Microbial Synthesis of Poly(β-hydroxyalkanoates) Containing Fluorinated Side-Chain Substituents

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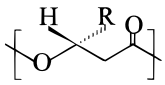
ABSTRACT: The preparation of novel fluorinated poly(β-hydroxyalkanoates), PHAs, was carried out using *Pseudomonas oleovorans* (ATCC 29347) and *Pseudomonas putida* (KT 2442) as biocatalysts. These organisms were first grown on 40 mM sodium citrate prior to studying polymer formation in the second stage using 1:1 molar mixtures of nonanoic acid (NA) and fluorinated acid cosubstrates. The following fluoro acids were synthesized and used in this study: 6,6,6-trifluorohexanoic acid (TFHxA), 6,6,7,7,8,8,8-heptafluorooctanoic acid (HpFOA), 6,6,7,7,8,8,9,9,9-nonafluorononanoic acid (NFNA), and 6,6,7,7,8,8,9,9,-10,10,11,11,11-tridecafluoroundecanoic acid (TDFUDA). In general, the use of NA/fluoro acid cosubstrate mixtures instead of only NA in second-stage cultivations resulted in little to no cellular toxicity as measured by values of colony-forming units per milliliter. The mol percent incorporations of fluorinated side chains was determined by ^{1}H and ^{19}F NMR spectroscopies, and peak assignments were made using two-dimensional reverse-detected heteronuclear multiplet quantum correlation (HMQC) as well as ^{1}H−^{1}H correlation spectroscopy (COSY). *P. putida* formed PHA after a 3-day second-stage cultivation time with 17.3 mol % fluorinated side chains using NA/NFNA as cosubstrates. For shorter second-stage cultivation times (1 day) where product yields were relatively higher, 0.3 g/L of product was formed that contained 6.4 mol % fluoroalkanoate side groups using *P. oleovorans* as the biocatalyst and NA/HpFOA as cosubstrates. The incorporation of 12.4 mol % fluoroalkanoate repeat units resulted in products which showed melting at higher temperatures (55−80 °C), crystallized at faster rates from the melt, and had higher heats of fusion. Investigation of the surface free energy of products by surface contact angle measurements showed only a modest increase from 87 to 94° for PHAs containing 0 and 17.3 mol % fluorinated side chains.

Introduction

The family of the microbial polyesters referred to as poly(β-hydroxyalkanoates), PHAs, has the general structure shown below, where the repeat unit chiral centers have (*R*)-stereochemical configurations[1,2] so that the polymers are isotactic and optically active.[3−5] Also, PHAs are normally built from 3-hydroxyalkanoates, although much work has been carried out in preparing PHAs containing 4-hydroxybutyrate repeat units.[6,7]



β-linked PHA structure, R = side-chain substituent

A number of reviews have been published that describe the wide range of 3-hydroxyalkanoate repeat unit structures that have been incorporated into product polyesters by bacterial polymerization systems.[8−13] Examples of 3-hydroxyalkanoate functional side groups incorporated by the medium side chain (*n*-propyl to *n*-nonyl) producing strain *Pseudomonas oleovorans* ATCC 29347 include vinyl,[14] cyano,[15] phenyl,[16−18] and phenoxy.[19] *Pseudomonas putida* (KT 2442) has been used to produce PHAs with medium-chain-length 3-hydroxyalkanoate repeat units which contain multiple double bonds.[20] *P. putida* was also found to be useful for the incorporation of 3-hydroxy-6-(cyanophenoxy)hexanoate repeat units.[21,22] Thus, side groups that vary significantly from the *n*-alkanoate structure in steric size and polarity have been incorporated into product polyesters.

Recently, PHAs containing monohalogenated side groups, specifically fluorine,[23] bromine,[24] and chlorine,[25] have been produced in *P. oleovorans* from ω-monohalogenated alkanes. Abe *et al.* used *P. oleovorans* cultivated on various mixtures of nonane and 1-fluorononane in bulk one-stage fermentations to produce copolyesters containing up to 24 mol % 3-hydroxy-9-fluorononanoate.[23] Interestingly, it was reported by these workers that incorporation of monofluorinated repeat units up to 27 mol % into PHA polymers resulted in a modest increase in the melting temperature (T_m) from 47 to 61 °C, a slight decrease in the glass transition temperature from −39 to −44 °C, and a large increase in the enthalpy of fusion (ΔH_f) from 3.1 to 7.9 cal/g. The authors did not offer an explanation for these interesting changes in polymer thermal properties.

Fluorochemistry to prepare fluoro polymers has proved useful in modulating performance characteristics relative to their hydrocarbon analogues to achieve higher heat- and oil-resistant materials having superior surface properties with low surface tension, high lubricity, and nonwetting properties.[26] More recently, there has been increasing interest in exploring fluorine-containing compounds for use as bioactive agents and in the development of materials for diagnostic devices.[27] Also, fluorinated amino acids have been used in cultivation media for the biosynthesis of designer fluorinated proteins. Tirrel *et al.*[28] have synthesized fluorinated proteins containing *p*-fluorophenylalanine units using a strain of *Escherichia coli* that required phenylalanine in the growth medium.

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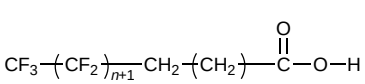
In this work, studies were carried out to determine whether microbial polyester-producing organisms would metabolize substrates with high degrees of flourination to form 3-hydoxyalkanoate repeat units with fluorinated side groups. Specifically, after growth of *P. oleovorans* (ATCC 29347) and *P. putida* (KT 2442) on sodium citrate in a first stage, the carbon sources 6,6,6-trifluorohexanoic acid (TFHxA), 6,6,7,7,8,8,8-heptafluorooctanoic acid (HpFOA), 6,6,7,7,8,8,9,9,9-nonafluorononanoic acid (NFNA), and 6,6,7,7,8,8,9,9,10,10,11,11,11-tridecafluoroundecanoic acid (TDFUDA) were cofed along with nonanoic acid in the second or polymer-producing stage. The ability of these bacteria to produce PHAs with fluorinated side groups from TFHxA, HpFOA, NFNA, and TDFUDA fluorinated acids was studied. The polymers formed were analyzed by ^{1}H and ^{19}F nuclear magnetic resonance (NMR) to establish the mole % of fluorinated repeat units. ^{13}C NMR analysis was also carried out to gain information on the repeat unit sequence distribution of fluorinated products. Assignments of NMR signals were made using information obtained from 2D experiments. Polymer molecular weights were measured by gel permeation chromatography (GPC). The effects of PHA flourine content on material thermal transitions and surface properties was investigated by differential scanning calorimetry (DSC) and surface contact angle measurements.

Materials and Methods

Synthesis of Carbon Sources. The general synthetic procedure used for the preparation of 5-fluorocarbon-substituted pentanoic acid carbon sources where the fluorocarbon chain length was varied from 1 to 6 is summarized below. To a 50-mL round-bottom flask, 0.1 mol of 4-pentenoic acid (Aldrich Chemical Co., 97%), a slight excess of perfluoroalkyl iodide (0.11 mol, Aldrich Chemical Co., 98%), and 0.2 g of azobis(isobutyronitrile) (AIBN, Eastman Kodak Co.) were added, and this solution was stirred and heated at 80 °C for 2 h. The reaction was followed by observing the olefin stretching band at 1653 cm^{-1} in the infrared. After removal of volatiles on a rotary evaporator, the yields of the corresponding iodo acids were greater than 95%. These products were hydrogenated over 5% Pd/C in ethanol with 2 equiv of sodium acetate added. The hydrogenation was conducted at room temperature and about 200 psi of hydrogen for ~16−18 h. The reaction mixture was filtered through diatomaceous earth rinsing with ethanol. The ethanol was removed using a rotary evaporator, and the residue was partitioned between ether and dilute aqueous HCl. The ether layer was washed with a saturated NaCl solution and dried over anhydrous sodium sulfate. The structures, melting points (peak values measured using a Seiko DSC Model DSC220C), and full and abbreviated names of the synthesized fluorinated acids are shown below. Spectra recorded by Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy (^{1}H, ^{13}C, and ^{19}F) of these products were consistent with that expected based on their structures.



n = 1; 6,6,6,-Triflourohexanoic acid (TFHxA, liquid)
n = 3; 6,6,7,7,8,8,8-Heptafluorooctanoic acid (HpFOA, liquid)
n = 4; 6,6,7,7,8,8,9,9,9-Nonafluorononanoic acid (NFNA, mp. 37 °C)
n = 6; 6,6,7,7,8,8,9,9,10,10,11,11,11-Tridecafluoroundecanoic acid (TDFUDA, mp. 55 °C)

Preparation of Growth Medium. The growth medium, medium E*, for *P. oleovorans* (ATCC 29347) and *P. putida* (KT 2442) was prepared using nanopure distilled water and contained the following (per liter):[29] $(NH_4)_2HPO_4$, 1.1 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g; 15 mL of 0.1 M $MgSO_4$; and 3 mL of a microelement solution. The microelement solution was as follows (per liter of 1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl_2 \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g. The pH of medium E* was adjusted to 7.0 using 1 N HCl, and the medium was sterilized by autoclaving at 121 °C for 20 min.

Strain Information and Long-Term Preservation. *P. oleovorans* (ATCC 29347) was obtained from the U.S. Department of Agriculture (Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, IL 61604), and *P. putida* (KT2442) was obtained from Prof. G. Eggink at the Agrotechnological Research Institute (ATO-DLO, Wageningen, The Netherlands). For long-term cell preservation (up to 2 years), liquid broth cultures of medium E* containing 40 mM sodium citrate as the sole carbon source were inoculated by transfer of either *P. oleovorans* or *P. putida* from agar plates; the cells were grown aerobically in 500-mL flasks (100-mL culture volumes) at 30 °C for 24 h in a shaker incubator (New Brunswick Scientific Co., Inc.; 250 rpm). Cultures were then diluted 1:1 with 20% sterile glycerol, 1-mL volumes were transferred to cryogenic vials, and the vials were frozen in a dry ice/ethanol bath and then transferred to a liquid nitrogen container for long-term storage. These preparations of *P. oleovorans* and *P. putida* cells served as inocula for all of the experiments described below.

Inoculation, Cell Growth, and PHA Production. To avoid inhibition of cell growth by fluorinated carboxylic acid carbon sources, a two-stage batch culture process was investigated.[21,22] In the first or cell growth stage, sterile medium E* (see above) amended with 40 mM sodium citrate as the sole carbon source was inoculated (0.4% v/v) using the contents of rapidly thawed cryovials (see above) containing one of the above organisms. First-stage *P. oleovorans* and *P. putida* cultivations were carried out to the early stationary phase (17- and 20-h culture times for *P. oleovorans* and *P. putida*, respectively[22]) in 500-mL flasks (100-mL culture volumes) at 30 °C in a shaker incubator (New Brunswick Scientific Co., Inc.; 250 rpm). For the second or PHA production stage, fluorinated acid/nonanoic acid (NA) 1:1 molar mixtures (total carbon source concentration equaled 15 mM) were added. Periodic monitoring of culture-viable cell numbers (CFU/mL) was carried out by removing 1-mL aliquots from cultures under aseptic conditions and using the spread plate method.[30] Cultures were terminated at 1-, 2-, 3-, 4-, and 5-day second-stage cultivation periods. The cells were harvested by centrifugation (Sorvall; 4 °C, 8000 rpm), washed with 0.02 M sodium phosphate buffer solution (pH 7.2), and lyophilized.

Polymer Isolation. Intracellular PHAs were isolated from the lyophilized cells by extraction with an excess of chloroform (15 mL per 1 g of biomass, 25 °C, 48 h).[17] Residual cell material was then removed by filtering, the polymers formed were precipitated by addition of chloroform solutions into cold methanol (1:10 v/v), and the precipitated polymers were washed with methanol and dried *in vacuo* (30 °C, 5 mmHg, 24 h).

Instrumental Procedures. A UNITY-500 NMR spectrometer was used for all of the NMR experiments described below. Proton (^{1}H) NMR were recorded at 500 MHz. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The experimental parameters were as follows: 0.5% w/v polymer in chloroform-*d*, temperature 298 K, 2.4-μs (14°) pulse width, 3-s acquisition time, and 8000-Hz spectral width. Carbon (^{13}C) NMR spectra were recorded at 125 MHz and the following parameters: 2.0% w/v polymer in chloroform-*d*, 298 K, 7.4-μs (67°) pulse width, 0.4-s acquisition time, 26 400-Hz spectral width, and continuous Waltz-modulated proton decoupling. The observed ^{13}C NMR chemical shifts in ppm were referenced relative to chloroform-*d* at 76.91 ppm. Fluorine (^{19}F) NMR spectra were recorded at 470 MHz on 2.0% w/v solutions in chloroform-*d* at 298 K, 4.6-μs pulse width (27°), 0.64-s acquisition time, and 100 000-Hz spectral width. Trifluorotoluene was added to the sample as a crossintegration reagent. For the COSY experiment (0.5% w/v polymer in chloroform-*d*), the data were collected in a 1024 × 256 data matrix and zero-filled to 1024 × 1024 using 8 scans per increment, a 4260-Hz sweep width, and a 1.1-s delay between transients. The data were processed using sine-bell weighting. Two-dimensional (2D) reverse-detected heteronuclear multiplet quantum correlation (HMQC) spectra were obtained (2.0% w/v polymer in chloroform-*d*) with spectral windows of 4260 Hz (^{1}H) and 12 771 Hz (^{13}C). The data were optimized for a one-bond scalar coupling constant of 140 Hz and used 90° pulses of 9.8 μs (^{13}C) and 14.8 μs (^{1}H). The delay time between scans was 1.0 s. The data matrix was zero-filled to 1024 × 1024 and processed with a Gaussian weighting function.

Fourier transform infrared (FT-IR) spectra of the fluorinated acid carbon sources (see above) were carried out by preparation of KBr pellets and recording spectra at 25 °C. FT-IR spectra were recorded using a Perkin-Elmer 1600 Series FT-IR.

To determine the mol % of 3-hydroxyheptanoate (HH), 3-hydroxynonanoate (HN), and other 3-hydroxyalkanoate repeat units in the products formed, the isolated PHAs were subjected to acid-catalyzed methanolysis to obtain the corresponding β-hydroxyalkanoic acid methyl esters. The method used for methanolysis and isolation of the methyl esters follows exactly as was previously described.[32] The volatile methyl esters synthesized by the methanolysis reaction were identified by comparison of their retention time with those of standard methyl β-hydroxyalkanoates.[32] A Perkin-Elmer 8500 gas chromatograph (GC) equipped with a Hewlett-Packard Ultra-2 capillary column (25 m × 0.2 mm × 0.33 μm) and a flame ionization detector was used. The chromatographic conditions follow exactly as was described previously.[31,32]

Elemental analyses (C, H, and F) of fluoro PHAs were carried out on samples which were dried to constant weight (48 h, 55 °C, 0.03 mmHg) in the presence of phosphorous pentoxide and then carefully stored in screw cap glass vials in a dessicator prior to analyses. The work was performed at Galbraith Laboratories Inc. (Knoxville, TN).

The molecular weights of the PHAs were measured by gel permeation chromatography (GPC) using a Waters Model 510 pump, Model 410 refractive index detector and Model 730 data module with 500-, 10^3-, 10^4-, and 10^5-Å ultrastyragel columns in series. Chloroform (HPLC grade) was used as an eluent at a flow rate of 1.0 mL/min. The sample concentrations and injection volumes were 0.3% (w/v) and 150 μL, respectively. Polystyrene standards with a low polydispersity (Polysciences) were used to generate a calibration curve.

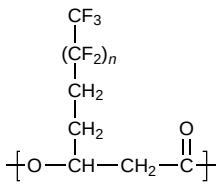
Thermal characterizations were carried out using a Du Pont 2910 differential scanning calorimeter (DSC) equipped with a TA 2000 data station. Between 3.0 and 10.0 mg of sample was sealed in aluminum pans, and analyses were carried out maintaining a dry nitrogen purge. The polymer samples were heated at a rate of 10 °C/min from ~25 to 100 °C (first heating scan), cooled rapidly by quenching in liquid nitrogen, and then analyzed again during a second heating scan from −120 to +100 °C. Data reported for the melting temperature (T_m) and enthalpy of fusion (ΔH_f) were taken from the first heating scan. Where multiple melting transitions were observed, the reported T_m was the peak melting temperature of the largest endotherm transition. ΔH_f values were taken as the cumulative value over the entire melting transition range. The reported glass transition temperatures (T_g) were the midpoint values measured during the second heating scans.

To characterize the surface properties of fluorinated PHAs, the surface contact angle through the profile of a liquid drop (water) placed on a polymer film surface was measured by the direct observation−tangent method.[33] To prepare highly uniform polymer films, polymer solutions (2 mg/mL chloroform) were filtered (0.45 μm) and then spin-coated onto glass slides (1000 rpm, 30 s of spinning, 25 °C). Reported surface contact angle values were obtained from the average of duplicate measurements at three different sites on the polymer films.

Results and Discussion

General Strategy for the Production of Fluoro PHAs. The fluorinated acids used in this work (see structures in the Materials and Methods section) were prepared by reaction of 4-pentenoic acid with a series of perfluoroalkyl iodides to form iodoflouro acid intermediates which were then deiodinated by hydrogenation (see Materials and Methods for details). The fluoro acids contain fluorocarbon segments of variable length and hydrocarboxylic acid segments of fixed length (terminally substituted pentanoic acid). Spectral analyses of the synthesized fluoro acids were consistent with that expected.

A chemoenzymatic route was investigated as a method to prepare a new series of target fluoro PHAs that contain repeat units as shown below.



n = 0, TF3HHxA
n = 2, HpF3HOA
n = 3, NF3HNA
n = 5, TDF3HUDA

The biocatalysts *P. oleovorans* and *P. putida* were selected for the enzymatic conversion of fluoro acids to fluoro PHAs since they have been shown in previous work by us[21,22] and others[14−20] to have considerable flexibility for the incorporation of a wide range of side-group structural types (see Introduction, above). The

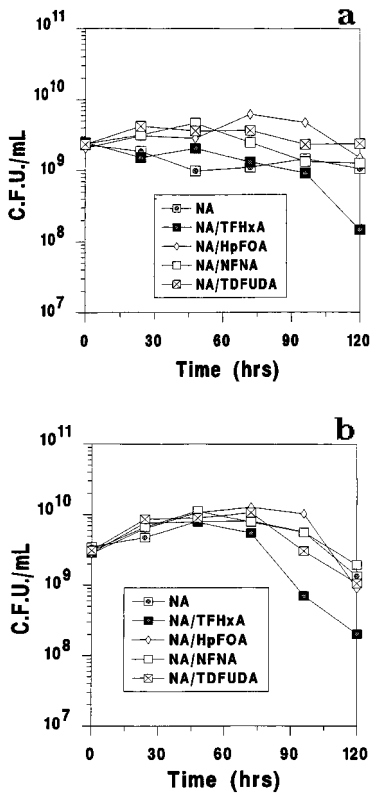


Figure 1. Number of viable cells in colony-forming units per mL (CFU/mL) as a function of time for second-stage shake flask cultivations of (a) *P. oleovorans* and (b) *P. putida* using NA, NA/TFHxA, NA/HpFOA, NA/NFNA, and NA/TDFUDA as carbon sources.

fluoro acids prepared herein were specifically designed with a spacer group (two methylene units) between carbon 3 (site of β-oxidation to a 3-hydroxyl functionality) and the functional fluorinated side group. The importance of providing a suitable spacer group for incorporation of the unusual side groups in PHAs has been demonstrated in previous work.[22]

Attempts to produce fluoro PHAs in second-stage cultivations using only the fluoro acids TFHxA, HpFOA, NFNA, and TDFUDA as the sole carbon sources gave no observable PHA formation over extended second-stage cultivation times (up to 5 days). Our efforts were then directed toward the formation of fluoro PHAs in second-stage cultivations by cometabolism.[21,34] Equimolar mixtures (total of 15 mM) of NA, a known PHA producing substrate,[31,32,35−37] with the fluorinated acids were investigated.

Prior to the second or polymer-producing stage, *P. oleovorans* and *P. putida* were first cultivated using 40 mM sodium citrate as the carbon source (see Materials and Methods).[22] This first stage of the fermentation, carried out to the early stationary growth phase, allowed the accumulation of ~0.9 and 1.2 g/L cell biomass for *P. oleovorans* and *P. putida*, respectively. Carrying out cell growth in the first stage circumvented problems that would likely have arisen due to differences in growth as a function of the fluoro acid/NA mixture used.

Cell Viability of *P. oleovorans* and *P. putida* in the Second-Stage Cultivations. *P. oleovorans* and *P. Putida* viability in colony forming units (CFU) per milliliter as a function of the second-stage culture time and the carbon source used were measured (see Figure 1, parts a and b, respectively). For both organisms and cultivation periods up to 120 h (5 days), the introduction of HpFOA, NFNA, and TDFUDA as cosubstrates resulted in no substantial loss in cell viability relative to cultures containing only NA. In fact, the former substrate mixtures showed slightly enhanced CFU/mL values to 72 h for *P. oleovorans* cultures (Figure 1a). Both *P. oleovorans* and *P. Putida* show substantial losses in cell viability relative to 15 mM NA cultivations at prolonged culture times (96 and 120 h) for NA/TFHxA. Thus, with the exception of TFHxA, the introduction of the fluoro acids as cosubstrates with NA did not result in any notable cellular toxicity as measured by CFU/mL for second-stage culture times to 120 h (5 days).

Table 1. Volumetric Polymer Yields, Molecular Weights, and mol % Fluorinated Repeat units in PHAs Formed by *P. oleovorans* in Second-Stage Cultivations Using NA and 1:1 Mixtures of NA with TFHxA, HpFOA, NFNA, and TDFUDA as the Carbon Sources

C source(s)	cult. time,[c] days	PHA yield, g/L	mol % fluorinated repeat units[a]	M_n, g/mol	M_w/M_n
NA only	1	0.41	0	174 000	1.90
	2	0.58	0	162 000	2.02
	3	0.36	0	144 000	2.01
	4	0.25	0	149 000	1.94
NA/TFHxA	1	0.21	2.4	121 000	1.80
	2	0.12	2.9	122 000	2.32
	3	0.01	4.9	133 000	1.84
NA/HpFOA	1	0.30	6.4	138 000	1.84
	2	0.17	3.3	119 000	2.38
	3	0.09	6.2	169 000	1.61
	4	0.02	3.0	nd[b]	nd
	5	0.02	3.1	nd	nd
NA/NFNA	1	0.33	1.9 (1.7)	110 000	2.04
	2	0.16	3.4 (3.0)	128 000	2.23
	3	0.08	3.7	151 000	1.81
	5	0.03	8.8	nd	nd
NA/TDFUDA	1	0.52	<1.0	130 000	2.59
	2	0.36	<1.0	139 000	2.05
	3	0.24	<1.0	146 000	3.34
	4	0.19	<1.0	102 000	3.32
	5	0.17	<1.0	117 000	2.31

[a] Measurements by ^{1}H and ^{19}F NMR spectral integration except those given in parentheses which are by elemental analysis (see Materials and Methods). The fluorinated repeat unit incorporated is the 3-hydroxyorganoate having the identical chain length as the fluorocarbon source used. [b] nd is not determined. [c] Cultivation period for the second or polymer-producing stage of the fermentation.

PHA Formation on NA/Fluoro Acid Mixtures. Since significant quantities of the provided carbon sources remained as insoluble particles after 5 days of cultivations, values of cell yields (g/L) and % PHA in cells were not obtained. The high solubility of the carbon sources in methanol facilitated their removal upon isolation of the products (see Materials and Methods). PHA formation is therefore reported herein as the volumetric yield or weight of isolated product corrected for a 1-L culture volume (g/L). Moreover, it is important to note that at the onset of the second stage, both microorganisms showed no evidence of PHA granule inclusions and, therefore, polymer formation. This was based on careful inspection of cells using a phase contrast microscope (1000×). Thus, polymer yields reported herein correspond to the polyester formed in the second stage of the fermentation after the addition of NA/fluorinated acid mixtures.[22]

In general, with the addition of fluorinated acid cosubstrates, PHA volumetric yields for both *P. oloevorans* and *P. putida* decreased relative to 15 mM NA fermentations. The magnitude of the decrease in PHA yield was a function of the organism, cultivation time, and the NA/fluorinated acid mixture (see Tables 1 and

Table 2. Volumetric Polymer Yields, Molecular Weights, and mol % Fluorinated Repeat Units in PHAs Formed by *P. putida* in Second-Stage Cultivations Using NA and 1:1 Mixtures of NA with TFHxA, HpFOA, NFNA, and TDFUDA as the Carbon Sources

C source(s)	cult. time,[c] days	PHA yield, g/L	mol % fluorinated repeat units[a]	M_n, g/mol	M_w/M_n
NA only	1	0.38	0	142 000	1.98
	2	0.21	0	141 000	2.01
	3	0.16	0	125 000	2.12
	4	0.11	0	128 000	2.04
NA/TFHxA	1	0.13	3.3	72 300	2.24
	2	0.01	nd [b]	nd	nd
NA/HpFOA	1	0.11	3.3	67 300	1.87
	2	0.01	nd	nd	nd
	3	0.05	2.0	103 000	2.07
	4	0.02	<1.0	98 000	2.54
NA/NFNA	1	0.02	8.0	114 000	2.59
	2	0.16	9.8 (8.2)	113 000	2.18
	3	0.01	17.3	109 000	1.82
	5	0.02	12.4	97 400	2.21
NA/TDFUDA	1	0.21	<1.0	67 400	2.02
	2	0.09	<1.0	94 200	2.53
	3	0.05	<1.0	nd	nd

[a] Measurements by ^1H and ^{19}F NMR spectral integration except those given in parentheses which are by elemental analysis (see Materials and Methods). The fluorinated repeat unit incorporated is the 3-hydroxyorganoate having the identical chain length as the fluorocarbon source used. [b] nd is not determined. [c] Cultivation period for the second or polymer producing stage of the fermentation.

2). Such a decrease in yield would be anticipated upon the addition of an unusual carbon source which deviates significantly from that of linear hydrocarbons,[38,39] *n*-alkanoic acids,[32] and other oil-related substrates.[40] Interestingly, NA/TDFUDA proved to be a striking exception to the above. Specifically, *P. oleovorans* PHA volumetric yields were similar in value (within 20% of the mean) when using either NA or NA/TDFUDA at cultivation times of 3, 4, and 5 days (approximately 0.20 g/L). *P. putida* also showed similar behavior for extended cultivation times (5 days). PHA yields formed by *P. oleovorans* using NA/HpFOA and NA/NFNA were similar throughout the 5-day cultivation period. In contrast, PHA yields formed by *P. putida* on NA/HpFOA were largest (0.11 g/L) at day 1 and then dropped rapidly to 0.01 g/L at day 2, while cultivations carried out using NA/NFNA showed a distinct maximum PHA yield at day 2 (0.16 g/L). For both organisms, PHA yields on NA/TFHxA were similar to those from NA/HpFOA to day 2, after which PHA yields on the former were generally lower. It should be noted that the low PHA yields at extended cultivation times using NA/TFHxA are consistent with the observed substantial decrease in cell viability (cell death) at corresponding culture times (see above and Figure 1).

NMR Characterization of Fluoro PHAs. A detailed NMR study was undertaken to characterize the fluoro PHA formed by *P. putida* after a 2-day cultivation period using NA/NFNA for polymer formation (see Table 2). The one-dimensional 500-MHz ^1H NMR spectrum (see Figure 2) shows signals at 1.85, 1.92, 2.08, and 2.62 which are not seen in spectra of PHAs formed from NA.[32,38] In the 2D $^1H-^1H$ COSY spectrum (not shown), the signals at 1.85 and 1.92 have crosspeaks with that at 5.18 ppm. Furthermore, there is a crosspeak between the signals at 2.62 and 5.18 ppm. Also, the signals at 1.85 and 1.92 are of almost equal intensity and the additive spectral integration of these two signals is approximately equal and 2 times that of the peak areas

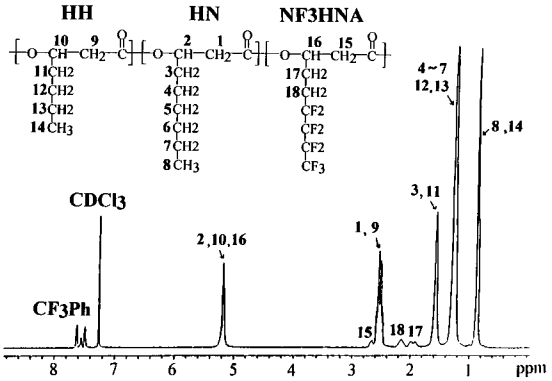


Figure 2. ^1H NMR (500-MHz) spectrum in chloroform-*d* of the PHA containing 9.8 mol % fluorinated repeat units produced by *P. putida* after a 2-day second-stage cultivation time using 7.5/7.5 NA/NFNA (mM) as cosubstrates.

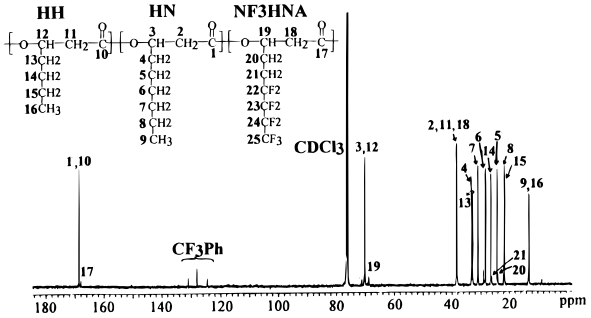


Figure 3. ^{13}C NMR (125-MHz) spectrum in chloroform-*d* of the PHA containing 9.8 mol % fluorinated repeat units produced by *P. putida* after a 2-day second-stage cultivation time using 7.5/7.5 NA/NFNA (mM) as cosubstrates.

corresponding to the signals at 2.08, and 2.62, respectively. Based on the above, the signals at 1.85/1.92, 2.08 and 2.62 are assigned to protons 17 (H-17), H-18, and H-15, respectively. It should be noted that protons H-17 are diastereotopic and, therefore, chemically nonequivalent. This nonequivalence results in two partially resolved signals with peaks at 1.85 and 1.92 which each correspond to one of the diastereotopic protons. The ^{13}C NMR spectrum of this product is shown in Figure 3. ^{13}C NMR signals due to 3-hydroxynonanoate (HN) and 3-hydroxyheptanoate (HH) repeat units were assigned based on a previous literature report for poly(HN-*co*-HH) produced from NA by *P. oleovorans*.[32] Also, we believe that the two weak ^{13}C signals at 8.95 and 71.80 ppm can be assigned to the methyl and methine carbons of 3-hydroxyvalerate repeat units present at low concentrations.[32] The 2D $^1H-^{13}C$ heteronuclear multiple quantum correlation (HMQC) spectrum of this product was also recorded (spectrum not shown). The spectrum reveals that protons H-17 at 1.92 and 1.85 ppm show correlations to the ^{13}C signal at 24.6 ppm, which is therefore assigned to carbon 20 (C-20). Also, the 2.08 ppm proton signal correlates with a carbon signal at 26.88, and this was assigned to C-21. However, fluorinated carbons C-22 through C-25 were not detected in the ^{13}C NMR spectrum. Based on model compounds such as 4,4,5,5,6,6,6-heptafluoro-1-(2-thienyl)-1,3-hexanedione and 2,2,2-trifluoroethanol,[41] ^{13}C NMR signals for C-22 through C-25 should be observed in the spectral region between 100 and 140 ppm. However, fluorinated carbons C-22 through C-25 are complex multiplets due to one bond and long-range coupling between ^{19}F and ^{13}C nuclei, and the sample concentration was insufficient to observe these fluorines.

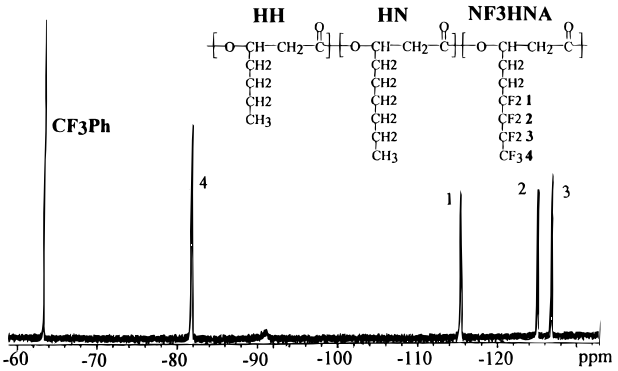


Figure 4. ^{19}F NMR (470-MHz) spectrum in chloroform-*d* of the PHA containing 9.8 mol % fluorinated repeat units produced by *P. putida* after a 2-day second-stage cultivation time using 7.5/7.5 NA/NFNA (mM) as cosubstrates.

In the carbonyl region of the ^{13}C NMR spectrum, two signals are observed at 169.25 and 168.61 that have relative signal integration ratios of about 10:1. Since the HH + HN:NF3HNA ratio is approximately 10:1, the minor resonance at 168.61 ppm was assigned to the carbonyl in NF3HNA (C-17). The carbonyls of the NF3HNA repeat units are sufficiently far from the C_4F_9 side chain that there should be no perturbation of the chemical shift due to long-range substitution effects. Therefore, chemical shift differences most likely result from changes in the main-chain conformation due to the presence of fluorinated relative to hydrocarbon side chains. The above analyses by both ^{1}H and ^{13}C NMR support that PHAs formed from NFNA/NA contain NF3HNA repeat units. Unfortunately, ^{13}C NMR analysis of this product did not provide information on the polymer repeat unit sequence distribution.

To determine the mol % PHA repeat units which contain fluorinated side chains, trifluorotoluene was added to the samples as an internal reference and was used as a cross-integration reagent for 500-MHz ^{1}H and 470-MHz ^{19}F NMR spectra of products. Observation of Figures 2 and 4 shows the phenyl and trifluoromethyl resonances, respectively, due to trifluorotoluene. The ^{19}F spectrum of the product also shows four additional ^{19}F signals at −126.5, −124.8, −115.1, and −81.4 ppm that were assigned to fluorine nuclei 3 (F-3), F-2, F-1, and F-4 of the NF3HNA repeat units (see Figure 4) based on ^{19}F spectral assignment made by others on related structures.[42] The moles of repeat units with fluorinated side groups was determined from the ^{19}F spectra by measuring the relative integration of the trifluoromethyltoluene signal at −63.2 and the signal due to CF_3 (F-4 in Figure 4) at −81.4 ppm. The total moles of repeat units was determined by comparison of the relative signal intensities of the ^{1}H signal due to trifluorotoluene (five hydrogens) and the methine protons (one hydrogen) H-2, H-10, and H-16 whose signals were not resolved. This information was then used to determine the mol % NF3HNA repeat units in the product. The identical strategy was used to determine the mol % TF3HHxA, HpF3HOA, and TDF3HUDA repeat units in polymers formed from mixtures of NA with TFHxA, HpFOA, and TDFUDA, respectively, and the results are presented in Tables 1 and 2. The ^{19}F and ^{1}H chemical shifts of TF3HHxA, HpF3HOA, and NF3HNA repeat units are listed in Table 3. The strategy used to assign ^{1}H and ^{19}F peaks for TF3HHxA and HpF3HOA followed exactly as was described above for NF3HNA and, therefore, will not be further elaborated. It should be mentioned that no evidence was found from ^{1}H and ^{19}F NMR analyses for the formation of fluorinated repeat units that differed in chain length from that used as the carbon source in fermentations. As seen in Table 3, fluorines Fa1 of the TF3HHxA repeat units show two signals at −66.79 and −66.88 ppm. It is likely that this results from differences in repeat unit sequence though, at present, this cannot be confirmed since a sufficient range of copolymer compositions is not in hand.

In a few cases, the mol % fluorinated side groups in products was also determined by elemental analysis (see Tables 1 and 2 and the Materials and Methods section). The estimations were made using the following assumptions: (1) fluorinated repeat unit structures in products have the identical chain length as the fluorocarbon source used (see discussion above); (2) the ratio of HN to HH units is 62:29 (based on GC analysis, see Materials and Methods section). The values of mol % fluorinated repeat units from elemental analysis were found to be in excellent aggreement with those from NMR measurements (see Tables 1 and 2).

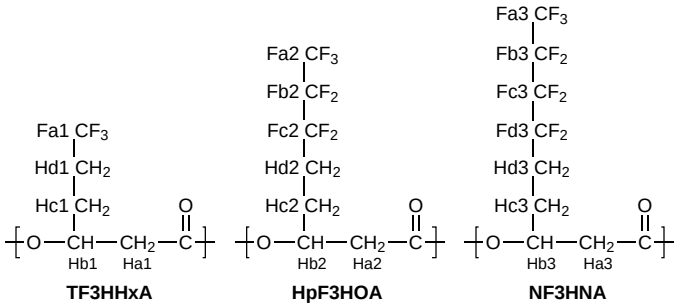
Relationship between the Fluoroalkanoate Structure and Fluoro PHA Formation. The mol % values of fluorinated PHA repeat units formed by *P. oleovorans* and *P. putida* as a function of the fluorinated cosubstrates and second-stage cultivation time are presented in Tables 1 and 2, respectively.

Inspection of Tables 1 and 2 shows that cultivation of both *P. oleovorans* and *P. putida* on 1:1 mixtures of fluorinated acids with NA resulted in substantial incorporation of fluorinated repeat units. The highest level of incorporation observed was 17.3 mol % for a 3-day cultivation of *P. putida* with NA/NFNA. When this cultivation was carried for 2 as opposed to 3 days, the PHA volumetric yield was significantly higher (0.16 relative to 0.01 g/L) but the mol % NF3HNA repeat units was lower (9.8 relative to 17.3 mol %). *P. putida* showed comparatively lower incorporation of shorter chain length fluorinated substrates. The maximum mol % fluorinated side chains formed by *P. putida* using NA/TFHxA and NA/HpHOA was 3.3. Unfortunately, due to low PHA yields, compositional analyses of PHAs formed by *P. putida* from NA/TFHxA at culture times of ≥2 days was not obtained.

Interestingly, the use of the longer chain fluoro acid TDFUDA for polymer formation by both organisms resulted in relatively high PHA yields but little to no incorporation of fluoroalkanoate repeat units (see Tables 1 and 2). It may be that the rigid and long (six carbons) fluorocarbon chain length of TDFUDA does not allow its metabolism to activated monomer and subsequent polymerization. Fatty acid β-oxidation of TDFUDA would produce a substrate of relatively shorter chain length but which now lacks a hydrocarbon spacer between the pendent fluorocarbon substituent and the 3-position of the chain. Thus, further metabolism of this substrate to the 3-hydroxylthiocoenzyme A activated monomer and subsequent polymerization would likely not be possible.

For short cultivation times where PHA yields were relatively high, the results obtained using *P. oleovorans* and NA/HpFOA were of interest (see Table 1). Specifically, after only a 1-day second-stage cultivation period, the PHA formed (0.3 g/L) had 6.4 mol % fluoroalkanoate side groups. This indicates that conversion of HpFOA to HpF3HOA repeat units occurs on a relatively rapid time scale when compared to the results using the other fluoroalkanoates for both organisms (see Tables 1 and

Table 3. ^{1}H and ^{19}F NMR Chemical Shifts of Fluorinated Repeat Units Incorporated into PHAs



TF3HHxA		HpF3HOA		NF3HNA	
Chemical Shifts in the ^{1}H NMR Analysis, ppm					
CH_2 (Ha1)	2.63	CH_2 (Ha2)	2.68	CH_2 (Ha3)	2.59
CH (HB1)	5.18	CH (Hb1)	5.18	CH (Hb1)	5.18
CH_2 (Hc1)	1.88, 1.93	CH_2 (Hc2)	1.82, 1.97	CH_2 (Hc3)	1.85, 1.92
CH_2 (Hd1)	2.15	CH_2 (Hd2)	2.14	CH_2 (Hd3)	2.08

TF3HHxA		HpF3HOA		NF3HNA	
Chemical Shifts in the ^{19}F NMR Analysis, ppm					
CF_3 (Fa1)	−66.79, −66.88	CF_3 (Fa2)	−81.2	CF_3 (Fa3)	−81.4
		CF_2 (Fb2)	−128.2	CF_2 (Fb3)	−126.5
		CF_2 (Fc2)	−116.0	CF_2 (Fc3)	−124.8
				CF_2 (Fd3)	−115.1

2). The yields of PHAs by *P. oleovorans* using NA/NFNA were almost identical to that using NA/HpFOA, while the mol % incorporated fluorinated side groups with the former was slightly lower (see Table 1). Interestingly, *n*-octanoic acid (OA) and NA were preferred carbon source chain lengths for PHA production by *P. oleovorans*.[32] Also, the selectivity of *P. oleovorans* for the production of 3-hydroxyoctanoate and 3-hydroxynonanoate repeat units from OA and NA, respectively, was almost identical.[32] Thus, in this work using *P. oleovorans*, the trend for the dependence of the fluoroalkanoate chain length on its incorporation into PHA and PHA yield was remarkably similar to that observed using *n*-alkanoic acids as sole carbon sources. The only exception to this that is not currently understood by us is the relatively high PHA yields that resulted from use of NA/TDFUDA mixtures.

In a number of cases above, the mol % incorporation of fluorinated side chains increased as the PHA yield decreased with extended cultivation times. Two factors which may contribute to such behavior are (1) the use of NA as the primary carbon source for PHA production so that the fluorinated carbon source will be utilized only after NA reaches relatively low media concentrations and (2) preferential degradation and cellular recycling of the PHA product fraction that contains *n*-alkanoate hydrocarbon side groups when the available carbon becomes insufficient to maintain cellular functions. A similar trend of increased mol % incorporation of functional side groups at extended culture time was seen by us in a previous study when cyanophenoxyalkanoates were used as carbon sources for polymer formation by *P. putida*.[21,22]

Molecular Weights of PHAs Produced from Multifluorinated Alkanoates. The molecular weights of PHAs isolated from *P. oleovorans* and *P. putida* grown on NA/fluorinated alkanoates were measured by GPC, and the results are presented in Tables 1 and 2. In all cases, even with fluorinated side-group contents up to 17.3 mol %, high molecular weight products were formed. M_n values of PHAs produced by *P. oleovorans* containing from 0 to 6.4 mol % fluorinated side chains ranged from 102 000 to 169 000 g/mol. PHAs produced by *P. putida* containing from 0 to 17.3 mol % fluorinated repeat units had M_n values that ranged from 67 000 to 114 000 g/mol. From a comparison of the product molecular weights as a function of the fluorinated side-group content, no readily interpretable trends were observed.

Table 4. DSC Measurements[a] of PHAs Formed with and without NF3HNA Repeat Units

mol % NF3HNA repeat units[b]	T_g[c] °C	T_m[d] °C	ΔH_f[e] cal/g	microorganisms, NA/NFNA, time[f]
0	−41	49	3.2	*P. oleovorans*, 15/0 (mM), 3 days
0	−41	48	3.2	*P. putida*, 15/0 (mM), 3 days
8.8	−42	51	5.9	*P. oleovorans*, 7.5/7.5(mM), 5 days
12.4	−41	51	8.0	*P. putida*, 7.5/7.5 (mM), 5 days

[a] The samples used in this study were from solution precipitation (see Materials and Methods). [b] Determined by ^{1}H and ^{19}F NMR spectroscopy. [c] The midpoint glass transition temperature measured during the second heating scan after samples were rapidly quenched from the melt. An additional T_g transition occurring between −28 and −25 °C was also observed for these samples. [d] Measured during the first heating scan where the value reported is the peak melting temperature for the largest endotherm transition [e] Measured during the first heating scan where the value reported is the cumulative heat of fusion taken over the entire melting range. [f] Cultivation period for the second or polymer producing stage of the fermentation.

Thermal Properties of PHAs Produced from Multifluorinated Alkanoates. Selected solution precipitated (see Materials and Methods) fluoro PHAs were characterized by DSC to determine whether the incorporation of fluorinated side groups influenced product thermal transitions. DSC thermograms for the first heating scans were used to determine T_m and ΔH_f values (see Table 4 and Figure 5). Second heating scans after rapid quenching of samples from the melt were also recorded to determine T_g values (see Table 4 and Figure 6). Inspection of Table 4 shows that incorporation of fluorinated side groups in PHAs leads to large increases in ΔH_f. Considering products of *P. oleovorans* which contained 0 and 8.8 mol % NF3HNA repeat units,

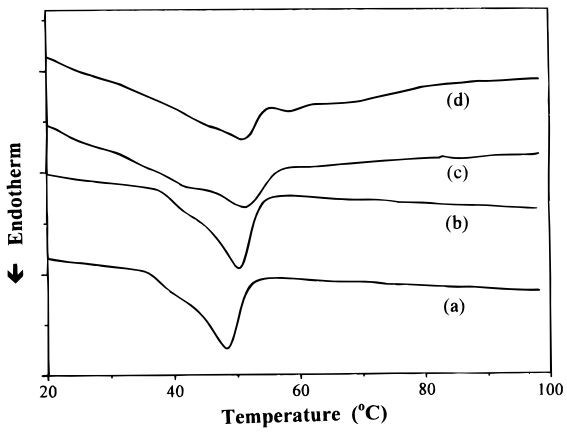


Figure 5. DSC thermograms (first heating scans) of the PHAs isolated from *P. oleovorans* and *P. putida* using either NA or NA/NFNA mixtures as carbon sources during second-stage cultivations: (a) 0 mol % NF3HNA (from *P. oleovorans*), (b) 0 mol % NF3HNA (from *P. putida*), (c) 8.8 mol % NF3HNA (from *P. oleovorans*), and (d) 12.4 mol % NF3HNA (from *P. putida*).

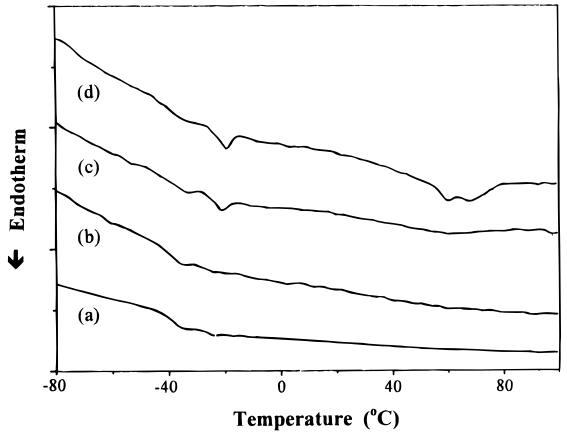


Figure 6. DSC thermograms (second heating scan after quenching from the melt) of the PHAs isolated from *P. oleovorans* and *P. putida* using either NA or NA/NFNA mixtures as carbon sources during second-stage cultivations: (a) 0 mol % NF3HNA (from *P. oleovorans*), (b) 0 mol % NF3HNA (from *P. putida*), (c) 8.8 mol % NF3HNA (from *P. oleovorans*), and (d) 12.4 mol % NF3HNA (from *P. putida*).

the ΔH_f values increased from 3.2 to 5.9 cal/g. Comparison of the first heating scans for these two products (thermograms a and c, respectively, Figure 5) shows that the sample containing 8.8 mol % NF3HNA has a relatively broader melting transition and a slightly increased T_m (see Table 4). Similarly, fluoro PHAs formed by *P. putida* that contain 0 and 12.4 mol % NF3HNA had ΔH_f (T_m) values of 3.2 cal/g (48 °C) and 8.0 cal/g (51 °C). Further inspection of thermogram d in Figure 5 for the 12.4 mol % NF3HNA sample shows that, unlike PHAs with 0 mol % NF3HNA, substantial sample melting is still observed at temperatures between 55 and 80 °C. Also, unlike the 0 mol % PHA samples, the 12.4 mol % product showed melting transitions at 60 and 68 °C during the second heating scan (thermogram d, Figure 6) so that NF3HNA repeat units accelerates crystallization during the cooling cycle. The above results are consistent with a nonrandom distribution of NF3HNA repeat units so that chains or chain segments exist with relatively high NF3HNA content.

Using the midpoint value of the T_g-step transition, there was no substantial change in the lowest temperature T_g as a function of NF3HNA content (values ranged from −42 to −41 °C, see Table 4). Furthermore, the T_g values of these products are similar to literature T_g values for PHAs that have predominantly *n*-hexyl side groups.[32] Another endotherm transition during second heating scans was observed at temperatures between −28 and −25 °C for all of the PHA samples studied (see Figure 6). The steplike character of the transition leads us to believe that it is an additional T_g. To our knowledge, two T_g transitions for PHAs produced by these microorganisms from *n*-alkanoic acids was not previously observed. Furthermore, this transition has an associated enthalpy of relaxation that increases in magnitude with increased NF3HNA content. It may be that the distribution of HN and HH repeat units formed from NA by the two-stage cultivation method deviates significantly from random statistics. Furthermore, a densification of the amorphous phase with increased NF3HNA content would explain the apparent increase in the enthalpy of relaxation. However, further work on additional samples by other techniques such as dynamic mechanical analysis will be needed to confirm these observations and better define this thermal transition.

In the results presented above, possible changes in the ratio of HN, HH, and HV caused by the use of NFNA could also be important in altering the thermal transitions of the products formed.[32] To study this further, a number of samples formed by *P. oleovorans* and *P. putida* having variable NF3HNA contents were degraded by acid-catalyzed methanolysis to their corresponding methyl esters and analyzed by GC (see Materials and Methods). The results of this work clearly showed that the products formed had almost identical molar ratios of *n*-alkanoate repeat units (data not shown). Therefore, it was concluded that the observed changes in thermal properties in this work can indeed by attributed to the polymerization of NF3HNA repeat units.

Previous work showed that PHAs containing *n*-pentyl, *n*-hexyl, and *n*-heptyl side chains crystallize with participation of both the main and side chains.[32] The above results that describe a substantial increase in ΔH_f values and melting at elevated temperatures when fluorinated side groups are incorporated may be due to phase separation of the relatively stiff fluorinated side groups. This is likely facilitated by a nonrandom distribution of NF3HNA repeat units or, in other word, the formation of nonrandom copolymers or copolyester mixtures (see above). Furthermore, phase separation of fluorinated side groups may accelerate crystallization rates and increase sample crystallinity. The possibility that phase separation of fluorinated side groups leads to a unique crystalline structure is currently under investigation.

It is interesting to consider work by Abe *et al.* (see ref 23 and the Introduction section) where the incorporation of up to 27 mol % 3-hydroxy-9-fluorononanoate repeat units in PHAs formed by *P. oleovorans* lead to substantial increases in T_m and ΔH_f. However, in that work, dramatic compositional changes in the ratio of HH to HN repeat units from 26:73 to 5:95 were claimed to occur along with 3-hydroxy-9-fluorononanoate incorporation. Thus, the observed changes in thermal properties observed in their work may be due in large part to changes in the degree of compositional homogeneity.

Surface Properties of PHAs Containing Multifluorinated Side-Chain Substituents. To investigate the surface properties of microbially synthesized PHAs containing fluorocarbon side-chain substituents,

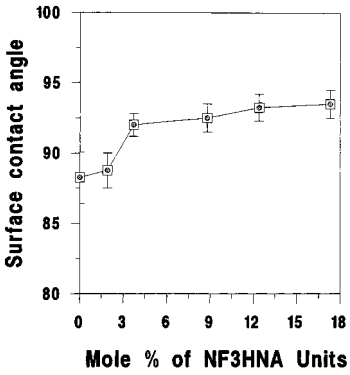


Figure 7. Surface contact angles of PHAs produced by *P. oleovorans* and *P. putida* using 7.5/7.5 NA/NFNA (mM) cosubstrates as a function of NF3HNA contents.

surface contact angles were measured using the direct tangent water-drop method (see ref 33 and Materials and Methods). In Figure 7, the surface contact angle is shown as a function of the NF3HNA mol %. There were no prominent effects of fluorine incorporation on the surface contact angle up to ~2 mol %. Upon increasing the NF3HNA content from 2 to 3.3 mol %, the surface contact angle increased from 87 to 92°. Further increase in the NF3HNA content up to 17.3 mol % led to only a modest increase in the surface contact angle to 94°. It is concluded from these measurements that the level of incorporation of fluorocarbon substituent groups achieved herein was not sufficient to cause dramatic alterations in the surface free energy. It is interesting to compare the maximum surface contact angle of the fluoro PHAs to other commercial polymers. High surface contact angles of ~108° were reported for the commercial fluoro plastic poly(tetrafluoroethylene) while other common plastics such as polystyrene, poly(vinyl chloride), poly(ethyleneterephthalate), and poly(methyl methacrylate) were reported to have surface contact angles of 91, 87, 81, and 80°, respectively.[43] Future work will consider methodologies that allow the bioengineering of PHAs that have higher side-chain fluorocarbon contents. It is hoped that these efforts will lead to bioderived PHA−fluoro polymers that have surface characteristics which will meet or exceed current commercial fluoro polymers.

Summary of Results

A chemoenzymatic route was used to prepare fluorinated polyesters. Specifically, achiral fluoro acids prepared by traditional chemical methods were converted into chiral monomers and polymerized by microbial catalysts to form high molecular weight bacterial polyesters containing fluorocarbon side-chain substituent groups. COSY and HMQC NMR experiments were used to aid in assigning ^{1}H and ^{13}C NMR signals of fluoroalkanoate repeat units. The highest level of incorporation observed was 17.3 mol % for a 3-day second-stage cultivation of *P. putida* using 7.5/7.5 NA/NFNA (mM) as cosubstrates. For shorter cultivation times where product yields were relatively higher, interesting results were found using *P. oleovorans* as the biocatalyst and NA/HpFOA as cosubstrates. Specifically, 0.3 g/L of product was formed that contained 6.4 mol % fluoroalkanoate side groups. The incorporation of 12.4 mol % NF3HNA repeat units resulted in products which showed melting at higher temperatures (55−80 °C), crystallized at faster rates from the melt, and had higher heats of fusion. The possibility that chains or chain segments enriched in NF3HNA are formed (nonrandom copolymers or copolyester mixtures) that phase separate to form unique crystalline organizations was proposed to explain the observed changes in fluoro PHA thermal properties. Investigation of the surface free energy of products by surface contact angle measurements showed only a modest increase from 87 to 94° for PHAs containing 0 and 17.3 mol % NF3HNA repeat units. Thus, to achieve dramatic changes in surface free energy by this route, work is currently in progress to develop suitable methods by which substantially higher fluoroalkanoate incorporations can be achieved.

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