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6. AUTHOR(S) David A. Tirrell			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Polymer Science and Engineering University of Massachusetts Amherst, MA 01003		8. PERFORMING ORGANIZATION REPORT NUMBER N/A	
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13. ABSTRACT (Maximum 200 words) The goal of this research program has been to establish strategies for producing protein-based materials that contain amino acids not normally found in natural proteins. Efforts have been focused on amino acids with fluorinated, electroactive or conformationally constrained side chains, and have allowed us to prepare protein-based polymers with unique properties, including low surface energies and sensitivity to electrochemical signals. These developments have expanded in a substantial way the range of materials properties accessible via chemical or biological synthesis of polypeptides and artificial proteins.			
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Genetic Engineering of Polymers Containing Non-Natural Amino Acids

FINAL PROGRESS REPORT

David A. Tirrell

Principal Investigator

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University of Massachusetts Amherst

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GENETIC ENGINEERING OF POLYMERS CONTAINING NON-NATURAL AMINO ACIDS

Statement of the Problem

The goal of this research program has been to establish strategies for producing protein-based materials that contain amino acids not normally found in natural proteins. Efforts have been focused on amino acids with fluorinated, electroactive, or conformationally constrained side chains, and have allowed us to prepare protein-based polymers with unique properties, including low surface energies and sensitivity to electrochemical signals. These developments have expanded in a substantial way the range of materials properties accessible via chemical or biological synthesis of polypeptides and artificial proteins.

Summary of Results

Previous progress reports have discussed our results on *p*-fluorophenylalanine¹, trifluoroleucine², azetidine-2-carboxylic acid³, thiaproline, dehydroproline⁴, and 3-thienylalanine⁵. All of these amino acid analogues have been successfully incorporated into artificial proteins expressed in bacterial hosts, via routes that exploit the promiscuity of the normal protein synthetic apparatus of the bacterial cell. During the most recent funding period, primary attention has been given to two new directions that are complementary to the first: i). chemical synthesis and structural analysis of poly(α ,L-glutamate)s with long fluoroalkyl side chains, and ii). development of new methods for site-directed incorporation of non-natural amino acids in *Escherichia coli*.

Poly(α ,L-glutamate)s with fluoroalkyl side chains. Fluorinated polymers exhibit special properties, including stability at high temperatures, toughness and flexibility at very low temperatures, nonadhesiveness, insolubility, chemical inertness, and, in some cases, biocompatibility. Incorporation of fluorinated amino acids has been proposed as a means to impart some of these properties to polypeptides. In work reported earlier this year in *Macromolecules*⁶, poly(α ,L-glutamate)s carrying C₈, C₁₀, C₁₂ fluorinated side chains were synthesized by polymerization of the corresponding amino acid N-carboxyanhydrides. Contact angle measurements with water were used to assess the effects of fluorination on the surface energies of films of the resulting polypeptides. The wettability of the polymers was found to decrease with increasing fluorine content, as expected. A remarkably high contact angle (121°) was measured for the homopolymer carrying C₁₂ fluorinated side chains, indicating a surface consisting of closely packed trifluoromethyl groups. Side chain crystallization, consistent with the formation of such a surface, was suggested by the results of x-ray diffraction and calorimetric measurements.

Site-directed incorporation of non-natural amino acids in E.coli. In this phase of our program we are developing *E. coli* expression strains for the site-directed incorporation of non-natural amino acids.

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Our approach entails encoding the position of the non-natural amino acid in the gene of choice by the "blank" amber stop codon (TAG); this stop codon is deciphered in translation by a yeast amber suppressor tRNA, which in turn is charged by its co-expressed cognate yeast tRNA synthetase (a so-called 21st pair) with an analogue provided in the growth medium. The long-term goal of this work is the creation of mutant yeast tRNA synthetases which can charge useful non-natural amino acids with high specificity to their cognate suppressor tRNAs. So far, we have identified the yeast seryl-tRNA synthetase (SRS) and the tRNA_{amber}^{ser} as a suitable 21st pair for our purposes. We are currently identifying active-site residues in the yeast SRS that make essential contacts to the serine substrate side chain. Such residues will be prime targets in our effort to change substrate specificity through protein engineering.

Our shorter term goal is a direct application of the 21st pair approach, namely the site-directed incorporation of p-fluorophenylalanine (pFPhe) as a useful ¹⁹F-NMR probe. The analogue pFPhe is a cytotoxic substrate for *E. coli*, but a pFPhe resistant mutant strain isolated a long time ago rejects this non-natural amino acid, owing to increased substrate specificity of its phenylalanyl-tRNA synthetase (FRS). We have equipped this resistant strain with a yeast tRNA_{amber}^{Phe} and yeast FRS. Co-expression of this 21st pair in the absence of analogue results in incorporation of about 95% Phe at the programmed position in the marker protein dihydrofolate reductase (DHFR), demonstrating that the yeast tRNA_{amber}^{Phe} is not mischarged by the other host synthetases. Preliminary data show that when an 8-fold excess of pFPhe over Phe is present in the growth medium, the programmed position in DHFR now contains about 70% pFPhe and about 30% Phe as determined by peptide sequencing. Even though complete substitution of Phe by pFPhe cannot be achieved in this system at this point, unlabeled species will be silent in the NMR measurement and will not complicate data acquisition with the labeled fraction of the sample.

Future experiments are designed to corroborate the preliminary finding, to show that only the programmed position contains the analogue, and to increase the expression yield about 10-fold from the current 0.8 mg/L of culture at OD₆₀₀ = 1.0. We also have indirect genetic evidence that suggests that p-chlorophenylalanine might be incorporated by the 21st yeast pair as well.

Our current experiments are directed toward *site-directed* insertion of non-natural amino acids into proteins. The synthetases and strategies developed here, however, can also be used, with some modification, for *uniform* labeling of protein-based polymer materials with non-natural amino acids. We intend to begin exploration of this possibility during the coming months, pending continued ARO support of this work.

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Publications

The papers and manuscripts resulting from this program are listed below.

1. Yoshikawa, E., Fournier, M. J., Mason, T. L., & Tirrell, D. A. (1994). Genetically Engineered Fluoropolymers. Synthesis of a Repetitive Polypeptide Containing p-Fluorophenylalanine Residues. Macromolecules, 27, 5471-5475.
2. Kothakota, S., Atkins, E. D. T., Mason, T. L., Tirrell, D. A., & Fournier, M. J. Genetically Engineered Fluoropolymers with Reduced Surface Energy, in preparation.
3. Deming, T. J., Fournier, M. J., Mason, T. L., & Tirrell, D. A. (1996). Structural Modification of a Periodic Polypeptide through Biosynthetic Replacement of Proline with Azetidine-2-carboxylic Acid. Macromolecules, 29, 1442-1444.
4. Deming, T. J., Fournier, M. J., Mason, T. L., & Tirrell, D. A. Biosynthetic Incorporation and Selective Chemical Modification of Functionality in Genetically Engineered Polymers, submitted for publication.
5. Dessipri, E., & Tirrell, D. A. (1994). Trifluoroalanine N-Carboxyanhydride: A Reactive Intermediate for the Synthesis of Low Surface Energy Polypeptides. Macromolecules, 27, 5463.
6. Dessipri, E., Tirrell, D. A., & Atkins, E. D. T. (1996). Fluorinated Poly(α ,L-glutamate)s. Macromolecules, 29, 3545-3551.
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8. Kothakota, S., Yoshikawa, E., Murphy, O. J., Mason, T. L., Fournier, M. J., & Tirrell, D. A. (1995). A Simple Assay for Screening Translational Activity of Non-Natural Amino Acids. Implications for Polymer Synthesis on Messenger RNA Templates. Journal of Polymer Science, Part A: Polymer Chemistry, 33, 1267.
9. Tirrell, J. G., Fournier, M. J., Mason, T. L., & Tirrell, D. A. (1994). Biomolecular Materials. Chemical & Engineering News, (December 19, 40).
10. Fournier, M. J., Mason, T. L., & Tirrell, D. A. (1995). Role of Molecular Genetics in Polymer Materials Science. New York: VCH Publishers.

Scientific Personnel Supported By This Project

Srinivas Kothakota, Ph.D. 1995, Biochemistry and Molecular Biology

Eugenia Dessipri, Ph.D. 1995, Polymer Science and Engineering

Michael Yu, Ph.D. candidate Polymer Science and Engineering

Timotny Deming, Postdoctoral Fellow, Polymer Science and Engineering (now Assistant Professor of Materials, University of California, Santa Barbara)

Rolf Furter, Research Assistant Professor, Biochemistry and Molecular Biology

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Report of Inventions

None to report.