librium curve as in the previous paper.[3] Kinetic parameters for the oxygen-binding was determined with stopped-flow and flash-photolysis spectrophotometers (Unisoku Co.) equipped with a kinetic data processor.

Solution Property Measurements. The viscosity was measured with an Ubellohde-type viscometer (JIS standard 0.03 and 0.005) at 37 °C. Osmotic pressure and colloidal osmotic pressure were measured with a membrane-type osmotic pressure apparatus (Knauer).

Acknowledgment. This work was partially supported by a Grant-in-Aid for Scientific Research on Priority Area of Macromolecular Complexes (No. 62612005 and 63612005) from the Ministry of Education, Science and Culture, Japan.

Registry No. 1, 92786-54-4; 2, 119480-89-6; (2)(3) copolymer, 119503-05-8; 3, 95721-44-1; 4, 119528-78-8; 5, 116462-06-7; imidazole, 288-32-4; 11-bromoundecanoic acid methyl ester, 6287-90-7; 11-(1′-imidazolyl)undecanoic acid methyl ester, 72338-54-6; 11-(1′-imidazolyl)undecanal, 116679-84-6; triethyl 4-phosphomocrotonate, 10236-14-3; oxygen, 7782-44-7.

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Sequencing Bacterial Poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) by Partial Methanolysis, High-Performance Liquid Chromatography Fractionation, and Fast Atom Bombardment Mass Spectrometry Analysis

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ABSTRACT: The partial methanolysis of poly(β-hydroxybutyrate) (PHB) and of poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) (P(HB-Co-HV)) has been performed, and reaction kinetics has been optimized in order to produce oligomers with molecular masses below the detection limit (about 2000 daltons) of the mass spectrometer used for the subsequent analysis. The fractionation of the methanolysis products was achieved by HPLC, and the fractions collected were analyzed by fast atom bombardment mass spectrometry (FAB-MS), in the presence of NaCl. FAB spectra of oligomers from PHB and P(HB-Co-HV) consist only of pseudomolecular ions MH^+ and MNa^+, allowing for the identification of the methanolysis products, the estimate of the copolymer composition, and the determination of the sequence distribution of monomeric units.

Introduction

The structural characterization of poly(β-hydroxybutyrate) (PHB) and poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) (P(HB-Co-HV)) has attracted much attention recently[1-3] because these materials are important members of a family of poly(β-hydroxyalkanoates) of microbial origin, which are potential sources of thermoplastic materials.[1-5]

Since the mechanical, physical, and processing properties of the P(HB-Co-HV) copolymers vary systematically with composition,[1,2] it is important to have accurate estimates of this parameter.

The composition of P(HB-Co-HV) copolyester samples can be determined by ^1H NMR spectra,[1-3] and the sequence distribution of monomeric units in P(HB-Co-HV) has been deduced analyzing the diad and triad sequences from their 125-MHz ^{13}C NMR spectra.[2,3]

However, since NMR can hardly discern beyond the triad level, the characterization of more complex sequence arrangements (such as partial blocks or higher order regularities) cannot be easily achieved without using complementary techniques.

Mass spectrometry is able to look at the mass of individual molecules in a mixture, and it is therefore an interesting alternative to NMR. Recently,[6] a pyrolysis MS method was used to obtain the sequence of P(HB-Co-HV).

The recent development of fast atom bombardment mass spectrometry (FAB-MS) has shown that a such technique is of considerable importance for the structural characterization of biopolymers. In the case of proteins, for instance, it allows us to perform direct analysis of the mixtures of peptides produced by the enzymatic degra-

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0024-9297/89/2222-2107$01.50/0

dation of native and modified proteins.[7,8]

FAB mass spectra of synthetic polymer samples have been reported,[9] and FAB-MS has been recently applied to the study of sequences in synthetic copolymers.[10]

In this report we describe the use of FAB-MS to identify the oligomers formed in the partial methanolysis, followed by HPLC fractionation, of PHB and P(HB-Co-HV) samples.

The FAB mass spectra of the oligomer mixtures show structurally significant peaks up to 2000 daltons (the cut-off of our magnet), allowing us to characterize both the composition and sequence distribution of the P(HB-Co-HV) copolyester units. Therefore, the sequence distribution of chain segments which contain up to 20 repeating units can be characterized. This represents a significant advance from the work carried out previously by ^{13}C NMR, which is only sensitive to triads.

Experimental Section

Materials. PHB (MW 400 000) and P(HB-Co-HV) (MW 750 000) copolyesters were produced by ICI Agricultural Division, U.K., and are also available from Aldrich Chemical Company.

All other chemicals were of the highest purifity commercially available and were used without further purification unless otherwise stated.

P(HB-Co-HV) Copolymer Composition. A nominal composition of 80/20 (mol %) was indicated for the PHB-PHV copolyester, as received. A 250-MHz ^1H NMR spectrum showed that the composition was 87/13 P(HB-Co-HV) based on the ratio of the methyl triplet at 0.895 ppm and the methyl doublet at 1.274 ppm. The peak assignments are according to the literature.[1,3]

Partial Methanolysis. A 1 N solution of HCl in dry methanol was prepared by bubbling gaseous HCl through redistilled anhydrous methanol. The amount of HCl dissolved was determined gravimetrically. The concentration was then adjusted by adding an appropriate volume of methanol. About 0.1 g of each sample was dissolved in 20 mL of $CHCl_3$, and 3 mL of a freshly prepared 1 N solution of HCl in methanol was added. The mixture was allowed to react at room temperature for 35–39 h, after which the solvent was evaporated. The residue was taken up with 2 mL of acetonitrile and transferred for HPLC analysis.

HPLC Fractionation. The fractionation of the methanolysis products was performed by HPLC, using a Varian VISTA 5500 HPLC system equipped with a Rheodyne injector with a 10-μL loop, a Varian 2050 UV detector, and a Micropack-MCH-N-Cap column (C18, Varian) of 15 cm × 4 mm. Ten microliters of the acetonitrile solution was injected, using an elution gradient starting with a 20/80 acetonitrile/water composition and ending with 100% acetonitrile in 40 min, with 1 mL/min flow and UV detection at 205 nm.

FAB Mass Spectra. A double-focusing Kratos MS 50S equipped with the standard FAB source and a DS 90 data system was used to obtain mass spectra. The FAB gun (Ion Tech) was operated with a 7–8-keV xenon beam. The instrument was scanned from m/z 2200 to 60, with a scan rate of 10 s/decade. The accelerating voltage was 6–8 kV. Cesium and rubidium iodides (50/50 w/w) were used for computer calibration. The resolution was approximately 3000.

Lyophilized samples to be analyzed were dissolved in acetonitrile. About 2 μL of the sample solution was placed on the copper target end of the direct insertion probe and mixed with 3-nitrobenzyl alcohol doped with NaCl.

Peak intensity values shown in the mass spectra represent the average of three separate mass spectra.

Results and Discussion

Fractionation and Identification of PHB Oligomers. The procedure adopted for the partial methanolysis and HPLC fractionation of PHB oligomers is a modification of that reported by Marchessault et al.[11] The methanolysis time was optimized in the present case to produce a high portion of oligomers with molecular masses below the detection limit of the mass spectrometer used (up to about 2000 daltons). The HPLC trace of the partial methanolysis products from PHB is shown in Figure 1.

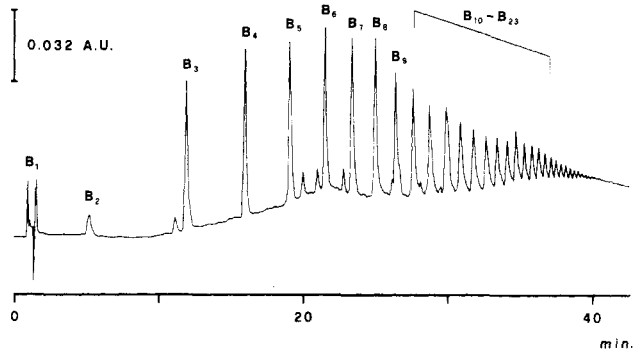


Figure 1. HPLC separation of the methanolysis products from PHB polyester: B_n = $H(OCH(CH_3)CH_2CO)_nOCH_3$ where n = 1–23.

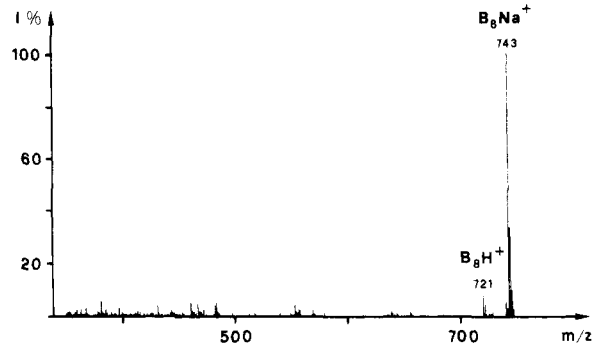


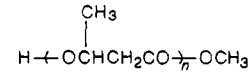
Figure 2. FAB mass spectrum corresponding to peak B_8 in the HPLC trace of the degraded PHB.

The PHB oligomers are well separated, allowing the collection of fractions corresponding to each peak in Figure 1 up to peak B_9. The last fraction collected contains, instead, peaks from B_{10} up to B_{23} all together. All the HPLC fractions collected from a single injection of 10 μL of acetonitrile solution were lyiophilized and analyzed by FAB-MS.

Each mass spectrum corresponding to peaks B_3–B_9 in the HPLC trace shows the presence of a single oligomer, as evidenced by the molecular ions MH^+ and MNa^+ in the FAB spectra.

A representative FAB spectrum is shown in Figure 2, corresponding to the octamer (chromatographic peak B_8 in Figure 1). Two pseudomolecular ions, B_8H^+ (m/z 721) and B_8Na^+ (m/z 743), appear in the spectrum in Figure 2. No other peaks due to the ion fragmentation of this oligomer are present up to the lowest masses, except for those due to the FAB liquid matrix.

The oligomers corresponding to peaks B_3–B_9 in Figure 1 were identified as follows:



where n = 3–9. The m/z values of the MNa^+ ions are, respectively, 313 (B_3Na^+), 399 (B_4Na^+), 485 (B_5Na^+), 571 (B_6Na^+), 657 (B_7Na^+), 743 (B_8Na^+), and 829 (B_9Na^+).

HPLC fractions corresponding to peaks B_1 and B_2 in Figure 1 did not yield FAB mass spectra, possibly because these components are too volatile and too low in concentration. They were tentatively assigned to monomer and dimer from their retention times.

The FAB mass spectrum corresponding to the fraction containing the HPLC peaks B_{10}–B_{23} in Figure 1 is reported in Figure 3. MNa^+ ions corresponding to PHB oligomers

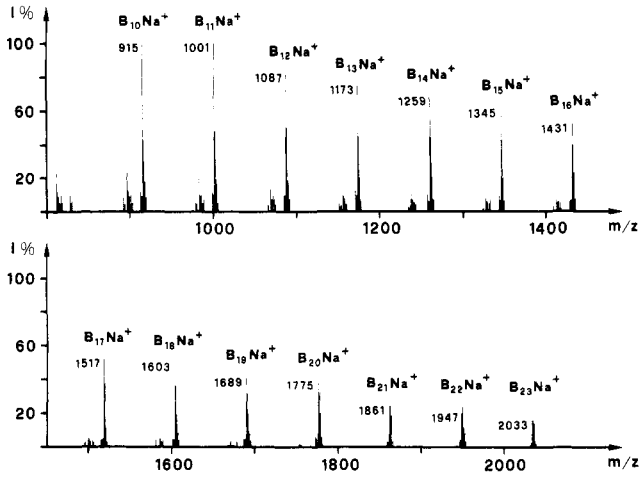


Figure 3. FAB mass spectrum corresponding to peaks B_{10}–B_{23} in the HPLC trace of the degraded PHB.

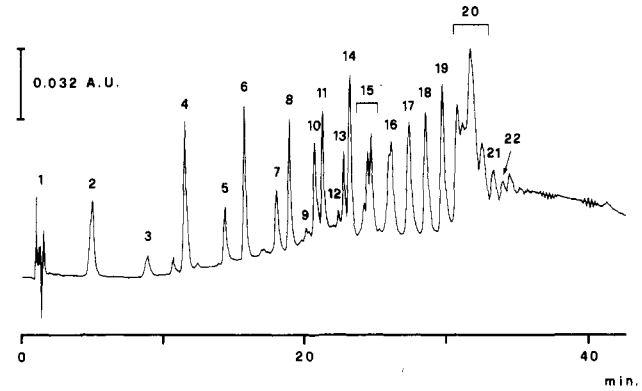


Figure 4. HPLC separation of the methanolysis products from P(HB-Co-HV) copolyester. Structural assignments for the oligomers identified are reported in Table I.

from B_{10} to B_{23} appear in the mass spectrum in Figure 3, and a quantitative correlation among the HPLC and FAB peak intensities is noticeable.

The absence of fragmentation of the pseudomolecular ions in the FAB spectra reported above is in fact the essential condition in order to obtain MS data suitable for semiquantitative mixture analysis. This leads to the expectation that FAB mass spectra of P(HB-Co-HV) copolyesters may allow a correct and realistic estimate of the repeating units distribution.

Fractionation and Identification of P(HB-Co-HV) Oligomers. The HPLC trace of the partial methanolysis products from the P(HB-Co-HV) copolyester is reported in Figure 4. Elution conditions were those used for PHB. The chromatogram in Figure 4 shows several peaks that were not well-resolved, due to the large number of P(HB-Co-HV) oligomers produced.

However, the FAB analysis of all the fractions collected allowed the identification of all the oligomers contained in the nonresolved HPLC peaks, because the FAB spectra are constituted only by the pseudomolecular ions corresponding to each oligomer. Structural assignments for the oligomers identified are reported in Table I.

FAB spectra representative of some of the fractions collected are reported in Figures 5 and 6. In Figure 5 are shown the FAB spectra corresponding to HPLC peaks 4, 5, and 6, respectively (Figure 4). Each fraction appears to contain one oligomer; intense peaks arising from the FAB liquid matrix are also shown in Figure 5, and they are identified by an ×.

The FAB spectrum in Figure 6 corresponds to a typical fraction containing mixtures of oligomers and has been

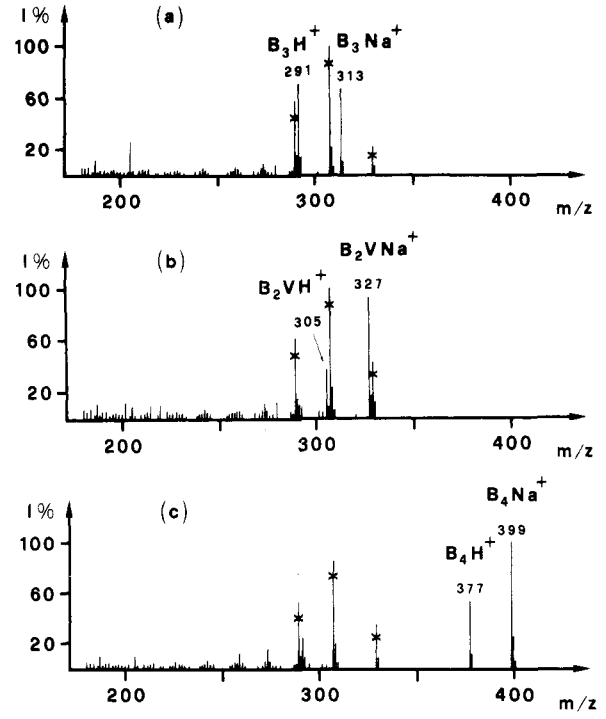


Figure 5. FAB mass spectra corresponding to (a) peak 4, (b) peak 5, and (c) peak 6 in the HPLC trace of the degraded P(HB-Co-HV) copolyester.

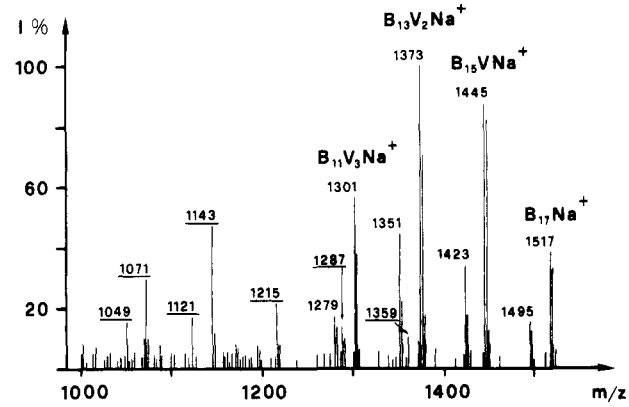


Figure 6. FAB mass spectrum corresponding to peak 22 in the HPLC trace of the P(HB-Co-HV) copolyester. Underlined m/z values correspond to MH^+ and MNa^+ ions of oligomers contained in the preceding chromatographic peak 21.

used to estimate the copolymer composition and sequence distribution (see below).

Copolymer Composition and Sequence Distribution. Assuming a quantitative FAB-MS correlation to the amount of oligomers produced in the partial methanolysis, the relative abundance of the MNa^+ ions present in the FAB spectrum of an HPLC fraction, which contains more than one oligomer, ought to reflect the copolymer composition. Interestingly, the latter can be reliably calculated by making the explicit assumption of a random distribution of monomeric units HB and HV in the copolymer.[12]

The statistical probability of each oligomer B_xV_y is given by

$$P_{x,y} = \binom{x+y}{y} P_B{}^x P_V{}^y \qquad (1)$$

where P_B and P_V are the molar fractions of HB and HV units in the copolymer. $P_B{}^x P_V{}^y$ is the probability to find a given B_xV_y sequence, assuming Bernoullian (random) statistics[12] (x and y are defined in footnote a of Table I).

Table I
Identification of the Methanolysis Products from PHB/PHV Copolymer by HPLC and FAB-MS Analyses

peak	B_xV_y[a]	MH^+[b]	MNa^+[b]	peak	B_xV_y[a]	MH^+[b]	MNa^+[b]
1	B	c	c	20	B_5V_4		885
	V	c	c		B_7V_3	935	957
2	B_2	c	c		B_6V_4	949	971
3	BV	c	c		B_9V_2	1007	1029
4	B_3	291	313		B_8V_3	1021	1043
5	B_2V	305	327		B_7V_4	1035	1057
6	B_4	377	399		$B_{11}V$	1079	1101
7	B_3V	391	413		$B_{10}V_2$	1093	1115
8	B_5	463	485		B_9V_3	1107	1129
9	B_2V_2	405	427		B_{13}	1151	1173
10	B_2V_2[d]	405	427		$B_{12}V$	1165	1187
	B_4V	477	499		$B_{11}V_2$	1179	1201
11	B_6	549	571		B_{14}	1237	1259
12	B_3V_2	491	513		$B_{13}V$	1251	1273
13	B_3V_2[d]		513		B_{15}	1323	1345
	B_5V	563	585	21	B_3V_6[d]		913
14	B_5V[d]	563	585		B_5V_5[d]		985
	B_7	635	657		B_7V_4[d]		1057
15	B_2V_3		527		B_9V_3[d]		1129
	B_4V_2	577	599		B_8V_4		1143
	B_6V	649	671		$B_{10}V_3$		1215
	B_8	721	743		$B_{12}V_2$		1287
16	B_3V_3	591	613		$B_{14}V$		1359
	B_5V_2	663	685		B_{16}		1431
	B_7V	735	757	22	B_6V_5[d]	1049	1071
	B_9	807	829		B_8V_4[d]	1121	1143
17	B_4V_3	677	699		$B_{10}V_3$[d]	1193	1215
	B_6V_2	749	771		$B_{12}V_2$[d]		1287
	B_8V	821	843		$B_{11}V_3$	1279	1301
	B_{10}	893	915		$B_{14}V$[d]		1359
18	B_3V_4		713		$B_{13}V_2$	1351	1373
	B_5V_3		785		$B_{15}V$	1423	1445
	B_7V_2	835	857		B_{17}	1495	1517
	B_9V	907	929				
	B_{11}	979	1001				
19	B_4V_4	777	799				
	B_6V_3	849	871				
	B_8V_2	921	943				
	$B_{10}V$	993	1015				
	B_{12}	1065	1087				

[a] $B_xV_y \equiv H[OCH(CH_3)CH_2CO]_x-[OCH(CH_2CH_3)CH_2CO]_yOCH_3$ where the first term is B and the second is V. [b] m/z values in the FAB mass spectra. [c] The methanolysis products corresponding to the first three chromatographic peaks do not give FAB mass spectra. The identifications have been made by the expected retention times considering those of the identified higher molecular weight homologues and by comparison with the chromatogram of methanolysis products of the PHB homopolymer (Figure 1). [d] Methanolysis products that were not well-resolved coming from the preceding chromatographic peak.

The binomial coefficient in eq 1 is the number of possible sequence arrangements of the B_xV_y oligomer. Starting with an arbitrary set of P_B and P_V values, it is possible to calculate the statistical abundances, $P_{x,y}$, for a series of oligomers actually present in a chromatographic fraction. The best match between the experimentally observed oligomer distributions and the calculated statistical abundances individuates the copolymer composition.

The results of the calculations are reported in Table II. The experimental data are the relative intensities of the MNa^+ ions appearing in the FAB spectra of HPLC peaks 15–22 in Figure 5. As it can be noted, the agreement between observed and calculated values is a function of the P_B/P_V ratio (Table II), and it is best for a ratio 86/14, almost coincident with a ^1H NMR estimate. This result also confirms the hypothesis on which the calculations are based: i.e., that the copolymer possesses a random distribution of monomeric units. However, with regard to composition analysis, this method appears rather indirect, in general, since it only works if the sample has a Bernoullian monomer sequence distribution.

Table II
Experimental[a] and Calculated[b] Relative Abundances of the Methanolysis Products from PHB/PHV Copolymer, Contained in Some Chromatographic Peaks

		calcd[b]			
	MS[a]	80/20	83/17	85/15	87/13
		Peak 15			
B_8	87	46	58	69	83
B_6V	100	100	100	100	100
B_4V_2	47	54	53	52	37
B_2V_3	6	11	9	6	4
		Peak 16			
B_9	71	40	51	60	73
B_7V	100	100	100	100	100
B_5V_2	58	82	65	55	45
B_3V_3	14	24	15	11	7
		Peak 17			
B_{10}	48	36	45	54	65
B_8V	100	100	100	100	100
B_6V_2	64	97	77	65	53
B_4V_3	27	38	24	17	11
		Peak 18			
B_{11}	62	28	41	48	58
B_9V	100	89	100	100	100
B_7V_2	75	100	89	75	62
B_5V_3	33	49	34	24	17
B_3V_4	7	9	5	3	2
		Peak 19			
B_{12}	57	23	36.5	44	53
$B_{10}V$	100	78	99	100	100
B_8V_2	72	100	100	85	70
B_6V_3	32	58	46	33	23
B_4V_4	7	15	9.5	6	3
		Peak 20			
B_{13}	56	19	30	40	48
$B_{11}V$	100	70	88	100	100
B_9V_2	94	100	100	95	79
B_7V_3	65	68	54	43	29
B_5V_4	29	22	14	9	5
B_3V_5	11	3	2	1	0.4
B_{14}	50	15	25	35	45
$B_{12}V$	100	63	80	95	100
$B_{10}V_2$	100	100	100	100	87
B_8V_3	77	78	62	52	37
B_6V_4	38	31	19	14	8
B_4V_5	17	6	3	2	0.8
B_2V_6	13	0.4	0.2	0.08	0.03
B_{15}	53	13	21	30	42
$B_{13}V$	95	57	73	86	100
$B_{11}V_2$	100	100	100	100	96
B_9V_3	72	88	70	59	46
B_7V_4	29	41	26	18	12
		Peak 21			
B_{16}	40	11	18	25	37
$B_{14}V$	92	53	67	79	96
$B_{12}V_2$	100	100	100	100	100
$B_{10}V_3$	64	98	78	65	54
B_8V_4	14	53	33	23	16
		Peak 22			
B_{17}	38	9	16	22	32
$B_{15}V$	87	45	62	73	89
$B_{13}V_2$	100	92	100	100	100
$B_{11}V_3$	57	100	85	72	59

[a] Relative intensities of MNa^+ ions in the FAB-MS spectrum. [b] Relative intensities of methanolysis products, calculated by eq 1 (see text) for the following four PHB/PHV copolymer compositions: 80/20, 83/17, 85/15, 87/13.

Acknowledgment. Partial financial support from the Italian Ministry of Public Education and from Consiglio

Nazionale delle Ricerche (Roma) is gratefully acknowledged.

Registry No. PHB, 26063-00-3; PHB (SRU), 26744-04-7; (HB)(HV) (copolymer), 80181-31-3.

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Sequence Distribution in Poly(dimethylsiloxane-*co*-methylvinylsiloxanes)

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Received August 8, 1988

ABSTRACT: ^{29}Si NMR studies of copolymerized mixtures of octamethylcyclotetrasiloxane and 1,3,5,7-tetramethyltetravinylcyclotetrasiloxane showed random sequence distribution of comonomer units at equilibrium. The same random sequencing seen in the copolymer chains could be seen in the vicinity of the chain ends as well as in the byproduced cocyclosiloxanes. This persisted over a wide range of compositions and equilibrium temperatures. The results, consistent with homopolymerization, infer the absence of enthalpic driving forces in the copolymerization. Chemical shift data from the ^{29}Si NMR are reported for various sequences of comonomer units.

Introduction

Sequence distribution of repeat units in copolymers, which is crucial in determining both their properties and applications, can be derived from an understanding of the kinetic copolymerization parameters that are typically arrived at from the Mayo–Lewis model which presumes an irreversible process.[1-3] Although copolymerizations of cyclosiloxanes are reversible, previous studies either ignored this or sought to circumvent the difficulty by limiting the copolymerization to low conversions[4,5] or by selecting strained ring monomers and mild catalysts which do not readily induce siloxane redistribution.[6,7] Such approaches are not germane to common practice where the copolymerizations are usually taken to equilibrium. The present study focuses on a siloxane copolymerization at equilibrium through interpretation of the ^{29}Si NMR spectra of equilibrated copolymers in terms of simple linkage probabilities.[8] These are calculated from the relative intensities of signals from various triad sequences and are expressed in terms of run number, sequence length, or simply probability.[9,10] Emphasis is on copolymers containing dimethylsiloxy (D) and methylvinylsiloxy (V) units made by equilibration of octamethylcyclotetrasiloxane (D_4) and 1,3,5,7-tetramethyltetravinylcyclotetrasiloxane (V_4) initiated by potassium silanolate at various compositions and equilibrium temperatures. The copolymers were chosen because of their importance in cross-linking silicone rubber[11] and because they supplement recent studies of poly(dimethylsiloxane-*co*-diphenylsiloxane) made with a similar initiator and presumably also taken to equilibrium.[12,13] The latter studies, however, did not consider the composition and sequencing of the byproduced cocyclosiloxanes which can be an important or even dominant part of the equilibrate. The present investigation focuses on these as well as on the comonomer units located in both the main chain and near the chain ends.

Table I
Cocyclotetrasiloxanes Synthesized for ^{29}Si NMR Peak Assignments

compd	unit	δ, ppm from TMS	rel intensity
D_4	D	−19.10	
$\overbrace{D_{(1)}D_{(2)}D_{(1)}V}$	D(1)	−18.58	2.0
	D(2)	−18.99	1.0
	V	−33.50	0.9
$\overbrace{DDVV}$	D	−18.44	2.0
	V	−33.02	2.0
$\overbrace{DVDV}$	D	−18.01	2.0
	V	−33.39	2.0
$\overbrace{DV_{(1)}V_{(2)}V_{(1)}}$	D	−17.91	1.0
	V(1)	−32.95	2.1
	V(2)	−32.55	1.1

Experimental Section

Spectra. ^{29}Si NMR spectra were obtained on a Varian XL-200 FT spectrometer at 39.74 MHz using proton noise decoupling and $CDCl_3$ as an interal lock. The 90° pulse width was determined to be 19 μs. Throughout the experiments a simple pulse sequence was used with a pulse width of 16 μs and an aquisition time of 0.8 s and 2.2-s delay. The spectral width was 10 000 Hz using 16K data points. Samples were prepared as 30 wt % solutions in $CDCl_3$. $Cr(AcAc)_3$ (0.8 wt %) was added as a relaxation agent and TMS as an internal reference standard. A 16-mm sample tube was used with accumulation of 300–8000 scans. These

0024-9297/89/2222-2111$01.50/0