequence of the tetramer. The recombinant plasmid with correct tetramer sequence was designated as pUC803-tetramer.

1.2.2.12 Purification of the Target Artificial Gene

pUC803-tetramer was digested with restriction enzyme BamHI and the digestion mixture was loaded on an 8 % polyacrylamide gel under constant 300 V. The band corresponding to the artificial gene was cut from the gel, and purified by the chop and soak method.

1.2.3 Expression of the Artificial Gene

1.2.3.1 Insertion of the Artificial Gene into pGEX-3X

pGEX-3X (10 μg, Figure 1.7) was digested with 5 μL BamHI in a total volume of 50 μL at 37°C overnight. NaOAc (5 μL, 3 M, pH 4.8) and 2 volumes of 100 %
Figure 1.7 Expression vector pGEX-3X.
ethanol were added to precipitate DNA. The collected pellet was washed, briefly dried on a SpeedVac and redissolved in 44 μL of buffer solution containing 50 mM Tris-HCl (pH 8.0), 1 mM ZnCl2, 1 mM MgCl2 and 1 mM spermidine. Calf intestine alkaline phosphatase (1 μL, 16 U) was added to the solution which was incubated at 37°C for 60 min. The dephosphorylation reaction was terminated by adding 10 μL 10X STE (100 mM Tris-HCl, pH 8.0, 1 mM NaCl, 10 mM EDTA), 35 μL ddH2O and 5 μL SDS (10%). After the reaction was heated to 70°C for 15 min, it was extracted with phenol/chloroform (100 μL, 1:1, v/v) twice and chloroform twice. The linearized vectors were further purified by the QIAEX extraction method from 1 % agarose gel.

The ligation of pGEX-3X and the artificial gene was carried out in the standard way. The dephosphorylated vector (~ 150 ng), the tetramer (~ 50 ng), and T4 DNA ligase (1 μL) were mixed in 20 μL of 50 mM Tris-HCl (pH 8.0), 20 mM DTT, 10 mM MgCl2 and 1 mM ATP. The incubation was done at 14°C overnight.

1.2.3.2 Transformation of DH5αF' with pGEX-3X-tetramer

Competent *Escherichia coli* strain DH5αF' cells were transformed with pGEX-3X-tetramer as described previously.

1.2.3.3 Verification of Construction

Recombinant plasmid DNAs (pGEX-3X-tetramer) were isolated from saturated cultures inoculated with colonies (20) using the same protocol as described above. *Bam*HI and *Ava*I digestion were used to determine the presence and the orientation of the tetramer insert. All 20 plasmids contained the tetramer and 7 of them incorporated inserts in the correct reading frame. One colony containing the
plasmid with correct insert orientation (designated as pGEX-3X.GZl) was used to do expression experiments.

1.2.3.4 Expression of pGEX-3X.GZl in Rich Medium

Cultures inoculated with single colonies of pGEX-3X.GZl transformed DH5αF', pGEX-3X transformed DH5αF' (a no insert control) and DH5αF (a no plasmid control) were allowed to grow overnight under antibiotic selection. A portion of each of these saturated cultures (500 μL) was used to inoculate 10 mL 2 X YT + amp which was then incubated at 37°C with vigorous aeration. These cultures were grown to OD₆₀₀ ~ 0.5 before isopropyl-β-D-thiogalactopyranoside(IPTG) was added to a final concentration of 0.4 mM. The culture growth was monitored for the next three hours.

Immediately before and after the addition of IPTG, portions (1 mL) of the growing culture were removed. The cells were separated from the medium by centrifugation at 13,500 x g for 1 min, washed with 1 mL YT, and lysed in a suitable amount of 1 X SDS gel-loading buffer (10 mM Tris-HCl, pH 7.5, 10 % glycerol, 1 % SDS, 1 % β-mercaptoethanol and 0.1 % bromophenol blue) such that cell concentrations were the same. Lysates were heated to 95°C for 10 min, and 20 μL of each was loaded on a 12 % discontinuous SDS polyacrylamide gel prepared by the method of Laemmli.70 The gel was run at 10 mA constant current for 12 hr, and then was stained with Coomassie brilliant blue R-250 for 2 hr and destained overnight.

1.2.3.5 Expression of pGEX-3X.GZl in Minimal Medium

Cells of DH5αF', pGEX-3X transformed DH5αF' and pGEX-3X.GZl transformed DH5αF' from frozen stocks were used to inoculate 5 mL fresh M9AA
medium supplemented with vitamin B1 (2 x 10^{-4} \%) and the cultures were allowed to grow overnight at 37°C with appropriate antibiotics. Portions (200 \mu L - 500 \mu L) of these saturated cultures were used to inoculate 10 mL M9AA in 200 mL Erlenmeyer flasks to make the initial OD600 0.10 - 0.15. The cultures were incubated at 37°C with vigorous aeration. When OD600 reached 0.6 - 0.8, a stock solution of IPTG was added to the medium to make a final concentration of 0.4 mM. [^{35}S]Methionine was added 5 min before the induction. Immediately before (t = 0) and at certain times after induction, 1 mL aliquots of cell culture were removed and cells were collected by centrifugation at 13,500 x g for 1 min. The supernatant was discarded and the cells washed with 1 mL 1 X YT to remove unincorporated radioactive methionine. Cells were again collected by centrifugation and lysed by adding a suitable amount (so that the cell concentration was 1.0 OD600/mL) of 1 X sample buffer (10 mM Tris-HCl pH 7.5, 10 \% glycerol, 1 \% SDS, 1 \% β-mercaptoethanol and 0.01 \% bromophenol blue). The lysates were analyzed on a 12 \% discontinuous SDS polyacrylamide gel by the method of Laemmli. The lysates were heated at 100°C for 3 min before being loaded. The gel was run at 10 mA for 12 hr and then fixed in fixation solution (50 \% methanol, 40 \% ddH2O and 10 \% acetic acid) for 2 hr. The gel was dried at 80°C for 2 hr and exposed to an X-ray film for 36 hr. The molecular weight markers are prestained. The protein corresponding to the new bands was designated as GST-ED(E17D)4E2.

1.2.4 Protein Purification

1.2.4.1 GST-ED(E17D)4E2 through Affinity Chromatography

A frozen stock of pGEX-3X.GZ1 transformed DH5αF' cells was used to inoculate 100 mL 2 X YT medium and were allowed to grow overnight at 37°C. This
saturated culture was mixed with 900 mL 2 X YT. The culture was incubated at 37°C with vigorous agitation. When OD600 reached 0.8, IPTG was added to make the final concentration 0.4 mM. After 2 hr of further fermentation, cells were harvested by centrifugation at 5,000 x g in 250-mL bottles. Pellets were resuspended in 20 mL ice-cold PBS (150 mM NaCl, 16 mM Na2HPO4 and 4 mM NaH2PO4, pH 7.3) and the suspension was transferred to a SS-34 centrifuge tube. The cells were lysed by three 1-min intervals of sonication (45 W). Triton X-100 (10 %) was added to the lysates to make the final concentration 1 %. The lysates were centrifuged at 13,000 x g for 20 min at 4°C and the supernatant transferred to a clean tube. The cleared solution was passed through a prepacked glutathione Sepharose® 4B affinity column (2 mL, Pharmacia LKB) which was first washed with 20 mL PBS to remove preservative and then equilibrated with 6 mL PBS + 1 % Triton X-100. After the supernatant was applied to the column, it was washed twice with 10 mL PBS. The fusion protein was eluted by adding 10 mL elution buffer (5 mM glutathione in 50 mM Tris-HCl pH 8.0) and the eluent was collected as five 2-mL fractions. Each fraction was lyophilized and redissolved in 500 μL ddH2O. Portions (10 μL) were mixed with 2 X SDS sample buffer and loaded on a 12 % SDS-polyacrylamide gel. The gel was run at 10 mA constant current for 12 hr, stained with Coomassie Brilliant Blue R-250 for 2 hr and destained overnight. Fractions 1, 2, and 3 contained most of the polymer GST-ED(E17D)4E2.

1.2.4.2 Enzymatic Digestion of GST-ED(E17D)4E2 by Factor Xa

GST-ED(E17D)4E2 (200 μg) was dissolved in 200 μL buffer consisting of 100 mM NaCl, 50 mM Tris-HCl pH 8.0, and 1 mM CaCl2. Factor Xa (10 μg) was added to the solution. The mixture was incubated at room temperature for 24 hr. Portions of
the digest at 12 and 24 hr time points were run on a 12 % SDS-polyacrylamide gel as described above.

1.2.4.3 Chemical Digestion of GST-ED(E17D)4E2 by Cyanogen Bromide

GST-ED(E17D)4E2 was dissolved in 88 % or 90 % formic acid in a round bottom flask. After complete dissolution, a suitable amount of water was added to make the formic acid concentration 70 % (the fusion protein concentration was ~ 2 mg/mL). The solution was purged with nitrogen for 5 min. At least equal weight of CNBr (with the protein) was added to the solution. The flask was connected to an aspirator and a nitrogen source to replace air with N2. The reaction flask was closed and the solution was stirred for 24 hr. The flask was then connected to an aspirator to remove partially the solvent, and to a vacuum pump (with liquid nitrogen trap) to remove the solvent completely. Water (at least equal volume with the original solution) was added carefully to the flask. The solvent was again removed under vacuum. The digested proteins were dissolved in TE or Tris-HCl, pH 8.0. All manipulations were done in an efficient hood (including weighing CNBr). Small scale digestions (protein < 1 mg) were done in a microcentrifuge tube in 70 % trifluoroacetic acid and several CNBr crystals were added without weighing.

1.2.4.4 ED(E17D)4E2 through Nondenaturing Polyacrylamide Gel

The powder from the CNBr digestion was dissolved in 100 mM Tris-HCl pH 8.0 and a portion of this solution was mixed with glycerol to make the final glycerol concentration 10 %. The mixture was loaded onto a 12 % polyacrylamide gel using 0.01M Na2HPO4 as the electrophoresis buffer. The gel was run at 25 mA for 2.5 hr and stained in 0.01 % methylene blue (pH 6.5) for 15 min. The gel was then destained
in ddH₂O for 8 hr with frequent changes of water. The bands corresponding to polymer ED(E₁₇D)₄E₂ were excised from the gel and put into a dialysis bag (molecular weight cutoff 8,000). Dialysis was first against 1 M NaCl to displace methylene blue and then against H₂O to remove salt. The solution in the dialysis bag was collected and lyophilized. The polymer purified this way was designated as gel purified I₁₁. The powder was dissolved in 100 mM Tris-HCl pH 8.0. About 15 µg of commercial polymers and 10 µg of polymer ED(E₁₇D)₄E₂ were loaded onto a 12 % polyacrylamide gel. The electrophoresis, staining and destaining procedures were the same as described above.

1.2.4.5 Large Scale Preparation of ED(E₁₇D)₄E₂

DH5αF' cells transformed with pGEX-3X.GZ1 were used to inoculate 5 mL 2X YT supplemented with 200 µg/mL ampicillin. The culture was allowed to grow at 37°C for 10 - 12 hr before it was used to inoculate another 250 mL 2X YT medium with the same concentration of antibiotics. This culture was grown at 37°C overnight and again used to inoculate 12 L YT medium + amp in a Microferm Fermenter (New Brunswick Scientific, Inc.). Antifoam A (~1 mL, Sigma) was added to the fermenter to suppress excess foam formation. Cells were grown (37°C) to OD₆₀₀ of 1.0 - 1.2 with vigorous magnetic stirring and aeration, and IPTG was added to 0.1 mM to induce protein synthesis. Cells were harvested 3 hr after induction in 1-L bottles by centrifugation at 4,000 rpm for 25 min (4°C), resuspended in 100 mL cold PBS (150 mM NaCl, 16 mM Na₂HPO₄ and 4 mM NaH₂PO₄, pH 7.3), transferred to a 250-mL centrifuge bottle and stored in the -10°C freezer.

Frozen cells were thawed by immersing the bottles in warm water. The viscous suspension in the bottle (in an ice bath) was subject to sonication three times, 5 min each (40 units power on a Branson Sonifier). Triton X-100 (10 %) was added to
each bottle to a final concentration of 1%. This mixture was vortexed well, and cell debris was removed by centrifugation at 13,000 rpm for 30 min (4°C). The turbid supernatant was transferred to new bottles and centrifugation was performed two more times to obtain a clear supernatant.

A cylindrical column (inner diameter = 2.5 cm) was packed with 10 mL glutathione-agarose bead slurry (Pharmacia). This column was washed with 100 mL PBS, and equilibrated with 30 mL PBS + 1 % Triton X-100. The supernatant was applied to the column which was then washed with 200 mL PBS + 1 % Triton to remove unbound materials. The beads were washed with 2 volumes of wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl) and 2 volumes of factor Xa cleavage buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl2). The beads were mixed well with 1.5 volumes of the cleavage buffer and 100 µg factor Xa (New England Biolabs) was added to the column. The incubation was carried out at room temperature for 24 hr with occasional mixing. The target protein segment was eluted out and the column was washed twice with 2 volumes of the same cleavage buffer.

Factor Xa and other potential contaminants were removed through anionic exchange chromatography. DEAE-Sephadex A-25 (5 g, Pharmacia) was equilibrated for at least 12 hr at room temperature in wash buffer. The “fines” in the supernatant were poured out several times during the equilibration and fresh buffer was added. The beads were mixed in a 250-mL centrifuge bottle with the target protein eluent which was cleared by centrifugation at 15,000 rpm for 30 min. The mixture was left at room temperature for 1.5 hr with occasional gentle swirling. The supernatant was taken out as much as possible using a pipet. The beads were washed twice with 100 mL wash buffer and the supernatant was removed by careful pipeting. The anionic exchange beads were further washed twice with 50 mL salt buffer (20 mM Tris-HCl, pH 8.0, 400 mM NaCl). After taking out the supernatant, 50 mL high salt buffer (20 mM Tris-HCl, pH 8.0, 2000 mM NaCl) was added to the beads. The mixture was
packed onto a column (ID = 1 cm) and the eluent was collected. This eluent was put into a dialysis bag (MWCO 3,500) and dialyzed against doubly distilled water for 24 hr with frequent changes of water. The contents of the bag were transferred to a 250-mL round bottom flask and water was removed by rotary evaporation. The materials were dried under vacuum.

The monodisperse derivative of PLGA, ED(E17D)4E2, was released from the eluted protein by cyanogen bromide cleavage. To the flask was added 42 mL 98.2 % formic acid. After dissolution of the materials, 16.92 mL of water was added. Approximately equal weight (with protein) of crystal CNBr was mixed with the solution. The reaction was carried out under argon for 24 hr. Unreacted CNBr and formic acid were removed under vacuum as described earlier. The CNBr cleavage products were resuspended in a minimum amount of 0.1 N NaOH solution and 50 mL water. The solution was added to a dialysis bag and dialyzed against doubly distilled water as described before. The contents were collected in SS-34 centrifuge tubes and any suspended materials were removed by centrifugation at 15,000 rpm for 30 min. Portions of this solution were lyophilized and the resulting powder was subject to amino acid analysis and FTIR characterization. The polymer purified this way was designated as column purified LI.

1.2.5 Analytical Techniques

1.2.5.1 Amino Acid Analysis

Amino acid composition analysis was obtained from the MCB Core Facility at the University of Massachusetts at Amherst. Phenyl isothiocyanate derivatization chemistry was used.
1.2.5.2 Elemental Analysis

Elemental analysis was performed at the Microanalysis Laboratory at the University of Massachusetts at Amherst.

1.2.5.3 Infrared Spectroscopy

Infrared spectra were recorded on a Perkin-Elmer Series 1600 FTIR spectrophotometer. About 2 mg polymer was mixed with 200 mg KBr to make a pellet. The pellet was dried under vacuum for at least 4 hr before the measurement.

1.2.5.4 Circular Dichroism

Circular dichroism (CD) spectra were obtained on an AVIV 60DS spectrophotometer at 25°C. Polymer (150 µg) was dissolved in 1 mL ddH₂O. Cells with 1 mm path length were used. The pH of the solution was measured with a semi-micro combination pH electrode (Aldrich). The spectra were recorded from 180 nm to 260 nm with 1.0 nm bandwidth and 1.0 sec averaging time.

The ellipticity \( \theta \) was recorded as millidegrees. The mean residue molar ellipticity \( \theta \) was calculated by the following equation:

\[
[\theta] = \frac{100 \theta}{C L}
\]

where \( L \) is the path length in centimeter, \( C \) is the residue concentration in decimole/cm\(^3\). The unit for \( \theta \) is degree, therefore \( [\theta] \) has the unit of deg cm\(^2\) dmole\(^{-1}\).

Both polymer I.1 (mol. wt. 9760) and polydisperse PLGA (mol. wt. 15,300 by LALLS, 17,500 by viscosity, Sigma) were used. The residue concentration was
obtained based on the average residue mol. wt. of 128.4 for I.1, and 152.1 for the polydisperse sample (sodium salt).

The helical fraction $f_H$ was calculated by the following equation:

$$f_H = \frac{[\Theta]_{222}}{-39500 \left(1-2.57/DP\right)} \times 100\%$$

A DP of 76 and a $\overline{DP}$ of 110 was used for I.1 and the polydisperse PLGA, respectively.

1.3 Results and Discussion

1.3.1 Design and Synthesis of the Oligodeoxynucleotide Duplex

The initial goal of this research was to synthesize monodisperse poly($\alpha$, L-glutamic acid) (PLGA) of discrete molecular weight, in its purest form, by recombinant DNA methods. This technique requires that the corresponding artificial gene be incorporated into an expression vector, and be subsequently transcribed into mRNA which is then translated to PLGA. Since current automated DNA synthesis technology still limits the length of oligodeoxynucleotides (ODN) to about 100 bases, high molecular weight PLGA can not be obtained without in vitro DNA multimerization of a smaller coding sequence. It is imperative to recover the DNA monomer and join this DNA segment unidirectionally to produce larger artificial genes. This requirement necessitates use of a restriction endonuclease, that can recognize a combination of codons for glutamic acid to generate nonpalindromic ends upon cleavage.

Two codons, GAA and GAG, code for glutamic acid. A search of restriction enzymes that can recognize various combinations of these two codons pointed to
MboII and MnlI (see Table 1.274). To construct large DNA coding sequences, application of these two enzymes requires long stretches of repetitive use of a single codon, a situation that might cause genetic instability.75 The one base overhang generated by digestion with these enzymes brought about concerns regarding effective multimerization. Recovery of DNA monomer fragments produced by MboII or by MnlI digestion might prove difficult because there are too many MboII sites (and even more MnlI sites) in commonly used plasmids.76 Synthesis of homopolymers of amino acids encoded by one or two codons by recombinant DNA techniques will frequently encounter these problems.

This predicament forced us to consider incorporation of foreign amino acids into PLGA. The thought was that after protein expression those amino acid residues should be converted back to glutamate through chemical modification. The search of restriction endonucleases recognizing Glu-Gln coding sequences yielded BbvI, a most expensive enzyme with low reaction efficiency, and which is present in many places in common plasmids. The search of restriction endonucleases recognizing coding sequences for Glu-Ser pairs produced Hinfl, an enzyme with a three-base overhang and many sites in common plasmids. This search also called attention to PleI and Ddel, with the former generating a single base overhang (GAGTC(4/5)) and the latter generating palindromic ends (C/TNAG).

The Glu-Asp combination led to restriction enzyme FokI, which recognizes GGATG, but cuts 9 nucleotides downstream to generate four-base cohesive ends. Since Asp is very close to Glu structurally, incorporation of an Asp residue at designated positions provided the best solution, at that time, to the synthesis of PLGA. The oligonucleotide sequence first designed had EcoRI and BamHI ends, and two FokI sites in between. As there are only two codons to arrange amid the FokI sites, and GAA is preferred to GAG in the ratio of 2:1,64,77,78 16 mixed bases were used to synthesize the coding and anticoding strands. After ligating the annealed and
The Table 1.2 Relevant restriction endonucleases discussed in the text. The recognition sequences are from 5' to 3'. A / indicates the cleavage site. The combinations of numbers are for cleavage sites that are outside the recognition sequences. For example, BbsI recognizes GAAGAC, but cuts two bases (any bases) downstream on the upper strand and six bases (any bases) downstream on the lower strand.76

<table>
<thead>
<tr>
<th>Restriction Endonucleases</th>
<th>Recognition and Cleavage Site (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AvaI</em></td>
<td>C/TCGAG, C/CCGGG</td>
</tr>
<tr>
<td><em>BamHI</em></td>
<td>G/GATCC</td>
</tr>
<tr>
<td><em>BbsI</em></td>
<td>GAAGAC(2/6)</td>
</tr>
<tr>
<td><em>FokI</em></td>
<td>GGATG(9/13)</td>
</tr>
<tr>
<td><em>MboII</em></td>
<td>GAAGA(8/7)</td>
</tr>
<tr>
<td><em>MnlI</em></td>
<td>CCTC(7/6)</td>
</tr>
<tr>
<td><em>NdeI</em></td>
<td>CA/TATG</td>
</tr>
</tbody>
</table>
phosphorylated oligonucleotide duplex into pUC18 digested with EcoRI and BamHI, and transforming the recombinant plasmid into DH5αF, few colonies appeared on an agar-YT plate. This may have been due to poor annealing efficiency of the oligonucleotides.

Of the plasmids isolated from four of these colonies, double-stranded sequencing did confirm that one plasmid had the correct sequence. This experiment provided us valuable information to design new coding and anticoding strands with fewer mixed bases (vide infra). Because there are many FokI sites in common cloning plasmids, it soon became apparent that it was very difficult to construct a plasmid to amplify multimers. Although an adaptor with BamHI ends and a unique FokI site in the middle was successfully constructed and its sequence verified, the poor cutting efficiency of this very expensive enzyme made multimerization a formidable task.

The discovery and marketing of a new enzyme, BbsI, by New England Biolabs in late 1990 proved to be a pivotal point of this research. BbsI works with high efficiency. Furthermore, there is no BbsI site in pUC18, which means it would be easy to construct a plasmid for amplification of multimers.

The design of the oligonucleotides reflected several salient features (Figure 1.8). BamHI ends are compatible with many common vectors, including pUC18 which was used in this work. A total length of 89 base pairs (89 is not a multiple of 3) ensures the insertional inactivation of β-galactosidase encoded by lacZ gene on pUC18, thus providing an easy method for detection of the insert by blue/white screening. The central 54 bp sequence flanked by two BbsI sites codes for 17 consecutive glutamic acids and one aspartic acid, therefore, one DNA monomer encodes a Glu17Asp repeating unit. This design would put Asp residues on the same helix face if the expected 185 α-helix is formed. The use of GAA is about as twice frequent as that of GAG, a ratio preferred by E. coli.64,77,78 Other important characteristics, such as positioning of the ATG codon and incorporation of the Ndel
Figure 1.8 Design of the oligodeoxynucleotide duplex coding for Glu17Asp.
Restriction sites are underlined. A total of five mixed sites was used. A* = A and G mixed on an equal feed (weight) basis, whereas T* = T and C mixed on an equal feed (weight) basis. The enzyme recognition sites are underlined.
site, are crucial to the construction of the transfer vector pUC803 and will be discussed in the next section.

The use of mixed bases at five sites was based on the construction of the FokI-ended DNA monomers. Although the FokI strategy had been abandoned, the sequence information did provide us a clue that relatively long stretches of GAA can be tolerated in bacterial cells. Most of the codon positions were fixed, leaving only five mixed bases to generate a population of oligonucleotides. This arrangement should result in efficient annealing.

Both coding and anticoding strands were obtained by solid-state synthesis and purified on a 10% denaturing polyacrylamide gel. These two strands were annealed through complementary base pairing to make a DNA duplex. The 5'-hydroxyl ends were phosphorylated by T4 polynucleotide kinase. This double-stranded DNA segment was joined with linearized pUC18, which was first digested with BamHI and, to decrease the chances of self-ligation, treated with phosphatase to remove 5'-phosphate groups. Transformation of competent *E. coli* cells by the recombinant pUC18 yielded white colonies on YT plates. Plasmids from those colonies were isolated and purified from saturated cell culture and subjected to double-stranded DNA sequence analysis. One plasmid, designated as pUC18-89, contained the correct insert sequence (Figure 1.9) and laid the basis for the following experiment.

### 1.3.2 Design and Construction of Transfer Vector pUC803

Several criteria should be used when designing a plasmid vector. A plasmid vector should be small in size and well characterized, and it should be easily propagated and carry selectable markers. A small plasmid is readily isolated from host cells and usually has multiple copies if it has a strong origin of replication. After recombining with a foreign DNA segment and introduction (usually with very low
Figure 1.9 The oligonucleotide duplex encoding one peptide repeat Glu17Asp. This DNA segment is from the recombinant plasmid pUC18-89. The enzyme recognition sites are underlined. The *BbsI* cutting sites are denoted by "I".
efficiency) into host cells, a vector should produce an easily identifiable phenotype (e. g., antibiotic resistance). pUC18 is an ideal plasmid that has an additional genetic marker that can be used for insertional inactivation screening. Inactivation can be realized by inserting a foreign piece of DNA at any of the unique restriction sites in its lacZ gene.

pUC803 was obtained by removing the 54 bp BbsI DNA fragment from pUC18-89, followed by recircularization. The resulting vector is equivalent to that which would be obtained by inserting an adaptor sequence (35 bp, Figure 1.10) into the BamHI site of pUC18. Although this construct destroyed the advantageous blue/white screening characteristics of pUC18, pUC803 remains a small (2721bp), high copy number plasmid and confers ampicillin resistance on the host. pUC803 acts as a transfer vector for the BbsI monomer, and was designed to screen and amplify multimers obtained from ligating the monomeric coding sequence. Features of the adaptor include: 1) the reading frame is compatible with powerful expression vectors pGEX-3X (vide infra) and pET-c series (pET-3c, pET-12c, pET-3xc, etc.)81,82; 2) the NdeI site provides a natural start for pET-3c so polypeptide can be expressed without fusing with other amino acids; 3) the upstream methionine allows cyanogen bromide digestion of N-terminal fusion fragments to release the poly(glutamic acid) portion; 4) the built-in stop codon ensures no C-terminal fusion, and 5) NdeI serves as an orientation check site for the pET series, while AvaI serves this function for pGEX-3X. The adaptor sequence was verified along with one DNA monomer by double-stranded sequencing of pUC18-89.

1.3.3 Preparation of Artificial Gene Segments

The BbsI DNA monomer (corresponding to Glu17Asp) isolated from pUC18-89 and purified by polyacrylamide gel electrophoresis was multimerized under the
Figure 1.10 Design of the 35-bp adaptor sequence that can incorporate a unique BbsI site into the polylinker region of pUC18. The enzyme recognition sites are underlined. The BbsI cutting site is denoted by "1".
catalysis of T4 DNA ligase. This reaction mix was run on an agarose gel (Figure 1.11). Although DNA fragments with degree of polymerization (DP) of 20 were clearly seen, most of the population centered around DP ~ 10. Individual multimers were recovered from the gel and then ligated into phosphatase-treated pUC803. Transformation of DH5αF' cells failed to yield any colonies under ampicillin selection.

In another experiment, the multimer ligation mix was directly added to a solution containing dephosphorylated pUC803. This reaction mixture was used to transform DH5αF'. BamHI analysis showed that of total nine colonies screened, only one colony carried plasmid with a tetrmeric insert, while most of the plasmids isolated contained only the monomeric DNA segment. Double-stranded sequencing confirmed the arrangement of four monomer repeats and the presence of all engineered restriction sites. BamHI digestion released the 251 bp artificial gene, which was purified on a 8% polyacrylamide gel and inserted into an expression vector.

1.3.4 Expression of the Artificial Gene as Fusion Protein

One of the expression vectors that was used for fusion protein production was pGEX-3X developed by Smith and Johnson.\(^3\) pGEX vectors facilitate expression, purification and detection of fusion proteins. The tac promoter makes sure protein synthesis is chemically inducible. Proteins are produced with a 26-kDa fragment of glutathione S-transferase as their N-terminal fusion and can be easily purified from crude cell lysate by affinity chromatography under mild elution conditions. Target proteins can be released either by thrombin protease digestion (pGEX-2T) or by factor Xa cleavage (pGEX-3X). Since we also incorporated an ATG codon just before the target segment into the artificial gene, the protein of interest can be released by CNBr cleavage of the fusion protein.
Figure 1.11 Multimerization of the BbsI DNA monomer fragment. The ligation reaction was analyzed on a 1.5 % agarose gel (Lane 3). Lanes 1, 2, and 4 are DNA molecular weight marker Lambda DNA-BstEII digest, 1 Kb DNA ladder, and 123 bp DNA ladder, respectively.
The purified *Bam*HI DNA fragment containing the tetrameric coding sequence was inserted into dephosphorylated pGEX-3X. This recombinant plasmid (pGEX-3X-tetramer) was transformed into DH5αF', and isolated and purified from a saturated cell culture. The presence of the artificial gene was checked by *Bam*HI digestion (releasing a 251 bp DNA segment) and the orientation of the insert was determined by *Ava*I digestion. Since pGEX-3X has one *Ava*I site (same as *Sma*I), digestion of a plasmid with correct orientation would generate an 11 bp segment which is too small to be detected. On the other hand, plasmid with incorrect insert orientation should produce a 250 bp fragment upon *Ava*I digestion. A plasmid with correct insert orientation was designated as pGEX-3X.GZ1 and used to perform protein expression experiments (Figure 1.12).

Small-scale protein expression was done in 2 X YT medium. The growth profiles of DH5αF', pGEX-3X transformed DH5αF', and pGEX-3X.GZ1 transformed DH5αF' are delineated in Figure 1.13. IPTG was added at time 0. After induction of protein synthesis, the growth rates of the transformed cells decline, while cells without plasmid continue to grow exponentially.

Figure 1.14 shows expression results of DH5αF' cells harboring pGEX-3X,GZ1 in 2 X YT medium. A new band with an apparent molecular weight of 36 kDa can be detected on the SDS-polyacrylamide gel after Coomassie Brilliant Blue (CBB) staining (Lanes 7-10 and 12). This band was absent before induction with IPTG, but was present after fusion protein synthesis had been initiated. The nascent polypeptide migrates at the expected position. The intensity of the protein band was significantly lower than that of the GST band in the control (Lane 5), which was believed to be partially a result of less efficient CBB binding to the fusion protein due to the long stretch of negatively charged glutamate residues. Another reason may be that the fusion protein was produced in low yield.
Figure 1.12 *BbsI*, *BamHI* and *AvaI* digests of pGEX-3X.GZ1 run on a 2% agarose gel. Lane 1, pBR322 DNA-*MspI* digest; Lane 2, uncut pGEX-3X.GZ1; Lanes 3, 4, and 5, pGEX-3X.GZ1 digested with *BbsI*, *BamHI* and *AvaI*, respectively; Lane 6, 1 Kb DNA ladder.
Figure 1.13 Cell growth profiles in protein expression experiment in rich medium. IPTG was added at time zero. Cells transformed with pGEX-3X.GZ1 were compared with those without plasmid, and those transformed with pGEX-3X.
Figure 1.14 Protein expression in rich medium. Crude cell lysates were analyzed by 12 % SDS-PAGE. The fusion protein of interest is indicated by the bar. Protein molecular weight markers are prestained (48.5, 36.5, and 26.6 kDa).
In order to see the new protein clearly, \[^{35}\text{S}]\text{methionine} was added to minimal M9AA medium shortly before IPTG induction. From Figure 1.15, it can be seen that a prominent new protein band was indeed present 5 min after protein synthesis was induced (Lanes 8-13). In contrast, no new band at equivalent position can be detected in protein mixtures from DH5\(\alpha\)F', DH5\(\alpha\)F' harboring pGEX-3X, and DH5\(\alpha\)F' transformed with pGEX-3X.GZ1 before adding IPTG (Lanes 1-7). The intensity of the fusion protein band was lower than that of GST, despite the fact that the cells producing the fusion protein were loaded at twice the cell number as those producing GST. This demonstrated that the fusion protein was in fact produced in low yield relative to glutathione S-transferase.

1.3.5 Protein Purification

Fusion protein from expression of pGEX-3X.GZ1 in 1 L of 2 X YT was purified on an affinity column packed with 2 mL swollen glutathione Sepharose\textsuperscript{®} 4B beads (gel capacity > 8 mg/mL). The absorbed GST fusion protein was eluted with 10 mL glutathione buffer and eluent was collected as 2-mL fractions. Concentration and purity were analyzed by 12 \% SDS-PAGE, and it was found that target protein was mainly in the first three fractions (Figure 1.16). Some low-level contamination by other proteins was also present. Purified fusion protein from 1 L rich medium weighed about 4 mg.

This yield is low compared with those of other repetitive artificial proteins obtained in this laboratory using pET protein expression systems. Creel\textsuperscript{50} was able to purify more than 100 mg protein from 1 L YT culture. The low yield of our GST fusion protein is partly due to the expression system. Even in best cases, yields of fusion protein from pGEX-3X system are no more than 10 mg/L. Typical yields are 1-3 mg/L, in some instances as low as 50 \(\mu\)g/L fusion protein can be isolated.\textsuperscript{64}
Figure 1.15 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. The band just above the GST is due to β-lactamase. Prestained molecular weight markers are 48.5, 36.5, and 26.6 kDa.
Figure 1.16 Purification of the fusion protein by affinity chromatography on glutathione-linked Sepharose 4B was analyzed on a 12% SDS-polyacrylamide gel. 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 reduced glutathione. Prestained molecular weight markers are 84.0, 58.0, 48.5, 36.5, and 26.6 kDa.
Although low protein yield is inherent to the pGEX construct, other possible explanations seem plausible. Since GAA and GAG are read by the same tRNA,85 a low turnover rate of tRNA due to long stretches of these two codons might account for the low yield. It was also found by the same authors that in their in vivo E. coli system, GAA and GAG were translated at a rate of 21.6 and 6.4 codons/s, respectively, and these rates did not change very much when glutamic acid was supplemented to the medium at a concentration of 50 μg/mL. They believed that exogenous glu did not affect substantially the charging levels of the tRNAglu.

Both factor Xa and CNBr cleavage of GST-ED(E17D)4E2 can be monitored by analyzing the mixture at different time on a nondenaturing polyacrylamide gel using 0.01 M Na2HPO4 as the electrophoresis buffer. Since the polyglutamate portion can not be stained by Coomassie Brilliant Blue efficiently, methylene blue, a cationic dye, was chosen to stain gels containing this highly negatively charged molecule. Methylene blue has been used to stain immobilized RNA86 and sodium poly(styrene sulfonate).87 We have found that this dye stains polyglutamate efficiently (μg protein per lane can be detected), but it stains other less acidic proteins poorly on native polyacrylamide gels. The gel analysis showed that either the enzymatic or the chemical digestion was complete in 24 hr (data not shown).

Small amount of ED(E17D)4E2 can be recovered from the stained polymer gel slices. GST-ED(E17D)4E2 was first cut with CNBr and the protein segments in the mixture were separated on a nondenaturing gel. The polypeptide diffused out off the gel matrix and can be confined in a dialysis bag. The target polymer (called gel purified I.1) was recovered by lyophilization and subject to electrophoresis and amino acid analysis.

Figure 1.17 compares the electrophoretic behavior of gel purified ED(E17D)4E2 generated by cyanogen bromide cleavage with that of chemically synthesized PLGAs (Sigma). In sharp contrast with the commercial samples (Lanes 1
Figure 1.17 Comparison of molecular weight distribution of purified target polymer I.1 with those of two commercial PLGA samples on a 12% polyacrylamide gel. Lane 1, PLGA of molecular weight (MW) 9,050 and polydispersity index (PDI) 1.20; Lane 2, I.1 (expected MW 9,760); and Lane 3, PLGA of MW 21,000 and PDI 1.38.
and 3), the derivative of PLGA from the biological source (Lane 2) migrates as a single tight band, demonstrating its uniformity in molecular length.

Relatively large quantity of I.1 was purified from several 12-L YT culture. Compared with the shake flask fermentation, in cases where 12-L fermenters were used, cells were grown to higher density (1.0 ~ 1.2 vs. 0.6 ~ 0.8); protein expression was induced by lower concentration of IPTG (0.1 vs. 0.4 mM); and cells were harvested later (3 h vs. 2 h). IPTG is expensive and one study of the pGEX system has shown that [IPTG] beyond 0.062 mM does not increase protein expression level.\(^8\) Pelleted cells were resuspended and disrupted by freeze-thaw cycle and sonication. Cell debris was removed by centrifugation. The fusion protein GST-ED(E17D)4E2 was absorbed on the GST affinity column and factor Xa digestion was carried out while the chimeric protein was still immobilized on the column. Two fragments were produced by the enzyme cleavage: GST and GIPHMED(E17D)4E2 (designated as I.2) (see Figures 1.7 and 1.8). Polymer I.2, along with factor Xa, was eluted from the column, leaving the GST portion remain absorbed on the gel matrix. I.2 was separated from the protease and other trace amount of proteins by anionic exchange beads, and from salts by dialysis.

The target polymer I.1 was obtained by digesting I.2 with CNBr, dialysis, and centrifugation (to remove GIPHhSL, where hSl = homoserine lactone,\(^7\) and salts). Electrophoresis on a 12 % nondenaturing polyacrylamide gel showed a single band (data not shown). This column purified I.1 was subject to amino acid analysis, elemental analysis, and FTIR and CD characterization. This large-scale preparation method was also adopted to make protonated ED(E17D)4E2 which was used as the precursor to make monodisperse derivatives of PBLG (Chapter 2).
1.3.6 Protein Characterization

1.3.6.1 Amino Acid Analysis

The amino acid analysis results of gel purified and column purified ED(E17D)4E2 are listed in Table 1.3. The mole percent of each amino acid was calculated from relative peak height.

<table>
<thead>
<tr>
<th>Expected (mole %)</th>
<th>Glutamic acid</th>
<th>Aspartic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel purified I.1</td>
<td>91.85</td>
<td>8.15</td>
</tr>
<tr>
<td>Column purified I.1</td>
<td>92.88</td>
<td>7.12</td>
</tr>
</tbody>
</table>

Although the results for I.1 purified on a native polyacrylamide gel are less satisfactory, those of the column purified I.1 are identical to the theoretical compositions well within the experimental error. This analysis confirmed that the overall amino acid components of I.1 are very close to the anticipated value of Glu71Asp5.

1.3.6.2 Elemental Analysis

The column purified I.1 was subject to combustion analysis. The expected values are based on the chemical formula of I.1 C375H524N76O229. The observed values are listed in Table 1.4.
Table 1.4 Elemental analysis of I.1

<table>
<thead>
<tr>
<th>Element</th>
<th>Expected (%)</th>
<th>Observed (%)</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>46.14</td>
<td>39.16</td>
<td>15.12</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>5.37</td>
<td>4.92</td>
<td>8.38</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>10.91</td>
<td>9.23</td>
<td>15.40</td>
</tr>
</tbody>
</table>

The large difference between the expected values and the experimental ones might be due to the presence of organic salts. Buffers were used during the whole purification procedure. A more extensive dialysis should reduce the amount of salts.

1.3.6.3 Infrared Spectroscopy

Polymer I.1 was first dissolved in water and then acidified with 0.1 N HCl solution. The solution was lyophilized and the resulting powder was dried under vacuum. The FTIR spectrum (Figure 1.18) clearly shows the absorbance of amide I at 1654 cm\(^{-1}\), and amide II at 1542 cm\(^{-1}\). This diagnostic measurement illustrates that when the polymer is in its acid form, it assumes an \(\alpha\)-helical structure.

1.3.6.4 Circular Dichroism

The CD spectra of the polymer I.1 and a polydisperse PLGA are presented in Figure 1.19 and Figure 1.20, respectively. The spectra for both polymers are very similar either in basic or acidic solution. From the spectra, we conclude that the conformation of polymer I.1, as well as the polydisperse counterpart, is random coil at pH \(\sim\) 8.0 as indicated by the strong negative molar ellipticity at 197 nm, and a slight positive one at 217 nm.
Figure 1.18 FTIR spectrum of polymer I.I in the α-helical form. See text for the preparation procedure.
Figure 1.19 Preliminary CD results for I.1 in basic and acidic solutions.
Figure 1.20 CD results for a polydisperse PLGA in basic and acidic solutions.
At low pH (~ 3.5), both polymers have two strong negative dichroic ratios at 208 and 222 nm, and a very large positive one at 191 nm. At pH 3.5, the molar ellipticity at 222 nm ([θ]222) is -37,400 deg cm² dmole⁻¹ for polymer I.1, and at pH 3.8, [θ]222 is -37,000 deg cm² dmole⁻¹ for the polydisperse PLGA. This gives the helical fraction of 98 % for I.1, and 96 % for the polydisperse sample. Both values are close to 100 % within the experimental error. Therefore, the CD result shows that the backbone of polymer I.1 adopts a complete helical conformation when its side chains are fully protonated.

1.4 Conclusions

A general route to synthesizing monodisperse derivatives of PLGA has been established. This strategy is based on recombinant DNA methodology. Polymer I.1 was successfully expressed as a fusion to the C-terminus of glutathione S-transferase in a bacterial host. The fusion protein yield is about 4 mg from 1 L 2X YT culture. Double-stranded DNA sequencing confirmed the artificial coding gene sequence. I.1 can be liberated from the fusion protein by CNBr digestion. Amino acid analysis of I.1 agrees very well with the expected composition. FTIR reveals the α-helix formation in solid state when I.1 is fully protonated. Preliminary CD experiments indicate this polymer undergoes a conformational transition from a random coil in high pH aqueous solution to a helix in low pH solution. A comparison of this polymer with two polydisperse PLGA samples by electrophoresis reveals that I.1 is much more uniform in terms of molecular length than its chemically synthesized counterparts. This biological route to PLGA provides homogeneous precursors of monodisperse PBLG derivatives.

GluAsp(Glu17Asp)₄GluGlu
I.1
1.5 References


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CHAPTER 2

SYNTHESIS OF MONODISPERSE DERIVATIVES OF
POLY(γ-BENZYL α,L-GLUTAMATE)

2.1 Introduction

From the viewpoint of physical chemistry, poly(γ-benzyl α,L-glutamate) (PBLG) is the most extensively studied biopolymer.¹ A number of reasons account for this fact: 1) the starting L-glutamic acid is the cheapest among the optically active α-amino acids; 2) high molecular weight PBLG can be easily prepared from its corresponding N-carboxy anhydride; 3) PBLG is soluble in many common organic solvents; 4) PBLG has the ability to exist in well defined conformations of long range chain order; and 5) PBLG is a model compound that can be used to establish and investigate new theories and novel aspects of (bio)polymer science.

2.1.1 Chemical Synthesis of PBLG

The majority of PBLG has been synthesized by polymerization of γ-benzyl L-glutamate (BLG) N-carboxy anhydride (NCA), although direct peptide coupling has been used to make the same polymer.² BLG is commercially available or can be synthesized by reacting L-glutamic acid with benzyl alcohol in the presence of hydrochloric acid. BLG-NCA is efficiently obtained by mixing BLG with excess triphosgene (solid), or diphosgene (liquid) at elevated temperature, usually at the boiling point of the solvent employed. (Older protocols require passing gaseous phosgene through BLG suspended in solvent.) BLG-NCA is purified by precipitation.
PBLG is synthesized by adding a small amount of triethylamine as initiator to a solution containing BLG-NCA and purified by precipitating it into methanol (qv Chapter 3).

2.1.2 Physical Chemistry of PBLG

2.1.2.1 Secondary Structures

PBLG is a simple protein analog and widely used as a model compound to gain insight into protein behavior. The α-helical secondary structural motif was proposed by Pauling and Corey, and it was Perutz who, by finding the crucial 1.5 Å reflection from oriented PBLG fibers, provided the first X-ray crystallographic proof for this model. This well defined structure persists in the solid state. PBLG assumes an α-helical conformation in many common organic solvents, even at very high temperature (e.g., ~200°C in m-cresol). In contrast, many organic polymers have little long range order in solution and assume disordered random coil structures. The robust nature of the α-helix results from the formation of intramolecular hydrogen bonds and the optimal packing of the residues in a helical chain. In the undisturbed state, the α-helix comprises 13-membered rings closed by intrachain hydrogen bonding and characterized by a spiral pitch of 5.4 Å. A total of 18 residues are required to complete 5 turns, which translates to a 27 Å repeat distance. α-Helical PBLG has a diameter of 15 Å and estimated persistence length on the order of 1000 Å (values vary from 700 Å by dipole moment and light scattering measurements to 1565 Å by sedimentation and dynamic light scattering measurements).

PBLG takes on a random coil conformation in strongly denaturing solvents such as trifluoroacetic acid (TFA) and dichloroacetic acid (DCA), since intramolecular hydrogen bonding is disrupted. An initially α-helical PBLG in a helicogenic solvent
can be transformed into a random coil PBLG by adding TFA or DCA. This helix-coil transition, having its analogy to the denaturing of proteins, has been studied for a long time, and will be discussed later. At high polymer concentrations, the preferred conformation of PBLG is \( \alpha \)-helical even in denaturing solvents.\(^1,5,10\)

Low molecular weight PBLG adopts a \( \beta \)-sheet conformation in weakly interacting solvents\(^11,12\) and this secondary structure can be disrupted by adding DCA. While films of low molecular weight PBLG do have a mixture of \( \alpha \)- and \( \beta \)-form, those of larger PBLGs produce mostly the \( \alpha \)-form, even when casting is from TFA. The difficulty of achieving \( \beta \)-sheet structure limits the mechanical strength of PBLG and precludes other attributes associated with extended sheet conformations.

2.1.2.2 Solution Properties

The highly anisotropic PBLG in many organic solvents aggregates due to its long persistence length and strong dipole moment (3.5 D/residue).\(^13\) Individual, non-interacting helices are only present in the most solvating, helicogenic amide solvents, e.g., dimethylformamide (DMF). The thermodynamic, hydrodynamic, dielectric, and spectroscopic properties of PBLG solutions have been studied extensively and are summarized by Block.\(^1\)

Liquid crystalline (LC) behavior of polymeric systems was first observed in concentrated (> 15%) PBLG solutions by Elliott and Ambrose.\(^14\) The LC phases are highly birefringent with a strong optical rotary power. The rheological property unique to LC solutions - the negative normal stress difference at medium shear rate - was first discovered by Kiss and Porter\(^15,16\) in the PBLG-\( m \)-cresol system.

Conformational transitions of PBLG in solution can be induced by adding denaturants or changing temperature. Interconversion of the \( \alpha \)-helical conformer of PBLG to random coil occurs over a narrow ranges of solvent composition and/or
temperature and can be readily detected by measurements of specific rotation, circular dichroism, NMR, viscosity, and calorimetry. The mechanism of solvent composition induced transition has been a subject of controversy. One possible explanation is that DCA or TFA associates, through competitive hydrogen bonding, with NH groups in the backbone, and C=O groups in the side chain as well as main chain.\textsuperscript{17,18} With solvent compositions not too far away from the transition, increasing temperature favors the more ordered \(\alpha\)-helix. This inverted thermal transition was elucidated by Cabani\textsuperscript{19} and others.\textsuperscript{20,21} The supramolecular textures of lyotropic liquid crystals of PBLG are listed in Table 2.1.\textsuperscript{22}

\subsection*{2.1.2.3 Solid State Properties}

Solid films of PBLG can easily form by solvent evaporation. In a majority of the cases, the polypeptide assumes an \(\alpha\)-helix conformation, unless PBLG is of low molecular weight, in which case both \(\alpha\)-helix and \(\beta\)-pleated sheet are observed. A piezoelectric film with highly oriented PBLG molecules has been obtained by slowly evaporating solvent under a magnetic field.\textsuperscript{23} Helical forms do differ depending on preparation procedures. For example, Uematsu et al.\textsuperscript{24} obtained a film consisting of 7/2 PBLG helices by casting a chloroform solution over a two or three month period. This metastable 7/2 helical form undergoes an irreversible phase transition to the more stable \(\alpha\)-helical form upon heating. Single crystals of PBLG were grown from chloroform, with a crystal size up to 2.0 x 0.3 x 0.05 cm.\textsuperscript{25} Ordering of \(\alpha\)-helices was observed when PBLG films were formed epitaxially on the surface of inorganic and organic single crystals. On NaCl and KCl crystal surfaces, the epitaxial structures of PBLG are aligned along the [110] substrate direction and built up of straight, non-folded \(\alpha\)-helical molecules aggregated either side-by-side (for high molecular weight fractions) or end-to-end (for low molecular weight fractions).\textsuperscript{26,27} PBLG grown on
Table 2.1 Textures of PBLG in different solvents. After Dupré and Samulski.22

<table>
<thead>
<tr>
<th>Texture</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteric</td>
<td>CHCl$_3$, CH$_2$Cl$_2$, dioxane, pyridine, $m$-cresol, DCA, DMF, quinoline, thionene</td>
</tr>
<tr>
<td>Nematic</td>
<td>CH$_2$Cl$_2$-dioxane (4:1 v/v)</td>
</tr>
<tr>
<td>Smectic</td>
<td>benzene</td>
</tr>
</tbody>
</table>
nylon 6 forms long needle-like objects inclined about 60 ± 2° to the drawing direction of the substrate molecules with average thickness of about 2000 to 3000 Å.28 Solid PBLG does not melt and is thermally stable even at temperatures above 160°C.29 The dynamic, mechanical, and relaxation processes have also been studied. PBLG conformation can be readily determined by measuring infrared absorbance which is structure dependent. Characteristic IR bands30 for polypeptides in general are shown in Table 2.2.

2.1.2.4 Surface and Interfacial Properties

Monolayers of PBLG on air-water or air-aqueous solution interface were studied by Malcolm.31 Infrared spectroscopy and electron diffraction of collapsed films removed from the surface were found to be consistent with a structure composed of condensed, ordered arrays of α-helices. Further compression leads to rolling of some α-helices onto the upper surface of the monolayer, and ultimately the formation of a bilayer. Jones and Tredgold32 studied orientation effects in Langmuir films of PBLG (28 kDa) by polarized IR spectroscopy. They found that PBLG molecules are oriented randomly in the monolayer at low pressures (2.5 mN/m), largely perpendicular to the compressing barrier (still a monolayer) at moderate pressures (5-10 mN/m), and predominantly parallel to the barrier in a collapsed bilayer at high pressures (25 mN/m). Samulski et al.33 investigated a ~20 kDa PBLG physisorbed on the [111] face of gold substrate and concluded that PBLG in the layers is α-helical and lies in the plane of the substrate. If the same PBLG is modified by coupling with lipoic acid (which has a specific binding interaction with gold) at its N-terminus, the transformed PBLG can self-assemble on gold substrate through chemisorption with the disulfide moiety. The α-helical conformation is retained, although ellipsometry measurements indicated the uneven nature of the self-assembled monolayer.
Table 2.2 Characteristic wave numbers and dichroism for the amide I and amide II bands in poly(α-amino acid)s.\textsuperscript{30} \(\|$ and \perp\) represent vibrational components parallel and perpendicular to the long axis, respectively.

<table>
<thead>
<tr>
<th>Conformation</th>
<th>Phase</th>
<th>Wavenumber/cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amide I</td>
</tr>
<tr>
<td>Disordered</td>
<td>—</td>
<td>1656 (s)</td>
</tr>
<tr>
<td>α-helix</td>
<td>(|$)</td>
<td>1650 (s)</td>
</tr>
<tr>
<td></td>
<td>(\perp)</td>
<td>1652 (m)</td>
</tr>
<tr>
<td>β-form, parallel chains</td>
<td>(|$)</td>
<td>1650</td>
</tr>
<tr>
<td></td>
<td>(\perp)</td>
<td>1630</td>
</tr>
<tr>
<td>β-form, antiparallel chains</td>
<td>(|$)</td>
<td>1685 (w)</td>
</tr>
<tr>
<td></td>
<td>(\perp)</td>
<td>1632 (s), 1670</td>
</tr>
</tbody>
</table>

(s) Strong, (m) medium and (w) weak.
Direct imaging of PBLG was achieved by using scanning tunneling microscopy (STM). In the experiment, PBLG was deposited from dimethylformamide on highly oriented pyrolytic graphite (HOPG) to form a thin film. The helical character of the PBLG molecules was clearly observed. The polypeptide conformation deduced from the STM images is consistent with an 113 helix with helical pitch of 16.5 Å, instead of the characteristic 185 helix. This result was presumed to be due to a different PBLG chain conformation caused by intrahelical interaction of the side chains. A more recent STM study of PBLG on HOPG brought about a direct correlation of the image features to the α-helical peptide model.

Surface and interface studies of rodlike molecules are likely to draw more attention.

Some of the important features of the PBLG helix are summarized in Table 2.3.

2.1.3 Overview of Chapter 2

Monodisperse polymers, in terms of sequence, composition, stereochemistry, and molecular weight, are fundamentally different from their polydisperse counterparts. In the traditional sense of monodispersity, it simply means polymers with narrow molecular weight distribution. However narrow the distribution, chemically synthesized polymers are still considered mixtures. Furthermore, the only mature anionic synthetic route to monodisperse products does not have control over stereochemistry (atactic configuration). Even when dealing with so-called monodisperse diblock copolymers of styrene and butadiene, Schwark found low molecular weight contaminants due to premature termination. Thus it is inevitable that a method to synthesize truly homogeneous population of polymers has to be defined. Monodisperse polymers play a very central role in accurately pinpointing structure-property relationships and fabricating designer polymers with precision.
Table 2.3 Physical characteristics of α-helical PBLG.36

<table>
<thead>
<tr>
<th>Physical character</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue molecular weight</td>
<td>129.11</td>
</tr>
<tr>
<td>Axial rise per residue</td>
<td>1.5 Å</td>
</tr>
<tr>
<td>Axial rise per helical turn</td>
<td>5.4 Å</td>
</tr>
<tr>
<td>Molecular length</td>
<td>DP × 1.5 Å</td>
</tr>
<tr>
<td>Core diameter</td>
<td>4.6 Å</td>
</tr>
<tr>
<td>α-Helix diameter</td>
<td>15 Å</td>
</tr>
<tr>
<td>Aspect ratio</td>
<td>DP/10</td>
</tr>
<tr>
<td>Density</td>
<td>1.27 g/cm³</td>
</tr>
<tr>
<td>Persistence length</td>
<td>≈1000 Å</td>
</tr>
<tr>
<td>Side-chain volume</td>
<td>≈ 2/3 of molecule</td>
</tr>
<tr>
<td>Macrodipole moment</td>
<td>DP × 3.5 Debye</td>
</tr>
<tr>
<td>Dielectric anisotropy</td>
<td>$\Delta \varepsilon = \varepsilon_\parallel - \varepsilon_\perp &gt; 0$</td>
</tr>
<tr>
<td>Diamagnetic anisotropy</td>
<td>$\Delta \chi = \chi_\parallel - \chi_\perp &gt; 0$</td>
</tr>
<tr>
<td>Birefringence</td>
<td>$\Delta n = n_\parallel - n_\perp = 0.025 &gt; 0$</td>
</tr>
</tbody>
</table>
Our primary goal is to make PBLG with uniform chain length and ranges of discrete molecular weights. The heterogeneity in PBLG molecular weight has complicated the explanation of many fundamental phenomena, the elucidation of which will have wide implication on practical application. These phenomena are associated with hydrodynamics,\textsuperscript{38} spectroscopy,\textsuperscript{39} conformation,\textsuperscript{40} liquid crystallinity,\textsuperscript{41} crystallization,\textsuperscript{42} and surface deposition.\textsuperscript{43}

Molecular weight variants of PBLG are also of significance. Polymer chemists are usually concerned about high molecular weight materials with, say, 50,000 atomic mass units. Traditional synthetic chemists have paid attention to molecules smaller than 2,000 amu.\textsuperscript{44} Midsized organic species has been neglected partly due to synthesis difficulties. Living organisms routinely make molecules of a wide array of sizes, tightly controlled structures and defined conformations. The recombinant DNA technique delineated in Chapter 1 has been used to make polypeptides of desirable lengths.\textsuperscript{45,46} Investigation on monodisperse oligopeptide or polypeptide variants not only can eliminate the ambiguity associated with polydispersity, but also can provide more useful quantitative information.

Many phenomena (aggregation, conformation transition, LC behavior, and crystallization) of polymeric system are dependent on molecular weight and its distribution. For example, crystallization of high molecular weight species is controlled kinetically, whereas that of small molecules is controlled thermodynamically. Where is the molecular weight demarcation? A series of chain variants might provide new insight into this problem.

On the application side, although large second and third order hyperpolarizability of PBLG solutions has been observed and piezoelectric films of PBLG have been prepared, the practical utilization has been lagging behind. These optical and electrical applications require long range order and uniform chain length. When Samulski et al. used a polydisperse PBLG to study self-assembly on gold
surface, they failed to observe chain orientation normal to the surface.⁴³ Although the exact reasons have not been found out, heterogeneous molecular weight may be a very important factor. More recently, Whitesell⁴⁷ fabricated directionally aligned polyalanine on the [100] face of gold, and concluded that the axes of the helices are aligned mainly normal to the surface. But the ellipsometry measurements on the polypeptide layer indicated an uneven thickness. This character is a result of the polymerization chemistry (the NCA method) they used. It is impossible to control polymer chain length through this statistical polymerization technique. Therefore, our endeavor to synthesize and characterize well-engineered polypeptides not only has direct bearing on many fundamental aspects of physical chemistry, but also has wide implication for uses such as chemical sensors, optical switches, and piezoelectric or ferroelectric devices.

Chapter 2 is based on the previous chapter. A general strategy using phenyl diazomethane to benzylate PLGA or its derivatives is described. Polymer II.1 is used as an example.

GluAsp(Glu₁₇Asp)₄GluGlu

II.1

Glu(OBzl)Asp(OBzl){[(Glu(OBzl)]₁₇Asp(OBzl)}₄Glu(OBzl)Glu(OBzl)

where OBzl denotes a benzyl ester.

II.2

The benzylated polymer (II.2), a derivative of PBLG, was analyzed by spectroscopic and chromatographic techniques. Results of nuclear magnetic resonance (NMR) spectroscopy of II.2 in chloroform/trifluoroacetic acid indicate essentially quantitative conversion of the carboxylic acid to the benzyl ester. NMR
spectra further show a conformational transition of II.2 from an α-helix in chloroform to random coil in a chloroform/TFA mixture. The FTIR spectrum demonstrate that II.2 adopts an α-helical structure in solid films. Gel permeation chromatography shows that the modified polymer has a much narrower distribution than its synthetic counterpart obtained through the NCA polymerization method.

2.2 Experimental Section

2.2.1 Materials

All reagents and materials used in the experiments described in this chapter are listed and their sources are indicated. Chemicals were used as received.

Benzaldehyde, 99+%  
Benzene, ACS certified  
Benzyl triethylammonium chloride, 99%  
Chloroform (d)  
Dimethyl sulfoxide (DMSO), ACS certified  
Hydrogen chloride, 0.1 N solution  
Methanol, HPLC grade  
Poly(γ-benzyl α,L-glutamate)  

MW: 15,600 (LALLS), 20,100 (viscosity)  
Polystyrene molecular weight standards  
Sodium hydroxide, ACS certified  
Tetrahydrofuran (THF), HPLC grade  
p-Toluenesulfonhydrazide, 97%  
Trifluoroacetic acid (d)  

Aldrich  
Fisher  
Aldrich  
Aldrich  
Fisher  
Fisher  
Fisher  
Sigma  
Aldrich  
Fisher  
J. T. Baker  
Aldrich  
Aldrich
2.2.2 Synthesis of Monodisperse Derivative of PLGA

A monodisperse derivative of PLGA, GluAsp(Glu17Asp)4GluGlu (II.1), was synthesized biologically and purified from 12 L YT culture as described in the Experimental Section of Chapter 1. After the last centrifugation step, the solution was transferred into a 250-mL round bottom flask. The pH of the solution was adjusted back to 2 by adding 0.1 N HCl solution. The mixture was stirred for 1 hr and the solvent was removed on a rotary evaporator. The material was dried under vacuum for at least 2 hr, and the benzylation reaction was to be carried out in the same vessel. The amount of polymer was ~ 10 mg.

2.2.3 Synthesis of Benzaldehyde Tosylhydrazone

*p*-Toluenesulfonhydrazide (20 g, 0.107 mol) was added to a 100-mL round bottom flask fitted with a magnetic stir bar and a condensor. Glacial acetic acid (25 mL) was mixed with the powder and the reaction flask was heated in an oil bath to reflux the acid. Benzaldehyde (13.1 mL, 0.128 mol) was added to the hot solution and the reflux was continued for one hour. The reaction mix was allowed to cool to room temperature and then further cooled in an ice bath, after which a white crystalline compound appeared. The product was filtered, washed successively with minimum amounts of cold acetic acid, a cold mixture of 1:1 acetic acid/water, and cold water, and then air-dried in a hood overnight. An off-white powder was obtained. Yield = 28.6 g, 91.7 %. Melting Point: 129 - 130°C.
2.2.4 Synthesis of Phenyl Diazomethane

To a 250-mL round bottom flask equipped with a magnetic stir bar and a condenser were added benzaldehyde tosylhydrazone (0.75 g) and benzene (40 mL). The flask was slowly heated to effect dissolution of the solid. After the reaction vessel was cooled to room temperature, 75 mL aqueous NaOH solution (14%) with 0.15 g benzyl triethylammonium chloride was added carefully. The flask was wrapped with aluminum foil to prevent exposure to light. The mixture was heated at 70°C under nitrogen for two hours before it was cooled to room temperature. The benzene layer (which contained the diazo compound) was separated from the aqueous phase in a separatory funnel and washed with distilled water until the pH of the aqueous washing layer was 7. The organic solution with deep orange color was dried over anhydrous sodium sulfate overnight at room temperature and filtered using gravity flow before being used as soon as possible. Typical yield (determined by titrating 10 mL benzene solution with 0.14 M trifluoroacetic acid in CH2Cl2 with the diazo compound as indicator): 0.307 g, 95.2%.

2.2.5 Synthesis of Monodisperse Derivative of PBLG

In a typical reaction, the monodisperse derivative of PLGA II.I (10 mg, 0.078 mmol carboxylic acid) was dissolved in its acid form in 50 mL DMSO in a 250-mL round bottom flask. After complete dissolution of the polymer, the phenyl diazomethane benzene solution (containing 0.307 g diazo compound, 2.60 mmol) was added to the reaction flask. The mixture was stirred under nitrogen for 30 min at room temperature. After wrapping the flask with aluminum foil, stirring was continued for 4 days. Benzene was removed by using aspirator and heating the solution to about 50°C. DMSO and unreacted phenyl diazomethane were removed by raising the
temperature to 70°C under vacuum. The reaction mixture was concentrated to
dryness. The solid product was first treated with tetrahydrofuran (THF) and any
undissolved particles were removed by centrifugation. The polymer (II.2) was
recovered by precipitating the THF solution in water. The precipitate was collected by
centrifugation, washed twice with a mixture of water and methanol (50/50, v/v), and
dried at 80°C in vacuo.

2.2.6 Analytical Techniques

2.2.6.1 Nuclear Magnetic Resonance Spectroscopy

Proton NMR spectra were recorded either on a Bruker 200 AC (200 MHz) or a
GE QE 300 (300 MHz) spectrometer. Samples were dissolved in CDCl₃ or
CDCl₃/TFA (d) mixed solvent, where a small amount of tetramethylsilane (TMS) was
added as the internal standard. Chemical shifts are reported as parts per million down
field from the standard.

2.2.6.2 Fourier Transform Infrared Spectroscopy

Infrared spectra were obtained on an IBM IR 32 Fourier Transform
spectrophotometer. Samples were dissolved in THF and the solution was cast on a
KBr plate. The film was dried overnight before the measurement.

2.2.6.3 Gel Permeation Chromatography

Gel permeation chromatography was performed on a Waters GPC Model 501
instrument which was equipped with three tandem Ultrastyragel™ columns (Linear,
Linear, and $10^3$ Å) and a refractive index detector. THF was used as the solvent at a flow rate of 1 mL/min. The measurements were carried out at room temperature. Polymer II.2 was dissolved in THF at a concentration of 0.5 mg/mL, the commercial PBLG 1.0 mg/mL, and six polystyrene (PS) standards were mixed and the concentration of each was 0.2 mg/mL. The molecular weights of the PS used were: 573,000, 184,200, 95,800, 32,660, 12,860, 2,727. The calculated molecular weights were based on PSs and were obtained by using a BASIC program on an IBM PC.

2.3 Results and Discussion

Polydisperse PBLG is usually conveniently synthesized through the NCA method. To make monodisperse PBLG, one route is to benzylate the carboxyl groups of monodisperse PLGA. We have successfully produced polymer II.1, a monodisperse derivative of PLGA. The subsequent reaction of II.1 with phenyl diazomethane should yield a monodisperse derivative of PBLG.

2.3.1 Synthesis of Monodisperse Derivative of PBLG

Polymer II.1 is a polycarboxylic acid. To make a benzyl ester from a carboxylic acid, the most efficient strategy is to use phenyl diazomethane. We followed a procedure that gives higher yield of phenyl diazomethane than other methods do. When this compound was used to esterify a polydisperse PLGA, quantitative conversion was achieved. Starting with $p$-tosyl hydrazide and following a similar procedure, we have made phenyl diazomethane in very good yield (Figure 2.1). A large excess of the diazo compound was used to benzylate II.1. The reaction product was analyzed by NMR, FTIR, and GPC. The results are
Figure 2.1 Synthetic schemes for converting II.1 into II.2 by reacting II.1 with phenyl diazomethane.
compared with those obtained from a commercial polydisperse PBLG of comparable average degree of polymerization.

2.3.2 Proton Nuclear Magnetic Resonance Spectroscopy

The $^1$H NMR spectra of II.2 in both helicogenic CDCl$_3$ and nonhelicogenic CDCl$_3$/TFA mixture reveals its chemical composition and conformational characteristics (Figure 2.2). A polydisperse sample of comparable molecular weight was also used in NMR experiments (Figure 2.3). Peaks labeled x are due to the solvent and the instrument used. We have observed similar peaks in NMR spectra of chemically synthesized PBLG. Peaks labeled a are probably due to impurities that are hydrocarbon rich. Although major effort had been devoted to using acetone, chloroform, and ether to extract the aqueous solution of II.1, and to precipitating THF solution of II.2 in ether, and washing the precipitate from water (see the Experimental section) with methanol and either, peak a remained in the spectra. Preparative HPLC may be useful to purify II.2 further.

The proton chemical shifts of polymers in CDCl$_3$ are compared in Table 2.4. They are very identical within the experimental error.

Table 2.4 Comparison of chemical shifts (ppm) in CDCl$_3$

<table>
<thead>
<tr>
<th>Polymer II.2</th>
<th>Polydisperse PBLG</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>8.3</td>
<td>NH</td>
</tr>
<tr>
<td>7.2</td>
<td>7.2</td>
<td>Ar-H</td>
</tr>
<tr>
<td>4.9</td>
<td>5.0</td>
<td>benzylic CH$_2$</td>
</tr>
<tr>
<td>3.8</td>
<td>3.9</td>
<td>$\alpha$-CH</td>
</tr>
<tr>
<td>2.1</td>
<td>2.2</td>
<td>$\gamma$-CH$_2$ and $\beta$-CH$_2$</td>
</tr>
</tbody>
</table>
Figure 2.2 $^1$H NMR spectra (200 MHz) of polymer II.2 in CDCl$_3$ (A) and in CDCl$_3$/TFA(d) (B).
Figure 2.3 $^1$H NMR spectra (300 MHz) of a polydisperse PBLG in CDCl$_3$ (A) and in CDCl$_3$/TFA(d) (B).
The chemical shifts in CDCl₃/TFA(d, 60/10, v/v) are listed in Table 2.5. They are identical for the two forms of PBLG within the experimental error.

Table 2.5 Comparison of chemical shifts (ppm) in CDCl₃/TFA (d) mixed solvent

<table>
<thead>
<tr>
<th>Polymer II.2</th>
<th>Polydisperse PBLG</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.8</td>
<td>NH</td>
</tr>
<tr>
<td>7.2</td>
<td>7.3</td>
<td>Ar-H</td>
</tr>
<tr>
<td>4.9</td>
<td>5.0</td>
<td>benzylic CH₂</td>
</tr>
<tr>
<td>4.5</td>
<td>4.6</td>
<td>α-CH</td>
</tr>
<tr>
<td>2.3</td>
<td>2.4</td>
<td>γ-CH₂</td>
</tr>
<tr>
<td>2.0</td>
<td>2.1</td>
<td>β-CH₂</td>
</tr>
<tr>
<td>1.8</td>
<td>1.9</td>
<td></td>
</tr>
</tbody>
</table>

The resonance shift of the α-proton towards low field from 3.8 to 4.5, when the solvent is changed from CDCl₃ to CDCl₃/TFA, is associated with the conformational transition of II.2 from an α-helix to a random coil.³⁹ It is not a consequence of polydispersity as suggested by Nagayawa and Wada.⁴⁰

A comparison of the area ratios of benzylic proton over the α-proton in different solvent systems is listed in Table 2.6. Theoretically, the area ratio should be 2. In the form of random coil (e.g., in CDCl₃/TFA), both II.2 and the polydisperse PBLG show they have twice as many benzylic protons as the α-proton. Therefore, we conclude that the -COOH groups of II.1 have all been transformed to -COOCH₂C₆H₅ by reacting with C₆H₅CHN₂. Quantitative conversion has been achieved.
Table 2.6 The ratio of the benzylic proton area/α-proton area in different solvents.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>in CDCl3</th>
<th>in CDCl3/TFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>II.2</td>
<td>2.85</td>
<td>2.01</td>
</tr>
<tr>
<td>Polydisperse PBLG</td>
<td>2.78</td>
<td>2.06</td>
</tr>
</tbody>
</table>

These proton area ratios in both helicogenic solvents (e.g., CHCl3) and chaotropic solvents (e.g., CHCl3/TFA) are intriguing and indicative of PBLG conformational transitions. Working with higher molecular weight PBLG (\(\bar{DP} \sim 160\), vs. 76 of II.2, and ~ 70 of the polydisperse PBLG), Bovey and Schilling\(^{54,55}\) failed to observe the α-proton resonance in CDCl3 (300 MHz). Only when 20/80 (v/v) TFA/CDCl3 was used did they measure the fully relaxed α-proton peak. Marlborough et al.\(^{56}\) used PBLG of an unspecified molecular weight and did not observe the fully relaxed α-proton in CDCl3 until 30/70 (v/v) TFA/CDCl3 was employed as the solvent. (No mention of the field was made.) Obviously, the resonance of the α-proton is related to molecular weight of PBLG. Bovey and his coworkers believed the complete rigidity of the polypeptide molecules accounted for the absence of the α-proton band. TFA, which interacts with the NH and C=O groups in the backbone and side chains, disrupts both inter- and intramolecular association of the PBLG macromolecules. Adding TFA gradually to a helicogenic solvent where PBLG is present, helps to expose first the benzylic CH2, then β-CH2 and γ-CH2, and finally the α-CH to the mixed solvent.

The signals are broader for II.2 than for the polydisperse PBLG under similar solvent conditions. One reason may be due to the field strength used (200 vs. 300 MHz). More probably the broader bands for II.2 are caused by the chemical structure of II.2 which contains five asp residues, each separated from another by 17 glu residues. The side chain of asp is one -CH2 unit shorter than that of glu. This might
disrupt the normal inter- and intrachain interaction. Therefore, the line broadening may be due to the different resonance of α-CH and β-CH₂ of asp and glu structural units.

2.3.3 Fourier Transform Infrared Spectroscopy

Infrared spectroscopy not only can provide information on the chemical identity of polypeptides, but also can reveal their secondary structures decisively and sensitively in the solid state. In particular, the amide I and amide II bands show strong polymer conformational dependence (Table 2.2).

PBLG overwhelmingly adopts an α-helical structure in solid state. The FTIR spectrum of II.2 reveals remarkable similarity to that of a conventional PBLG and demonstrates conclusively that polymer II.2 assumes an α-helical conformation in solid state film by the presence of the amide I band at 1651 cm⁻¹, amide II at 1547 cm⁻¹, and amide V at 614 cm⁻¹ (Figure 2.4). The characteristic bands and their assignments are listed in Table 2.7.

As in NMR spectra, II.2 gives broader lines, which is once again believed to be due to the structural difference between asp and glu. The combined absorbance is due to the vibration of asp atoms as well as that of glu atoms.

2.3.4 Gel Permeation Chromatography

To illustrate its molecular homogeneity, polymer II.2 was analyzed by gel permeation chromatography (A) along with a polydisperse sample (B). THF was used as it has been employed in other polypeptide systems. Chromatogram A (Figure 2.5) shows a very narrow peak compared with B. The polymer II.2 has an expected molecular weight of 16,600 and a theoretical polydispersity index (PDI) of 1.00. The
Figure 2.4 Fourier transform infrared spectra of II.2 (A) and a polydisperse PBLG (B).
Table 2.7 The FTIR characteristic bands of II.2 and their assignments.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>3291</td>
<td>Amide A, N-H stretch</td>
</tr>
<tr>
<td>2955</td>
<td>CH₂ stretch</td>
</tr>
<tr>
<td>1731</td>
<td>Ester C=O stretch</td>
</tr>
<tr>
<td>1651</td>
<td>Amide I</td>
</tr>
<tr>
<td>1547</td>
<td>Amide II</td>
</tr>
<tr>
<td>1453</td>
<td>CH₂ + phenyl</td>
</tr>
<tr>
<td>1325</td>
<td>Amide III</td>
</tr>
<tr>
<td>1212</td>
<td>Phenyl</td>
</tr>
<tr>
<td>1166</td>
<td>C-O-C stretch</td>
</tr>
<tr>
<td>1027</td>
<td>Phenyl</td>
</tr>
<tr>
<td>697</td>
<td>Phenyl</td>
</tr>
<tr>
<td>614</td>
<td>Amide V</td>
</tr>
</tbody>
</table>
Figure 2.5 Gel permeation chromatograms of II.2 (A) and a polydisperse PBLG (B), along with polystyrene standards (C). The molecular weights of the monodisperse PS standards are (i - vi): 573,000, 184,200, 95,800, 32,660, 12,860, and 2,727, respectively.
commercial PBLG has two average molecular weight values furnished by the supplier: 15,600 by low angle laser light scattering and 20,100 by viscosity. Based on polystyrene standards, II.2 has $M_w=10,400$, $M_n=10,000$, and therefore PDI=1.04; the commercial PBLG has $M_w=6,300$, $M_n=3,900$, and PDI=1.62. For the PBLG derivative II.2, the fact that its experimental $M_n$ is lower than the calculated value is attributed to higher intrinsic viscosity due to its rigid rod nature in THF, and the discrepancy between the experimental PDI (1.04) and the theoretical PDI (1.00) is thought to arise from limitations inherent to the GPC technique.

2.4 Conclusions

We have used phenyl diazomethane to esterify a monodisperse poly($\alpha$,L-glutamic acid) derivative. The resulting polymer II.2 is a derivative of PBLG. NMR spectra show quantitative conversion of the carboxylic acids to benzyl esters. The polymer undergoes a conformational transition from helicogenic to chaotropic solvent. The polymer chain in solid state assumes an $\alpha$-helical conformation as proved by FTIR. GPC reveals a much more uniform chain length of polymer synthesized through a method integrating recombinant DNA technique and subsequent chemical modification using a highly reactive diazo compound, than that obtained solely from chemical synthesis. Monodisperse rodlike polypeptides will be used as model compounds to study conformation transition and liquid crystal phase behavior. These polymers are likely to be used as building blocks for complex molecular architecture.
2.5 References

1. Block, H. Poly($\gamma$-benzyl-L-glutamate) and Other Glutamic Acid Containing Polymers; Gordon and Breach: New York, 1983.


40. Nagayawa, K.; Wada, A. Biopolymers 1975, 14, 2489.

47. Whitesell, J. K.; Chang, H. K. *Science* 1993, 261, 73.


CHAPTER 3

MEMBRANE SENSITIZATION BY SYNTHETIC POLYPEPTIDES

3.1 Introduction

3.1.1 Phospholipid Vesicles

Biological membranes are components that separate a cell from its surroundings, provide anchoring points for some proteins, and enable proteins to coordinate their catalytic activities.¹ For eukaryotic cells, membranes also define the boundaries of the intracellular compartments. The major structural elements of biomembranes are lipids. Of the amphiphilic lipids, phospholipids are the most abundant.

Phospholipids consist of polar phosphodiester head groups and double nonpolar fatty acid tails. Depending on the lengths of the hydrocarbon chains and temperatures, phospholipids, when dispersed in aqueous solution, form closed bilayer structures, with an internal aqueous compartment as shown in Figure 3.1. In the vesicles (liposomes) the hydrophobic fatty acid chains form a core and the hydrophilic head groups contact the aqueous environment. Vesicular structures can be obtained following different methods.² Multilamellar vesicles (MLV) are produced by simply vortexing the lipid dispersion in aqueous solution at temperatures above their transition points (vide infra). The sizes of MLVs are typically (in diameter) 100 nm to 5000 nm³,⁴,⁵, therefore MLV preparations appear turbid due to scattering of light. Small unilamellar vesicles (SUV) can be obtained by sonicating MLV suspensions
Figure 3.1 Vesicular structures made up of phospholipids dispersed in aqueous solution. The drawing is from Fendler.¹²
and their sizes range from 20 nm to 50 nm. The vesicles can be further separated by gel filtration to yield a more homogeneous size distribution.

Phospholipids undergo an order-to-disorder transition as temperature is increased above their characteristic melting temperature. This process can be studied by differential scanning calorimetry (DSC). Figure 3.2 shows the thermal phase transition in phospholipid bilayers by DSC and the associated changes in the membrane structure.

Although in actuality any single biomembrane can contain well over 100 different lipid species, proteins, and glycolipids, pure phospholipids have been used to make model membranes in order to study membrane-protein, membrane-synthetic polymer and membrane-membrane interactions. Those studies will ultimately lead to the understanding of fundamental phenomena such as endocytosis, exocytosis, and trafficking of cellular components. Lipid assemblies have been used as templates for mineralization and other mimetic studies. These ensembles also offer potential for targeted and controlled release.

3.1.2 Membrane-Protein or Membrane-Peptide Interaction

Most proteins either exist in solution or are embedded in lipid membranes. Biomembranes are essentially impermeable to charged molecules. It is proteins that, through their association with membranes, mediate functions such as transporting molecules across the cell, and receiving and transducing chemical signals. These so-called membrane proteins are amphiphilic in nature. Their polar surfaces interact with the aqueous solution and the lipid head groups while their nonpolar surfaces keep close contact with the nonpolar interior of the lipid bilayer. Membrane proteins diffuse rapidly in the two-dimensional plane of the membrane. They do not flip between the two layers.
Figure 3.2 Phase transitions in phospholipids as investigated by differential scanning calorimetry. As the temperature passes $T_m$, the hydrocarbon chain conformation changes from an ordered all-trans state ($L_{β′}$) to a disordered fluid-like state ($L_{α}$). Some phospholipids undergo a pretransition where the lipid molecules form a periodic lamellar phase ($P_{β′}$).
Bacteriorhodopsin, a 27 kDa membrane protein found in *Halobacterium halobium*, converts the energy of light into a transmembrane proton gradient that facilitates solute transport and ATP synthesis. Myelin basic protein (MBP) and its interaction with lipid bilayer membrane play an important role in the compaction and stabilization of the myelin sheaths found in the central nervous system. Using $^{31}$P NMR, Roux et al. was able to show that human MBP disrupted phosphatidylcholine MLVs. The authors drew the analogy of MBP to cytolytic melittin which brings about the formation of protein-associated disk-shaped micelles or small vesicular structures upon binding to lipid bilayers. Szoka and his coworkers observed pH-dependent egg PC bilayer destabilization and pH-dependent fusion of small PC vesicles induced by a 30-residue amphiphilic peptide with major repeat unit of Glu-Ala-Leu-Ala. The morphological changes of vesicles were believed to be driven by the conformational transition of the peptide from random coil to $\alpha$-helix as the pH was lowered to 5.0. Lear and DeGrado20 chemically synthesized a 20-residue peptide representing the N-terminus of influenza hemagglutinin-2 and found the peptide exhibited vesicle fusion activity.

3.1.3 Membrane-Synthetic Polymer Interaction

The bilayer structure of the anionic lipid dipalmitoylphophatidylglycerol (DPPG) can be transformed into a new morphology where the lipid chains interdigitate when a cationic polymer polyionene-6,6 is present either before or after the lipid hydration.21,22 The lamellar repeat period of the bilayer decreases from 6.0 nm to 4.9 nm. Another cationic polymer poly(L-lysine) (PLL) does not change the lamellar repeat of DPPG bilayers although the phase transition temperature of the latter increases from 40 to 50$^\circ$C.23 In the presence of PLL, PC-phosphatidylserine (PS) bilayer membranes fuse to form larger vesicles.
Tirrell and coworkers have been addressing the interaction of synthetic polymers with cell membranes and model membrane systems.\textsuperscript{24} They mostly use poly(2-ethylacrylic acid) (PEAA) to sensitize phospholipid vesicles. At pH \sim 6.5, PEAA undergoes a conformational transition from an extended random coil to a compact globule when the solution pH changes from above 6.5 to below this value.\textsuperscript{25} The presence of PEAA in PC or PG vesicle suspension renders the bilayer membrane pH-sensitive. The interaction of PEAA with membranes when pH is lowered is indicated by decreased optical density, a broad endothermic transition, and increased permeability of lipid bilayers. The interaction of PEAA and its copolymers with phospholipid can be modulated by glucose concentration, light, and UV irradiation. The disruption of vesicles is believed to be due to the lipid solubilizing effect of the hydrophobic PEAA globule at low pH.

3.1.4 Calcein as a Fluorescent Agent Used for Controlled Release Study

Calcein, bis\([N,N\text{-}bis(\text{carboxymethyl})\text{-}\text{aminomethyl}]\text{fluorescein}\) (Figure 3.3), is a water-soluble, self-quenching fluorescent molecule.\textsuperscript{26} Solutions of relatively high calcein concentration \(> 10^{-2}\) M self-quench because calcein has a high internal absorption of fluorescence. Calcein compares favorably to 5(6)-carboxyfluorescein (CF), another widely used fluorescent compound, since the former carries higher net negative charge and has higher molecular weight, and therefore increased latency in liposomes. Calcein fluorescence does not depend on pH in a wide pH range and is not perturbed by the presence of detergents.
Pyrene

8-Anilino-1-naphthalenesulfonic acid, ammonium salt (ANS)

Calcein

Dilauroylphosphatidylcholine (DLPC, $T_m = -1.8^\circ\text{C}$)

Figure 3.3 Chemical structures of some of the materials used in the research.
3.1.5 Pyrene and ANS as Probes for Hydrophobicity

Molecular interactions and formation of specific microenvironments play central roles in the functions of biological macromolecules. Many insights can be gained by designing and constructing synthetic polymers as simple model compounds, and studying their structure-property relationships. A popular approach for increasing hydrophobicity of a water-soluble polymer has been to attach chemically hydrophobic moieties (e.g., alkyl chains, (per)fluoroalkyl chains) to its backbone.\(^{27}\) For a hydrophobically modified polyelectrolyte, the hydrophobicity can also be modulated by changing solution pH.

The association of hydrophobic groups on polymers can lead to formation of hydrophobic domains. Fluorescence spectroscopy has been found to be a versatile method to investigate macromolecular association in aqueous solution.\(^{28}\) Among the many probes, pyrene has been widely used due to its strong medium sensitive emission and high propensity for excimer formation. The former characteristic will be used for this current research.

Nakajima\(^{29}\) and Thomas and coworkers\(^{30}\) have found that the fluorescence emission spectrum of pyrene shows five distinctive peaks. They observed that the fluorescence intensity of peak 1 at 373 nm, \(I_1\), exhibits strong solvent polarity dependence. On the other hand, the fluorescence intensity of peak 3 at 383 nm, \(I_3\), demonstrates little solvent dependence. \(I_1\) increases as the solvent polarity becomes larger. The ratio \(I_1/I_3\) has been used to characterize the polarity of microenvironment. The higher the \(I_1/I_3\), the larger the polarity of the domain surrounding pyrene.

Since pyrene is a very nonpolar molecule, it preferably exists in the core of hydrophobic domains. Another fluorescent probe, 8-anilino-naphthalene-1-sulfonic acid, ammonium salt (ANS), is likely to be found at the interface of polar and nonpolar domains in a microheterogeneous system. The wavelength at maximum
fluorescence emission ($\lambda_{\text{max}}$) blue shifts as the molecule detects more hydrophobic environments.31

3.1.6 Overview of Chapter 3

The ability of certain synthetic water-soluble polymers to control the structure and permeability of vesicles offers novel approaches to drug delivery and surface targeting,32,33 catalysis,34,35 reactivity control,36,37 and many other possible applications. Should such systems show results in clinical trials, the synthetic polymers must be biodegradable and biocompatible.

We have synthesized polymers based on amino acid units which should be degraded by a host of enzymes. Specifically, we synthesized poly($\alpha$,L-glutamic acid) (PLGA) using the standard N-carboxy anhydride (NCA) polymerization technique and the subsequent debenzylation. PLGA was then modified by coupling with hexylamine. The hydrophobicity of the modified polymers depends on the amount of hexylamine units and can be tuned by solution pH. The polymer-dilauroylphosphatidylcholine (DLPC) MLV interaction was monitored by optical density change and the polymer-egg yolk phosphatidylcholine (EYPC) SUV interaction by a calcein release experiment. The fluorescent probes pyrene and ANS indicate hydrophobic domain formation as the polymer concentration increases or as the solution pH decreases. These domains are responsible for solubilizing the lipid molecules, causing catastrophic structural reorganization of lipid molecules from bilayer membrane to mixed micelles.
3.2 Experimental Section

3.2.1 Materials

The following is a list of all reagents used in experiments described in this chapter. Sources for these chemicals are also indicated. All materials are used as received unless otherwise indicated.

8-Anilino-naphthalene-1-sulfonic acid, ammonium salt (ANS), 99+%

γ-Benzyl α,L-glutamate

Calcein

Dichloroacetic acid (DCA)

1,3-Dicyclohexylcarbodiimide (DCC), 99%

Diethyl ether (ether)

L-α-Dilauroylphosphatidylcholine (DLPC)

Dimethylformamide (DMF), anhydrous, 99+%

1,4-Dioxane, anhydrous, 99+%

Egg yolk phosphatidylcholine (EYPC)

n-Hexylamine, 99%

Hydrogen bromide, 99+%

Methanol, ACS certified

Petroleum ether

Phosgene, 1.0 M in toluene

Pyrene recrystallized from boiling ethanol

Sepharose CL-4B

Fluka
Sigma
Aldrich
Aldrich
Fisher
Sigma
Aldrich
Sigma
Aldrich
Aldrich
Sigma
Aldrich
Fisher
Aldrich
Fluka
Pharmacia
Tetrahydrofuran (THF), anhydrous, 99.9%
Triethylamine, gold label, 99+%  
Trifluoroacetic acid (TFA), 99%
Triton X-100, scintillation grade

3.2.2 Synthesis of γ-Benzyl α, L-Glutamate (γ-BLG) N-Carboxy Anhydride (NCA)

To a three-necked round bottom flask were added 50 mL anhydrous THF and 5.00 g (21.1 mmol) γ-benzyl α, L-glutamate. The suspension was heated to 60°C, then 25 mL phosgene-containing toluene (48.0 mmol COCl₂) was cannulated into the reaction vessel. The temperature was raised to 65°C. In about 5 min, the suspension became a clear solution. After an hour and half, this solution was poured into 0.5 L petroleum ether. The corresponding NCA precipitated immediately. After storing the mixture in a freezer for one day, the crystals was rapidly filtered and washed with 100 mL petroleum ether three times. After the product was further dried under vacuum, a white powder (III.1) was obtained. Yield: 5.01 g, 90.3%. Melting point: 95 - 96°C, lit. 97°C. Anal. calcd. for C₁₃H₁₃NO₅: C, 59.31%; H, 4.98%; N, 5.32%. Found: C, 59.34%; H, 4.96%; N, 5.29%. IR (THF cast film) cm⁻¹: 3327, 2962, 1857, 1786, 1732, 1171, 1107, 924. ¹H NMR (200 MHz, CDCl₃) δ: 7.35 (s, 5H, Ar-H), 6.60 (s, 1H, N-H), 5.12 (s, 2H, benzylic -CH₂), 4.39 (t, 1H, α-CH), 2.60 (t, 2H, γ-CH₂), 2.20 (m, 2H, β-CH₂).

3.2.3 Synthesis of Poly(γ-benzyl α, L-glutamate) (PBLG)

γ-BLG NCA (10.33 g, 39.3 mmol) was dissolved in 390 mL anhydrous dioxane in a three-necked round bottom flask which had been purged by nitrogen for
three cycles using a Firestone valve. After the mixture became a clear solution, the initiator (I) triethylamine (109.6 μL, 0.0795 g, 0.786 mmol, [NCA]0/[I]0 = 50) was charged into the flask via a syringe. The reaction was carried out at room temperature for 48 hr. The reaction solution was poured into a large excess of ethanol, filtered (aspirator) and washed with ethanol. The white fibrous product (III.2) was then dried in vacuo at 50°C for 24 hr. Yield: 5.56 g, conversion: 61.0%. Anal. Calcd. for [C12H13NO3]n: C, 65.74%; H, 5.98%; N, 6.39%. Found: C, 65.56%; H, 5.90%; N, 6.36%. IR (CHCl3 cast film) cm⁻¹: 3289, 2955, 1733, 1652, 1549. ¹H NMR (200 MHz, CDCl3/TFA (d), 60/10, v/v) δ: 7.30 (Ar-H), 5.10 (benzylic -CH2), 4.61 (α-CH), 2.45 (γ-CH2), 2.01 (β-CH2). [η] (DCA, 25.0°C), 1.88 dL/g, giving $\bar{M}_v$ = 356,000 (corresponding to $\bar{DP}_v$ of 1,600). Molar absorptivity at 260 nm (CHCl3, 23°C): $\epsilon$ = 180 L/Mol·cm.

3.2.4 Synthesis of Poly(α-L-glutamic acid) (PLGA)

PBLG (2.00 g) was dissolved in 100 mL TFA in a three-necked round bottom flask. A condensor topped with a CaSO₄ drying tube was attached to one neck. A NaOH trap was placed downstream from the drying tube. Nitrogen was used to remove most of the air from the reaction flask. After complete dissolution of the polymer in TFA, HBr (anhydrous) from a lecture bottle was introduced into the flask. The HBr flow rate was controlled such that only slowly did bubbles escape from the trap. After passing the HBr through the solution (ca. 30 min), the gas flow was stopped. The reaction solution was stirred for two hr at room temperature. Excess HBr was removed by connecting the flask to an aspirator. The solution was then slowly poured into three volume excess of ethyl ether. Precipitation occurred immediately. The product was filtered and washed three times with ether to remove TFA and benzyl bromide. Traces of TFA and benzyl bromide were further extracted
with ether by a Soxhlet extractor for 36 hr. The white powder product (designated as III.3) was vacuum dried at 56°C for 24 hr. Yield: 1.17 g, 99.0%. Anal. Calcd. for [C₅H₇N0₃]ₙ: C, 46.51%; H, 5.46%; N, 10.85%. Found: C, 44.75%; H, 5.24%; N, 10.18%. If assume 3 % H₂O in the sample, found: C, 46.13%; H, 5.06%; N, 10.49%.

IR (KBr pellet) cm⁻¹: 3293, 3079, 2960, 1718, 1653, 1545. ¹H NMR (200 MHz, D₂O, pD > 7) δ: 4.77 (α-CH), 2.61 (γ-CH₂), 2.40 (β-CH₂). [η] (0.01 M phosphate buffer containing 0.1 M NaCl, pH 7.05, 25.5°C), 1.664 dL/g, giving Mₘ = 94,000 (corresponding to DPw of 730). Molar absorptivity at 260 nm (0.1 M Na₂HP0₄, 23°C): ε = 1.15 L/Mol·cm.

3.2.5 Synthesis of Hydrophobically Modified PLGA

PLGA (0.50 g, 3.88 mmol repeating units) was added to a 250-mL round bottom flask flushed with nitrogen. Anhydrous DMF (80 mL) was cannulated into the flask to dissolve the polymer. At the same time DCC (0.32 g, 1.55 mmol) and hexylamine (205 µL, 0.157 g, 1.55 mmol) were dissolved in 10 mL DMF in a small vial. After the polymer had been completely dissolved, 2 mL of the DCC/hexylamine DMF solution was introduced to the flask via a syringe. The reacting solution was stirred for 48 hr at room temperature before it was added dropwise to 1 L anhydrous ethyl ether. Polymer precipitated very slowly. After 2 hr of stirring, the solvent was decanted and the remaining ether was removed by an aspirator. Polymer was again dissolved in 100 mL methanol. The solution was dialyzed extensively against methanol for 48 hr in a Spectra/Por 4 dialysis tube (molecular weight cutoff: 12,000-14,000). The methanol solution was then reprecipitated dropwise in 1 L ethyl ether. The solvent was decanted. This modified polymer (designated as III.4) was dried first by an aspirator and then in a drying pistol at the boiling point of acetone. Anal. Calcd. for [C₅H₇N0₃ + 0.08 (C₆H₁₃N(-O))]ₙ: C, 48.47%; H, 5.98%; N, 11.14%. Found:
C, 48.54%; H, 6.46%; N, 10.85%. IR (TFA cast film) cm⁻¹: 3287, 3070, 2942, 1682, 1655, 1552, 1445.

Polymer modified with 15 mol % hexylamine was made in a similar way, except 3.75 mL of the DCC/hexylamine DMF solution was used. This polymer was designated as III.5. Anal. Calcd. for [C₅H₇NO₃ + 0.15 {C₆H₁₃N(-O)}]n: C, 50.04%; H, 6.38%; N, 11.37%. Found: C, 50.54%; H, 6.90%; N, 10.98%. IR (TFA cast film) cm⁻¹: 3286, 3063, 2937, 1653, 1550, 1446.

3.2.6 Polymer-DLPC MLV Interaction

Dilauroylphosphatidylcholine (DLPC, 20 mg) was suspended in 10 mL 0.05 M Na₂HPO₄. This mixture was vortexed vigorously on a vortex mixer for 5 min at room temperature. One mL of this suspension was mixed with equal volume of polymer solution (2.0 mg/mL in 0.05 M Na₂HPO₄). The pH of the mixture was measured, and the optical density (OD) at 500 nm was taken on a UV/Vis spectrophotometer using the phosphate buffer as background. The pH was changed to lower values by adding 1.0 N HCl solution. The OD was measured after each pH was adjusted. All measurements were done at room temperature.

3.2.7 Polymer-EYPC SUV Interaction

Calcein loaded SUVs were made as follows: a chloroform solution of EYPC (0.25 mL, 100 mg EYPC/mL chloroform) was first subject to rotary evaporation to remove chloroform, and then vacuum dried for 2 hr. Two mL of a 200 mM calcein in phosphate-buffered saline (PBS, 0.05 M NaCl + 0.05 M Na₂HPO₄, pH 7) was added to the container. The sample was agitated on a vortex mixer at room temperature for 5 min and sonicated (Branson Sonifier Model 185 Cell Disruptor) in a water-ice bath for
40 min at 35 W. The sample was then passed through a Sepharose CL-4B column (ID = 2 cm, H = 25 cm) at room temperature using the same sodium phosphate buffer as eluent. Fractions (1.5 mL each) were collected. Fractions 15-29 were combined. Solutions of SUV (1 mL, ~ 0.5 mg EYPC) and polymer (1 mL, 2 mg/mL in PBS) were put into a vial and the pH was changed to the desired values. The fluorescence intensity I of the sample at 520 nm was monitored as a function of pH with excitation at 490 nm and 5 nm slit widths for both excitation and emission. The fluorescence intensity after adding a few drops of 10 % Triton X-100 to the solution was measured as I∞, and the fluorescence intensity of SUV alone as I0. The percentage of calcein release was calculated as the following:

\[
\text{% calcein release} = \frac{I - I_0}{I_\infty - I_0} \times 100
\]

3.2.8 Hydrophobicity Studies

In a typical case, 1.0 mL polymer solution (2.0 mg/mL in PBS), 1.0 mL PBS, and 2 μL of an acetone solution of pyrene (2 x 10^-3 M) were added to a small vial, and the mixture was adjusted to different pH in each vial by adding a small amount of 1.0 N HCl solution. The mixture was stirred at room temperature overnight to ensure homogeneous dispersion of pyrene. Emission spectra were recorded at room temperature using 337 nm excitation wavelength with 3 nm excitation slit width and 2 nm emission slit width. The concentration dependence experiment was done by diluting the polymer stock solution to 0.5, 1.0, 1.5, and 2.0 mg/mL using PBS. The emission spectra of pyrene were recorded the same way as described above.

For the experiments with ANS as the fluorescent probe, 1.0 mL polymer solution (2.0 mg/mL in PBS) was mixed with 1.0 mL of 0.60 mM ANS in PBS solution. The pH of the mixture was adjusted by adding 1.0 N HCl solution. The
polymer-ANS solution was stirred overnight at room temperature. The fluorescence emission spectra were recorded using 377 nm excitation and 5 nm excitation and emission slit widths. Solutions of different polymer concentration were obtained by diluting a concentrated polymer stock (4.0 mg/mL in PBS) to 1.0, 2.0, 3.0, and 4.0 mg/mL with PBS. This solution was mixed with an equal volume of ANS PBS solution. The spectra were obtained using the same measurement conditions.

3.2.9 Analytical Techniques

3.2.9.1 Melting Point

Melting point was measured on a Fisher-Johns Melting Point apparatus (Fisher Scientific) and reported as uncalibrated values.

3.2.9.2 Elemental Analysis

Elemental analysis was obtained from the Microanalysis Laboratory at the University of Massachusetts at Amherst.

3.2.9.3 pH of Solution

pH was measured on a Corning Model 155 pH/ion meter equipped with a semi-micro combination pH electrode (Aldrich). Before each experiment, the pH meter was calibrated using pairs of pH 4, 7, or 10 standard buffers (Fisher).
3.2.9.4 Optical Density

A Beckman DU-7 UV/Vis Spectrophotometer was used to measure the absorbance of polymer solutions and optical densities of polymer-MLV mixtures.

3.2.9.5 Fluorescence Intensity Measurement

Fluorescence emission studies at different pH and polymer concentrations were performed on a Perkin-Elmer MPF-66 Spectrophotometer. The excitation and emission slit widths were adjusted for different experiments.

3.2.9.6 Infrared Spectroscopy

IR spectra were recorded on Perkin-Elmer Series 1600 or Series 2000 FT-IR spectrophotometers. KBr pellets or cast films were used. Cast films were dried for at least 4 hr under vacuum before the spectra were taken.

3.2.9.7 Nuclear Magnetic Resonance Spectroscopy

NMR spectra were obtained on a Varian spectrometers XL-200 spectrometer. $^1$H NMR spectra (200 MHz) were recorded using either tetramethylsilane (TMS) or 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS), as the internal standard, and chemical shifts are reported as parts per million downfield from the standard.
3.2.9.8 Intrinsic Viscosity Measurement

Intrinsic viscosity measurements on PBLG were conducted in dichloroacetic acid (DCA) in a Cannon-Ubbelohde dilution type viscometer (size 75). The initial solution concentration was 1.00 g/100 mL. This solution was filtered before measurement at 25.00 ± 0.05°C. The temperature was monitored with an Omega digital thermometer. The solution was diluted several times during the measurement. The viscosity average molecular weights were calculated through the following equation:39

\[ [\eta] = 2.78 \times 10^{-5} \overline{Mv^{0.87}} \text{ (dL/g)} \]

Intrinsic viscosity measurements on poly(L-glutamic acid) were conducted in 0.1 M NaCl and 0.01M sodium phosphate aqueous solutions (pH = 7.05, 25.50 ± 0.05°C). Measurements were performed in a dilution type Cannon-Ubbelohde viscometer (size 50) with the initial polymer concentration of 0.3 g/dL. The following intrinsic viscosity-weight average molecular weight relation was used to estimate the molecular weight of PLGA:40

\[ [\eta] = 0.474 \times 10^{-5} \overline{Mw^{1.115}} \text{ (dL/g)} \]

3.3 Results and Discussion

To develop amino acid-based, pH tunable, polymer-phospholipid bilayer system was the objective of this research. We have selected a polyelectrolyte, poly(α,L-glutamic acid), whose conformation in aqueous environment can be changed from random coil at high pH to helix at low pH.41 Hydrophobic character was added
to this polymer through derivatizing the free carboxyl group with hexylamine. The hydrophobic associating properties of the modified polymers can be modulated by adjusting solution pH. The hydrophobic association provides domains that can solubilize lipid molecules and induce changes from membrane to mixed micelles. PLGA modified with 8 mol % hexylamine (III.4) and PLGA modified with 15 mol % hexylamine (III.5) were used to sensitize lipid bilayer membranes in a pH-dependent fashion.

3.3.1 Synthesis of Hydrophobically Modified PLGA

The synthetic route to hexylamine modified PLGA involved phosgenation of γ-benzyl α,L-glutamate to make the highly reactive NCA monomer, polymerization to produce high molecular weight PBLG, debenzylation to convert the polymer to PLGA, and DCC assisted coupling of hexylamine to PLGA. The reactions were carried out using published procedures with some modification.

γ-Benzyl α,L-glutamate N-carboxy anhydride (III.1) was synthesized in good yield using phosgene (Figure 3.4). Traditionally, gaseous phosgene was used. We used a safer method by adding phosgene toluene solution to THF suspension of γ-benzyl α,L-glutamate. Trichloromethyl chloroformate (diphosgene, a liquid)\(^42\) or bis(tricholoromethyl) carbonate (triphosgene, a solid)\(^43\) are gaining popularity. Other methods to make α-amino acid N-carboxy anhydrides have also been reported.\(^38,44\)

Polymerization of NCAs can be initiated either by nucleophiles or by bases, although explicit mechanisms remain elusive.\(^45,46,47\) Triethylamine, like other tertiary amines or strong bases, initiates polymerization by a so-called activated monomer mechanism.\(^48\) Polymerization under these conditions proceeds much faster than that initiated either by primary amine or secondary amines, and the average
Figure 3.4 Synthetic scheme to make III.1.
degree of polymerization (DP) is usually much higher than [NCA]₀/[I]₀. Poly(γ-benzyl α,L-glutamate) (III.2) was produced by this mechanism as illustrated in Figure 3.5. A proton is abstracted from III.1, yielding nucleophile III.1⁻, which in turn attacks a new III.1 to elongate the chain. Other polypeptides made this way may encounter solubility problems as the polymer size increases. PBLG is highly soluble in many organic solvents. This is the most popular route to the making of high molecular weight polypeptides and their derivatives. Elemental analysis and spectroscopic data confirmed the expected structure.

To remove the benzyl group from PBLG in order to produce PLGA, we used the well established procedure of Blout et al.⁴⁹ which introduced HBr (g) into TFA solution of PBLG. This route is efficient and introduces little racemization. In comparison with PBLG, the near zero molar absorptivity at 260 nm indicates complete debenzylzation. (A commercial PLGA made from poly(γ-methyl α,L-glutamate) has a molar absorptivity of 1.01 L/Mol-cm under the same measurement condition.) The DP of polymer was reduced from 1,600 before debenzylzation to 730 after the reaction as determined by the intrinsic viscosity measurements. This is also in agreement with the earlier observation that about one chain scission occurs per one thousand repeating units.⁴⁹ Different amounts of hexylamine were coupled to PLGA with the help of DCC. Other coupling modifications also used N-hydroxybenzotrizole (HOBt)⁵⁰ in addition to DCC. Since we were using relatively small amounts of hexylamine and polymer for long reaction times (Figure 3.6), the actual amount attached to the polymer was close to the feed as judged by the elemental analysis.

3.3.2 Polymer-DLPC MLV Interaction

Multilamellar vesicle suspensions scatter light due to the large size of vesicular aggregates. As shown in Figure 3.7, upon adding polymer III.5 to a suspension of
Figure 3.5 Polymerization of III.1 yields III.2 through an activated monomer mechanism. The reaction proceeds from N-terminus to C-terminus.
Figure 3.6 Synthesis of polymers III.4 and III.5 starting with III.2.
Figure 3.7 The optical density change when the pH was decreased in the polymer-MLV mixture.
DLPC MLV preparation, the optical density drops from around 1.2 to 0.4. The mixture is almost clear. III.5 acts as a polysoap that solubilizes hydrophobic moieties in the aqueous environment. The mixture further clarifies when the pH is lowered. In contrast, PLGA alone has no effect on the optical density of the mixture over the whole range of pH before it precipitates out of the mixture at pH 4.2. The OD of the polymer III.4-DLPC MLV mixture shows pH dependence. As the mixture is acidified, the turbid suspension becomes clear. Although the mixture is not totally transparent before the polymer precipitation pH, a significant change of the optical density within a narrow range is apparent. The transition point is about 5.2 in this polymer-DLPC system.

3.3.3 Polymer-EYPC SUV Interaction

Multilamellar vesicles have onion-like structures, and an optical density decrease can be due to the gradual “peeling off” of bilayers, thus reducing the sizes of MLVs, or to the complete solubilization of a fraction of the MLV population. We used another sensitive technique to investigate the polymer-lipid interaction. The highly fluorescent molecule calcein was entrapped in the interior of vesicles. Upon sonication and gel filtration, SUV were obtained. Due to the high concentration (200 mM) of calcein, its fluorescence emission is quenched inside the vesicles, therefore the calcein-loaded SUV solution is not fluorescent. Adding polymers III.4 and III.5 at pH 7 does not affect the fluorescence intensity very much as shown in Figure 3.8. When the solution becomes acidic, the fluorescence intensity gets stronger until the maximum value is reached (adding Triton X-100 does not further increase the intensity). This is due to the release of calcein from the interior of the vesicle to the extravesicular space. The midpoint for the polymer III.4-EYPC SUV system is 5.2,
Figure 3.8 Polymer-SUV interaction is dependent on pH, and is indicated by an increase of fluorescence intensity of solution containing initially self-quenched calcein.
which correlates very well with the polymer III.4-DLPC MLV system. The midpoint for the polymer III.5-EYPC SUV system is ca. pH 6.

3.3.4 Association of Modified Polymers

Along the backbone of polymer III.4 and III.5, there are ionizable carboxy groups and hydrophobic hexyl groups. If random derivatization is assumed, for polymer III.4, γ-N-hexyl α,L-glutamine units are separated on average by 12 α,L-glutamate units, while the hexyl units are separated by 6 glutamate units in polymer III.5. There are two forces dictating the polymer chain conformations. The hexyl groups tend to associate to form micellar structures in order to minimize disruption of the water molecular structure around them (a phenomenon called the hydrophobic interaction\textsuperscript{51,52}). The ionic groups tend to separate due to Coulombic repulsion. As the carboxylate groups are protonated, the repulsion becomes weak, which helps the association of the hexyl groups. (We are considering a simplified picture here. Presumably the methylene units and the carbonyl group on each structural unit are also contributing to the overall interaction. The acidified polymers might assume a helical structure which produces a very large dipole. The polarized molecules also confer interactions).

We used pyrene and 8-anilino-naphthalene-1-sulfonic acid, ammonium salt (ANS), to investigate the hydrophobic domain formation in the polymer system alone. Lipid molecules were not present since they form a very hydrophobic membrane layer where pyrene and ANS can be located. Figures 3.9 and 3.10 indeed demonstrate that as the solution pH decreases or the polymer concentration increases, pyrene probes detect more hydrophobic domains. At the same polymer concentration in basic media, the solution containing III.5 has more hydrophobic domains or has domains that are more hydrophobic compared with the solution containing III.4.
Figure 3.9 pH dependence of $I_1/I_3$ of pyrene in solution containing III.4 or III.5.
Figure 3.10 Polymer concentration dependence of I$_1$/I$_3$ of pyrene in solution containing III.4 or III.5.
The concentration effect is more pronounced for **III.5** solution at concentrations below 1.0 mg/mL. After that, I1/I3 levels off, indicating that further addition of polymer does not significantly change the character of the hydrophobic domains. Interestingly, **III.4** solution at 2 mg/mL does not appear as hydrophobic as **III.5** solution at 1 mg/mL, although the overall amount of the hexyl units are approximately the same. This indicates that attaching hydrophobic groups to the polymer backbone does help them to associate.

ANS also reports micelle formation in solutions containing either **III.4** or **III.5**, depending on solution pH or polymer concentrations (Figures 3.11 and 3.12). The hydrophobic microdomain formation in **III.4** solution shows strong pH dependence with a transition point of 4.8. The **III.5** solution has pH dependence within a narrow range with a transition point of 5.9. The large blue shift of the wavelength at maximum fluorescence emission demonstrates that increasing polymer concentration also aids in micelle formation.

A comparison of Figure 3.9 with Figure 3.11 is worthwhile. The midpoint for the microdomain formation in **III.4** solution (1.0 mg/mL) is ca. pH 6.0 as detected by pyrene, pH 4.8 as reported by ANS. Since pyrene is located at the core of the hydrophobic domain, it senses the micellization first. At this pH, ANS is still located at the interface.28 Only when the pH drops further, does ANS detect hydrophobic domain formation.

### 3.4 Conclusions

We have developed a method to make amino acid-based, hydrophobically associating polymers and studied their interaction with phospholipid bilayer membranes. While PLGA has no effect on the sensitization of vesicles, the 15 mol % hexylamine modified polymer interacts with vesicles over a broad pH range. PLGA
Figure 3.11 The wavelength at the maximum fluorescence emission of ANS as a function of pH in solution containing III.4 or III.5.
Figure 3.12 The wavelength at the maximum fluorescence emission of ANS as a function of polymer concentration.
with 8 mol % hexylamine interacts with lipid bilayers in a strongly pH-dependent manner. The lipid solubilizing behavior of the modified polymers is due to their hydrophobic domain formation in aqueous environment as solution pH decreases.

3.5 References


CHAPTER 4

CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

The engineering of novel materials at the molecular level is the final goal of this project. We have developed a general strategy that combines recombinant DNA techniques, chemical modification, and physical characterization. This approach has allowed us to synthesize a monodisperse polyelectrolyte IV.1 in a bacterial host (yield: 1 mg/L 2 X YT culture) and a monodisperse liquid crystalline polymer IV.2.

\[
\text{GluAsp(Glu}_{17}\text{Asp)}_{4}\text{GluGlu}
\]

\[
\text{IV.1}
\]

\[
\text{Glu(OBzl)Asp(OBzl)}_{17}\text{Asp(OBzl)}_{4}\text{Glu(OBzl)Glu(OBzl)}
\]

\[
\text{IV.2}
\]

where OBzl denotes a benzyl ester.

Double-stranded DNA sequencing confirmed the artificial gene sequence encoding IV.1. The amino acid analysis of IV.1 agrees with its expected overall composition. Circular dichroism indicates that IV.1 undergoes a pH-dependent conformational transition in aqueous solution. This agrees with our \textit{a priori} expectation that a polypeptide with Glu\textsubscript{17}Asp as the major repeat (P\textsubscript{\alpha,Glu}=1.59, P\textsubscript{\alpha,Asp}=0.99, see Table 1.1 for an explanation) should adopt an \alpha-helical structure when the carboxylate groups are protonated. The electrophoresis result shows IV.1 is composed of much more uniform polymer chains than chemically synthesized PLGA.
The reaction of phenyl diazomethane with IV.1 leads to polymer IV.2. Proton 
NMR confirmed quantitative conversion of the -COOH on IV.1 to -COOCH₂C₆H₅. 
Again to demonstrate our ability to predict the secondary structure based on the 
conformational propensities of amino acids, the NMR shows that IV.2 assumes an α-
helical conformation, and transforms into a random coil; and FTIR indicates that IV.2 
self-assembles into an α-helical structure in solid films. GPC results demonstrate that 
the polymer IV.2 shows a much narrower chain length distribution than its synthetic 
counterpart.

The chemically synthesized PLGA modified with 8 mol % hexylamine can 
disrupt DLPC MLV and EYPC SUV in a strongly pH-dependent manner. The 
polymer modified with 15 mol % hexylamine interacts with lipid bilayers even in 
basic aqueous solutions. The hydrophobically modified polymers self-associate to 
form hydrophobic domains that are responsible for their lipid membrane solubilization 
behavior.

4.2 Future Work

Synthesis of IV.1 in a reasonably large quantity is a top priority. More 
quantitative CD measurements at different pHs are needed to compare the molar 
ellipticity of IV.1 with that of chemically synthesized PLGA. Crystallization studies 
might provide evidence to support that IV.1 crystallizes differently from polydisperse 
PLGA of high molecular weight.¹

Sufficient amounts of IV.1 can help to make large quantities of IV.2. 
Conticello and Tirrell² are studying the liquid crystalline (LC) properties of IV.2 and 
their preliminary result indicates that the uniform helical rods form an anisotropic 
phase in benzyl alcohol. Studies of chain variants of IV.2 may clarify the LC 
behavior predicted by theory³ but complicated by polymer polydispersity.⁴ The
marriage of the recombinant DNA techniques and chemical modification can truly generate smart materials\textsuperscript{5} based on \textit{polymers without dispersity}.

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