Synthesis of biopolymers: proteins, polyesters, polysaccharides and polynucleotides

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The synthesis of proteins, polyesters, polysaccharides and polynucleotides can be adapted to produce new macromolecular materials. Proteins of designed sequence, and with specific chemical functions, conferred by the incorporation of unnatural amino acids, have been prepared in genetically engineered bacteria. Polyesters, useful as biodegradable thermoplastics, have been made in bacterial hosts, and more recently, in transgenic plants. Polysaccharides, made either chemically or enzymatically, are being explored for biomedical applications, and synthetic polynucleotides could eventually serve as scaffolds in the construction of nanoscale materials.

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Abbreviations

PHA	polyhydroxyalkanoate
PHB	poly-3-hydroxybutyrate

Introduction

Biopolymers have long been of interest to materials scientists because of their unique structural properties. Evolutionary constraints have resulted in classes of materials that are particularly suited for specific functions. Examples include proteins, which play structural or catalytic roles, polysaccharides, which may provide structural integrity in addition to energy storage, and polynucleotides, which in their natural environments, direct the synthesis of proteins and thus determine the characteristics of organisms.In addition to the very specific relations between structure and function, another aspect of biopolymers intriguing to materials scientists is the precision with which they are made. In contrast to conventional synthetic polymers, which show a distribution of monomer sequences and molecular weights, proteins and nucleic acids are of defined length and have precisely determined monomer sequences. Because the sequence and length of proteins are determined by the genetic material (DNA [deoxyribonucleic acid] and [ribonucleic acid] RNA) in the cell, genetic engineering allows the fidelity of protein synthesis to be harnessed to produce new polymers having precisely defined chemical and physical properties.

The advent of recombinant DNA technology has contributed greatly to the investigation of natural polymers as materials. Genes encoding new protein-based polymers can be designed and prepared by chemical synthesis, or alternatively, genes encoding natural proteins of interest can be isolated from the organisms in which they occur and cloned. In either case, natural or artificial proteins can then be produced in genetically engineered bacteria.

In this article we will discuss recent advances in the synthesis of several structural proteins, including spider silk, aquatic insect silks, and artificial silk-like proteins, in addition to artificial proteins that contain unnatural amino acids which have the potential to introduce unique materials properties. We will then discuss, firstly, the use of bacterial energy storage polyesters as novel, biodegradable plastics that can be 'engineered' to have useful materials properties, secondly, the use of polysaccharides, to study problems in polymer synthesis such as regioselectivity and stereochemistry, and, thirdly, polynucleotides, which have potential application in the fabrication of nanoscale devices.

Proteins

The chemical and materials properties of silks of the spider, *Nephila clavipes*, [1–5] the silkworm, *Bombyx mori*, [2,6,7] and other insects, notably *Manduca sexta* and *Sesamina nonagroides*, [8,9] have been studied extensively. The rigidity and high tensile strength of *B. mori* silk fibroin can be attributed to the fact that the protein consists mainly of hydrogen-bonded, stacked antiparallel β-sheets. The β-sheets are formed from repeating sequences of the amino acids (largely GlyAlaGlyAlaGlySer) and give rise to a rigid, crystalline protein.

Artificial proteins made up of folded-chain lamellar crystals of controlled thickness and surface chemistry have been successfully prepared by biological synthesis in bacterial hosts [10,11]. The rationale for the design of these proteins is the choice of a repeating amino acid sequence (alternating alanine and glycine residues) that allows the formation of β-strands, with reverse turns introduced by the periodic insertion of amino acids with charged or bulky side chains (e.g. glutamic acid). The β-strands associate in arrays of hydrogen-bonded sheets which then stack to form lamellar crystals. The thickness of the crystal is determined by the length of the β-strands, that is, by the periodicity of insertion of the bulky amino acid residues. The surface properties of the crystalline protein are determined by the nature of the bulky side chains.

These chain-folded lamellar crystals are synthesized in *Escherichia coli* cells transformed with synthetic genes encoding the desired amino acid sequences. The process

from gene synthesis to protein production has been described in detail [10]. Briefly, oligonucleotides encoding the desired amino acid sequence are prepared using solid phase organic synthesis, and are then ligated to form a population of DNA multimers. Multimers of appropriate size are inserted into a bacterial expression vector, which is then used to transform *E. coli* cells. The transformed cells yield the target protein under appropriate fermentation conditions.

A family of periodic polypeptides that has been shown to adopt β-sheet secondary structure has the amino acid sequence $([AlaGly]_xGluGly)_n$ where x = 3–6 and n = 14, 20, 28 or 36 [10,11]. Evidence for the formation of β-sheets is obtained by infrared spectroscopy, Raman spectroscopy, and cross-polarization magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy [10]. The infrared spectrum of $([AlaGly]_3GlyGlu)_{36}$ for example, shows vibrational modes characteristic of the β-sheet structure, and additional vibrations indicating the presence of a regularly alternating chain direction characteristic of antiparallel β-sheets. Other features of the spectrum indicate the presence of secondary structures unrelated to antiparallel β-sheets; these structures are probably reverse β- or γ-turns. Raman spectroscopy and CP/MAS NMR spectroscopy of $([AlaGly]_3GlyGlu)_{36}$ further confirm the antiparallel β-sheet structure and both kinds of spectra contain weaker signals that have been attributed to β or γ-turn structures. X-ray diffraction patterns of crystal mats of a series of polymers, $([AlaGly]_xGlyGlu)_n$, where x = 3, 4, 5, 6 and n = 14, 20, 28 and 36, respectively, give strong evidence for a crystalline antiparallel β-sheet architecture with a chain-folded lamellar structure as the basic crystalline unit [10]. The unit cell dimensions are consistent with those previously reported for silk fibroins.

The incorporation of unnatural amino acids into artificial proteins provides a means of expanding the range of functional groups usually found in proteins, and of placing these groups at predetermined sites along the polypeptide chain. Recently, proteins containing selenium, fluorine, potentially conductive moieties, and proline analogues have been obtained by substituting unnatural amino acids for their naturally occurring counterparts during protein synthesis in genetically engineered bacteria [12–14,15•,16]. A method for determining the potential for incorporating unnatural amino acids into recombinant bacterial proteins has been described [12].

Selenium proteins, containing the repeat sequence $([GlyAla]_3GlySeMet)$, are expected to provide accessible selenium groups at the protein crystal surface because the placement of a bulky residue following the GlyAla dyads is favorable for introducing a turn into the β-strand [13]. The bulky selenium atoms should thus be available for chemical modification, for example, alkylation to form trialkylselenonium halides, or oxidation to form reactive selenoxides.

Polymers containing fluorinated amino acids are expected to have many of the characteristics of conventional fluoropolymers, including good hydrolytic stability, excellent solvent resistance, low coefficient of friction, and low surface energy. *p*-Fluorophenylalanine [14] was substituted for phenylalanine in proteins containing the repeat sequence $([AlaGly]_3PheGly)$, to an extent of 95–100% during protein synthesis in an *E. coli* host strain which was dependent on phenylalanine for growth. Evidence for β-sheet structure was obtained from Fourier transform infrared spectroscopy (FTIR) and wide angle X-ray scattering analysis.

3-Thienylalanine (3-TA) was chosen for incorporation into genetically engineered proteins because of its similarity to the 3-alkylthiophenes [15•], which when polymerized are excellent organic conductors, showing conductivities of about $2000\,S\,cm^{-1}$ after doping. The incorporation of 3-thienylalanine into genetically engineered proteins may provide a means for electrodepositing proteins on electrodes or for fabrication of enzyme-based sensory elements. Electron absorption and NMR spectra of $([GlyAla]_3GlyPhe)_{13}$, in which 3-thienylalanine is substituted for phenylalanine, indicate that the highest extent of substitution achieved to date is approximately 80% [15•].

Repeating polypeptides of the sequence $([AlaGly]_nGluGly)$ adopt chain-folded, crystalline architectures, as described above [10,11]. However, the introduction of proline residues into the repeat sequence, as in $([AlaGly]_nProGluGly)$, produces proteins having disordered structures in the solid state [17]. To determine whether replacement of proline with a smaller amino acid residue would allow β-sheet formation, azetidine-2-carboxylic acid (Aze) was substituted for proline in polypeptides of repeating sequence $([AlaGly]_3ProGluGly)$ [16]. Aze substitution for proline was estimated by NMR spectroscopy to be approximately 40%, which is sufficient to allow the formation of antiparallel β-sheets as indicated by FTIR spectroscopy [16].

In addition to the production of artificial proteins, natural proteins, such as spider silk, are being synthesized using synthetic genes [1,18•]. The dragline silk of spiders has the tensile strength of Kevlar® and seven times the elasticity [1]. The structures of the proteins found in dragline silk are similar to that of *B. mori* silk fibroin in that glycine and alanine are the predominant amino acids, but in addition to these, there are amino acids with bulky side chains, such as tyrosine, glutamine, arginine and leucine [1]. As spiders do not produce silk in conveniently large packages and do not lend themselves to domestication, historically spider silk has not found wide application. With the introduction of recombinant DNA techniques, however, the potential for large-scale production of spider silk is being investigated.

Several groups have been exploring the production of spider silk using recombinant DNA methods. Randolph

V Lewis has constructed genes composed of multiple repeats of cloned DNA fragments encoding consensus repeats of the main dragline silk protein [1]. Using bacteria transformed with this gene, pure artificial silk protein has been produced and is now being spun into fibers. A similar gene synthesis strategy has been used for spider silk by Prince *et al.* [18•] and for silk from the midge, *Chironomus tentans*, by Case and Smith [19,20]. Circular dichroism measurements of synthetic spider dragline silk indicate that the protein contains substantial β-sheet structure [18•], but preliminary infrared spectra of midge silk show an absence of vibrational modes characteristic of β-sheets [19]. The secondary structure of synthetic midge silk is currently under investigation.

Polyesters

Several kinds of bacteria synthesize polyhydroxyalkanoates (PHAs) as osmotically inactive energy storage compounds [21,22]. These polymers have attracted interest because they are biodegradable thermoplastics and a wide variety of bacteria produce them in bulk under appropriate fermentation conditions. The physical properties of bacterial PHAs have been studied extensively [23]. The predominant polymer synthesized by *Alcaligenes eutrophus*, poly-3-hydroxybutyrate (PHB), is brittle and undergoes thermal decomposition just above its melting point, thus limiting its commercial usefulness. Recently, other types of PHAs have been produced both by regulating the feed composition and growth conditions of the host bacteria and by genetic engineering techniques. One of these, poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (P[HB-co-HV]), is less brittle and more processable than PHB, and is now marketed under the name of Biopol for use as biodegradable packaging.

Several laboratories have been investigating the efficient production of bacterial polyesters. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) is being prepared in *Alcaligenes eutrophus* by continuous production methods as opposed to batch fermentations [24]. The compositions of PHAs produced in bacterial cultures can be influenced by the composition of the medium or by the choice of organism. For example, poly(3-hydroxybutyrate-*co*-3-hydroxycaproate) [25] and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [26] are synthesized by *Bacillus cereus*, *Comomonas testosteroni*, [25] and by *Alcaligenes latus* [27], when the organisms are fed the appropriate monomers. Bacteria of the genus *Pseudomonas* have been known to incorporate a variety of monomers with different functionalities into bacterial polyesters. Recent examples of such novel bacterial polymers include the polymerization of 5-(4'tolyl)valeric acid by *P. oleovorans* to produce polymers containing aromatic substituents [28••], the incorporation of medium [29••] and long [26] chain fatty acids into PHA by *Pseudomonas* sp. A33 and by *P. aeruginosa*, respectively, and the synthesis of PHA containing cyano and nitrophenoxy substituents by *P. putida* and *P. oleovorans* [30••]. Additional studies of PHA storage granules have yielded insight into the relations between granule structure and efficiency of polymer synthesis [31•].

In efforts to find cheaper carbon sources for polymer production, PHB [32,33] and P(HB-*co*-HV) [34] syntheses are being studied in recombinant *E. coli*. An alternative route to inexpensive production of PHAs is through the use of transgenic plants. Considerable work has been done with *Arabidopsis thaliana* [35•,36] into which the genes encoding the PHB synthesizing system of *Alcaligenes eutrophus* have been introduced. In early experiments, PHB was produced throughout the plant, leading to stunted growth and low seed production, but targeting the polymer synthesis to plastids (sites of accumulation of storage compounds and biosynthesis of fatty acids which are potential substrates for PHA synthesis) appears to allow polymer to be synthesized in harvestable quantities without compromising plant growth [36].

Polysaccharides

The synthesis and postmodification of polysaccharides are often undertaken to study basic problems in polymer synthesis such as stereochemistry [37•], regioselectivity [38–40] and substituent effects [41]. Routes to these stereoregular polymers often take advantage of enzymes which normally catalyze the hydrolysis of polysaccharides, for example, cellulase which has been used to prepare an artificial xylan [37•] and phosphorylases, used to prepare model cellulose polymers [42–44]. Regiospecific modifications to sugar monomers which can then be polymerized to produce novel materials can also be carried out using enzymes [38,39]. In addition, enzymatic postmodification of polysaccharides, such as regioselective acylation [40], may be another useful route to new polymers.

Potentially useful materials properties of poly(galactoside acrylates), such as water absorbance and biocompatibility [45•], and reinforcing effects of cellulose fibers in synthetic polymer matrices [46] have been reported. To prepare the poly(galactoside acrylates), the regioselective acryloylation of α-methyl galactoside was carried out enzymatically, followed by free radical polymerization and cross linking [45•]. The resulting cross-linked networks formed hydrogels. Films made from composites of styrene-butyl acrylate copolymers and cellulose fibers isolated from tunicate exoskeletons showed improved mechanical properties when compared to polymer films not containing cellulose fibers, especially above the glass transition temperature [46].

Synthetic polysaccharides may eventually be used in drug delivery systems. A series of water soluble polymers containing clusters of sugar monomers were chemically synthesized and their binding activities toward various plant lectins were assessed [47]. Preliminary results indicate that the binding characteristics of these polymers

appear to be dependent on the spacing of the sugar clusters; further investigations are in progress.

In the search for inhibitory agents of the human immunodeficiency virus (HIV) it has been observed that polyanions suppress HIV infectivity. Toward this end, sulfated alkyl malto-oligosaccharides were chemically synthesized and were found to exhibit both anti-HIV activity and anticoagulant activity [48]. The oligosaccharides with the most potent anti-HIV activities, however, were also the most cytotoxic compounds. Work is in progress to reduce the cytotoxicity of these compounds while retaining the anti-HIV activity.

Polynucleotides

The structural engineering of DNA may have potential use in the fabrication of nanoscale devices. DNA polymers are unusually suited to engineering in three dimensions and could thus have applications as scaffolds for the ordering of other materials. Recently, branched and knotted forms of DNA have been synthesized and characterized [49–52]. Further study of these three dimensional structures will likely lead to new insights about their functions in cells (where they were first observed) as well as to construction of nanoscale materials.

Conclusion

Biopolymers with interesting materials properties can be produced by a variety of methods, including natural processes and traditional chemical reactions. The natural processes for fabricating biopolymers range from synthesis in genetically engineered bacterial hosts (applicable primarily to proteins) to the use of enzymes to modify natural substrates for subsequent polymerization, or for reactions on polymers synthesized by traditional chemical reactions. As efficient synthetic methods continue to develop, it is expected that the materials applications for biopolymers will become more widespread.

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