Biomacromolecules **2006,** *7,* 2210−2216

Sequence Distribution in Microbial Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Co-polyesters Determined by NMR and MS

Ema Žagar,*,† Andrej Kržan,† Grażyna Adamus,‡ and Marek Kowalczuk‡

National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia, and Centre of Polymer Chemistry, Polish Academy of Sciences, 41-800 Zabrze, Poland

Received March 3, 2006; Revised Manuscript Received May 5, 2006

The microstructure of bacterial poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolyesters (PHBV) as well as a mixture of two PHBV copolyesters of different comonomer composition and sequence distribution was studied by ^{13}C NMR based on dyad and triad analysis and multistage electrospray ionization mass spectrometry (ESI-MS^n). Both techniques gave results that were in good agreement for all investigated samples. The effect of microstructure on PHBV thermal properties was investigated from the melting behavior of samples. A PHBV copolyester with randomly distributed hydroxyvalerate units (12.0 mol % HV) showed a single melting peak, whereas samples with nonrandom composition distribution showed multiple melting peaks in their thermograms. Such complex melting behavior suggested that the 12.9 and 27.1 mol % PHBV copolyesters were actually blends of several copolymers with widely different comonomer-unit composition.

1. Introduction

Polyhydroxyalkanoates (PHAs) are thermoplastic aliphatic polyesters produced by microorganisms as intracellular energy and carbon storage materials. They represent an interesting group of biodegradable polymers that have recently received much attention, particularly as environmentally friendly materials produced from renewable resources[1] and are expected to find various applications, e.g., in packaging, agriculture, marine, and medical fields. PHAs are based on a number of different hydroxyacid monomers, which differ in the length of the alkyl side chain. Apart from the poly(3-hydroxybuyrate) (PHB) homopolymer, PHA copolymers are even more interesting due to the possibility of regulating copolymer physical and thermal properties by varying molecular structure, average comonomer composition, and comonomer composition distribution.[2−4]

Among the variety of PHA copolyesters, poly(3-hydroxybuyrate-*co*-3-hydroxyvalerate) copolyesters (PHBV) are of particular importance. Studies on these copolyesters have covered the reaction kinetics of PHBV production, melting and crystallization behavior, solid-state structure, mechanical properties, and enzymatic degradation processes dependent on the copolymer chemical composition and comonomer composition distribution (CCD).[5−34] Since the physical properties of PHA copolymers depend on average comonomer composition as well as CCD, it is important to be able to determine these parameters accurately and precisely.

The CCD of PHBV is usually determined by ^{13}C NMR spectroscopy based on dyad sequence analysis.[5,6,15,22−32] The extent of the deviation of the copolymer composition from the statistically random composition distribution can be proposed by the parameter D i.e., the dyad sequence distribution defined as

$$D = F_{\mathrm{BB}}F_{\mathrm{VV}}/F_{\mathrm{BV}}F_{\mathrm{VB}} \tag{1}$$

where subscripts BB, VV, BV, and VB represent butyrate−butyrate, valerate−valerate, butyrate−valerate, and valerate−butyrate dyad sequences, respectively. The D value for statistically random copolymers is 1.0. “Blocky” and alternating copolymers have D values much larger than 1 and very close to 0, respectively. The term “blocky” indicates that the copolymer is a true block copolymer, a mixture of copolymers with different compositions, or a mixture of poly-3-hydroxybutyrate (PHB) and poly-3-hydroxyvalerate (PHV) homopolymers. The parameter D is not very sensitive to the broadness of CCD, whereas it is sensitive to the bimodal (or multimodal; very large D) CCD.[22,31] Some PHBV copolymers produced by various bacteria have been reported to have D values close to 1,[5,28,31] whereas others showed D values much larger than 1. Such PHBV copolyesters are considered to be mixtures of random copolymers with different comonomer-unit compositions, which was confirmed by solvent/nonsolvent fractionation[11,12,14,15,20,22,28,31,32] studies. Such fractionation of original copolymers with D close to 1, which can be regarded as random copolymers according to D, revealed broad CCD. It could thus be concluded that values of parameter D much higher than 1 clearly indicate bimodal (or multimodal) CCD but give little information on the width of CCD.

More precise information on CCD can be obtained by the analysis of a higher order sequence distribution, i.e., the triad sequence distribution.[6,11,35−39] The degree of randomness in the copolymer chain based on the triad sequence distribution analysis can be calculated from the coefficient R (see the Appendix).[39] The parameter R has a value of 1 for a completely random distribution of HB and HV units in the copolymer chain and 0 for a diblock copolymer.

The copolymer CCD can also be determined by mass spectrometry (MS) using various “soft” ionization techniques, which enable the production of gas-phase ions from a wide variety of (co)polymers with little or no fragmentation during ionization.[40,41] The relative intensities of the mass peaks appearing in the mass spectrum corresponding to the individual copolymer chains depend on the copolymer composition and on the type of distribution of comonomer units in their chains.

---

* Corresponding author. Phone +386-1-4760200. Fax +386-1-4760300. E-mail ema.zagar@ki.si.

† National Institute of Chemistry.

‡ Polish Academy of Sciences.

10.1021/bm060201g CCC: $33.50

To decode the relative intensities of the peaks related to the individual copolymer molecules and to determine their composition and sequence distribution, the theoretical mass spectra of copolymers (based on the Bernoullian or first- or second-order Markovian chain statistic) are generated and compared with those experimentally acquired with the aid of "soft" MS ionization techniques. This method has been successfully applied for the characterization of several copolymer systems including microbial and synthetic aliphatic copolyesters.[42,43]

The drawback of MS characterization of high molar mass (co)polymers is that with increasing mass the mass resolution decreases, while the number of potential isobaric structures increases. Furthermore, many MS instruments (such as those equipped with a quadrupole mass analyzer, a magnetic sector, a cyclotron cell, or an ion trap) are limited in the range of mass they can detect. To deal with these limitations of MS instruments, the original high molar mass copolymer is usually subjected to partial degradation to obtain lower molar mass analogues possessing the same composition and sequence distribution as the original one. Several methods of partial degradation have been evaluated for different copolymers including partial hydrolysis, methanolysis, aminolysis, photolysis, ozonolysis, and methoxidation.[43] However, in the case of partial copolymer degradation, the analysis needs an additional step in which the sequence of the undegraded copolymer is reconstructed from the data obtained on the partially degraded sample.

One application of the ESI-MS method for the determination of the comonomer unit composition and composition distribution in bacterial PHA (co)polymers based on the analysis of their oligomers obtained by controlled, partial, alkaline depolymerization was reported recently.[43−45] Controlled depolymerization of natural PHAs catalyzed by KOH/18-crown-6 complex or tetrabutylammonium hydroxide due to the partial saponification of ester linkages and an elimination reaction, respectively, leads to the formation of oligomers with the same composition and sequence distribution as the starting materials containing carboxylic and olefinic end groups as revealed by ^{1}H NMR and ESI-MS analysis.[44−46] Additional information about the structure of (co)polymers can be provided by multistage mass spectrometry (MS^n). Using this technique, the molar masses and structural analysis of mass-selected macromolecular ions were recently determined, thus elucidating the chemical nature of the copolymer and its end groups.[44] For block, graft, and random copolymers related to biological copolymers, the distribution of comonomers in the mass selected macromolecules was determined by ESI-MS^n and MALDI-MS/MS experiments.[47−50]

We present an integrated study of the composition and sequence distribution of various bacterial PHBV copolyesters and the mixture of two individual PHBV samples using high-resolution 600 MHz NMR spectroscopy (on the dyad and triad level) correlated with multistage ESI-MS^n structural investigations on the molecular level. Structural studies using ESI-MS^n technique were performed based on analyses of low molar mass PHBV oligomers obtained via controlled depolymerization of high molar mass PHBV copolyesters. The influence of distribution of comonomer-unit composition on the thermal properties of copolymers was investigated by DSC measurements. To our knowledge this work represents the first attempt at correlating detailed PHBV copolyester sequence distributions based on NMR and MS characterization.

2. Experimental Section

2.1. Materials. PHBV with a 12.9 mol % HV was biosynthesized using *Ralstonia eutropha* fed on hydrolyzed sucrose. It was supplied by Biocycle, PHB Industrial S/A, Brazil. PHBV copolymers with 12.0 and 27.1 mol % HV were provided by the Institute of Biotechnology and Bioprocess Engineering, Graz University of Technology (Graz, Austria). The copolyesters were biosynthesized by an osmophilic bacterium fed with hydrolyzed whey. The content of hydroxyvalerate comonomer units (HV) in the individual PHBV copolymer was determined by ^{1}H NMR spectrometry. The 1:1 mixture by weight of 12.0 and 27.1 mol % PHBV samples was prepared by dissolving the constituent copolymers in $CHCl_3$. The solution was poured onto a glass Petri dishes, where the solvent was allowed to evaporate. The mixture contains 18.9 mol % HV monomeric units.

2.2. SEC-MALS Measurements. Sample solutions for SEC-MALS measurements were prepared by dissolving the samples at 60 °C for 1 h in acid free $CHCl_3$ at a concentration of 2×10^{-3} g mL^{-1}. The molar mass averages (MMA) and molar mass distributions (MMD) of PHBV copolymers were determined according to the procedure described in our previous paper.[51]

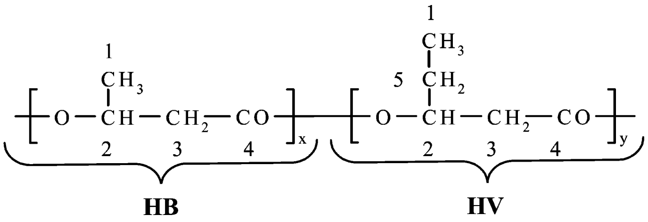
2.3. NMR Spectrometry. The solution ^{1}H and ^{13}C NMR spectra of PHBV copolymers were carried out on a Varian Inova 600 MHz spectrometer in the pulse Fourier Transform mode. The 600 MHz ^{1}H NMR spectra were recorded at 30 °C using 5×10^{-3} g mL^{-1} polymer solutions in $CDCl_3$ with 6.0 s pulse repetition, 8000 Hz spectral width, and 80 accumulations. The 150 MHz ^{13}C NMR spectra were recorded at 30 °C using 25×10^{-3} g mL^{-1} polymer solutions in $CDCl_3$ with 5.0 s pulse repetition, 30 000 Hz spectral width, and 15 000 accumulations. Tetramethylsilane (Me_4Si, $\delta = 0$) was used as an internal chemical shift standard.

2.4. ESI-MS^n Characterization. 2.4.1. Preparation of Oligomers. The PHBV oligomers were obtained via partial saponification of the original PHBV copolyesters dissolved in $CHCl_3$. The reactions were performed in the presence of a 0.375 mol L^{-1} aqueous solution of *tert*-butylammonium hydroxide at 35 °C. The chloroform fractions containing low molar mass oligomers were protonated using the acid ion-exchange resin Dowex and worked up as described previously.[44,49] The obtained oligomer samples were analyzed by SEC, ^{1}H NMR (Table 1), and ESI-MS^n spectrometry.

2.4.2. ESI-MS^n Experiments. Electrospray mass spectrometry analyses were performed using an LCQ ion trap mass spectrometer (Finnigan, San Jose, CA) and an Esquire ion trap with orthogonal ESI source (Bruker, Germany). PHBV samples were dissolved in chloroform/methanol mixture (10/1; v/v). The solution was introduced to the ESI source by continuous infusion by means of the instrument syringe pump at a rate of 3 μL min^{-1}. The LCQ ESI source was operated at 4.25 kV and the capillary heater was set to 200 °C. For ESI-MS^n experiments, mass-selected mono-isotopic parent ions were isolated in the trap with an isolation width of 2 m/z and activated by collision with a 33% ejection RF-amplitude at standard He pressure. The experiments were performed in negative-ion mode.

2.5. Thermal Characterization. Sample films used for the DSC measurements were prepared by casting PHBV chloroform solutions onto glass Petri dishes. The solvent was allowed to evaporate under vacuum at room temperature. So obtained films were melted at 180 °C and subsequently isothermally crystallized at 30 °C for 1 month. All thermal analyses were carried out using a differential scanning calorimeter (DSC) Perkin-Elmer Pyris 1 under a nitrogen atmosphere. Isothermally crystallized samples were pre-sealed (approximately 1 mg) in aluminum pans. The samples were heated from −50 to +200 °C (first heating scan) at a heating rate of 100 °C min^{-1}. From the obtained DSC curves the peak melting temperatures, T_m, were determined. After rapidly quenching, the samples were reheated at a heating rate of 20 °C min^{-1} (second heating scan). From the second thermal diagram the samples glass transition temperatures, T_g, were estimated.

Scheme 1. Schematic Structure of PHBV Copolyesters



$$\left[-O-\underset{2}{CH}(\overset{1}{CH_3})-\underset{3}{CH_2}-\underset{4}{CO}-\right]_x\left[-O-\underset{2}{CH}(\overset{5}{CH_2}\overset{1}{CH_3})-\underset{3}{CH_2}-\underset{4}{CO}-\right]_y$$

Table 1. SEC-MALS Results for 27.1, 12.9, and 12.0 mol % As-produced PHBV Copolymer Samples

PHBV	$\bar{M}_n \times 10^5$ g mol^{-1}	$\bar{M}_w \times 10^5$ g mol^{-1}	$\bar{M}_z \times 10^5$ g mol^{-1}	$\bar{M}_w/\bar{M}_n$	$\bar{M}_z/\bar{M}_w$	HV content,[a] mol %
12.9 mol %	0.738	1.562	2.978	2.12	1.91	12.9
27.1 mol %	0.454	1.635	3.587	3.60	2.19	27.1
12.0 mol %	12.92	14.44	15.76	1.12	1.09	12.0

[a] Determined by ^{1}H NMR spectrometry.

3. Results and Discussion

3.1. NMR Spectrometry of PHBV Copolymers. The determination of copolymer composition by ^{1}H NMR spectrometry indicated that the as-produced PHBV copolyesters contained 12.0, 12.9, and 27.1 mol % HV comonomer units (Table 1). The 12.0 mol % PHBV copolymer showed much higher molar mass averages and a narrower molar mass distribution than the corresponding values for 12.9 and 27.1 mol % PHBV copolymers (Table 1).

The sequence distribution of hydroxyvalerate (HV) and hydroxybutyrate (HB) comonomer units in each PHBV copolyester was determined from proton-noise decoupled ^{13}C NMR spectra from the relative peak intensities of the carbonyl (4HB, 4HV, Scheme 1) and methylene carbon resonances of HV units (5HV, Scheme 1). These signals were split into several peaks due to the sensitivity of carbon nuclei to different sequences of HB and HV units. The carbonyl region showed four peaks, arising from different dyad sequences (VV, VB, BV, and BB) of connecting HB and HV units. The triad sequences were determined from a resonance of the HV side-chain methylene carbon (5HV) composed of four peaks assigned to VVV, BVV, VVB, and BVB HV-centered triad sequences. The relative intensities of each ^{13}C resonance were determined from the peak areas. From the relative intensities of carbonyl and side-chain methylene carbon resonances, the dyad and triad sequence distributions of HB and HV units in the as-produced PHBV copolymers were estimated. The mole, the dyad, and the HV-centered triad molar fractions of HB and HV in the respective samples are summarized in Table 2.

The degree of randomness in sequence distribution was estimated by the parameter D (eq 1) on the dyad level and by the parameter R (eq 2) on the triad level (Table 3). The obtained D values were 1.21, 1.95, and 8.10, whereas the R values were 0.97, 0.93, and 0.58 for PHBV copolymers with 12.0, 12.9, and 27.1 mol % HV units, respectively. Within the experimental uncertainty of the measured dyad and triad fractions (±3 mol %), both D and R values were close to 1 for the 12.0 mol % PHBV sample implying that it is a random copolymer. For the other two samples the obtained D parameters were higher, and R parameters lower, than 1. According to the obtained results as well as previous reported results on fractionation of PHBV copolymers with $D > 1$,[11,12,14,15,20,22,28,31,32] we have concluded that 12.9 and 27.1 mol % as-produced PHBV copolymers are mixtures of random copolymers with different comonomer unit compositions. The obtained results show that the deviation of D and R values from 1 increase with increasing HV content in the copolymer. In our case, this relationship appears to be linear; however, to prove that this is a general principle (for a particular microorganism and/or specific polymerization conditions), a detailed additional study would be needed.

Table 3. Parameters D (eq 1) and R (eq 2), Experimental Number Average Sequence Lengths of HV Units (L_V^E, eq 3), Number Average Sequence Length of Randomly distributed HV units in copolymer (L_V^R, eq 6), Ratio between the Concentration of HB and HV Units (k, eq 7) for 12.0, 12.9, and 27.1 mol % PHBV Copolymer Samples, and 1:1 by Weight Mixture of 12.0 and 27.1 mol % PHBV Copolymers

PHBV	D	R	L_V^E	L_V^R	k
12.9 mol %	1.94	0.93	1.24	1.15	6.75
27.1 mol %	8.10	0.58	2.38	1.37	2.69
12.0 mol %	1.21	0.97	1.18	1.14	7.33
mixture	4.40	0.67	1.84	1.23	4.29

3.2. ESI-MS^n Characterization of PHBV Copolymers. The sequence distribution of comonomeric units in PHBV copolyesters was also evaluated by ESI-MS analyses of low molar mass PHBV oligomers obtained via controlled, partial, alkaline depolymerization of the original high molar mass samples. The composition of partially degraded PHBV copolymers was determined from their ^{1}H NMR spectra. The obtained results revealed that the content of both monomeric units in partially degraded samples is similar to that of the original ones (Table 4).

Table 4. Molar Mass Averages, Molar Mass Distribution, and Composition of Degraded PHBV Copolymers

PHBV	$\bar{M}_n$[a]	$\bar{M}_w/\bar{M}_n$[a]	composition[b] HB/HV
27.1 %	700	1.4	72.4/27.6
12.9 %	600	1.6	86.9/13.1
12.0 %	650	1.5	87.8/12.2
mixture			80.9/19.1

[a] Determined by SEC in chloroform at 35 °C using polystyrene standards. [b] Determined from ^{1}H NMR spectra of degraded PHBV samples.

In addition to proton signals of PHBV copolyester chains, the ^{1}H NMR spectra of oligomers showed the signals assigned to the olefinic end-groups arising from the elimination reaction

Table 2. Experimental Monomer, Dyad, and Triad Sequence Mole Fractions[a] in 12.0, 12.9, and 27.1 mol % PHBV Copolymer Samples and 1:1 by Weight Mixture of 12.0 and 27.1 mol % PHBV Copolymers

PHBV	F_B[b]	F_V[b]	F_{BB}	F_{BV}	F_{VB}	F_{VV}	F_{VVV}	F_{BVV}	F_{VVB}	F_{BVB}
12.9%	0.871	0.129	0.771	0.101	0.102	0.026	0.006	0.019	0.020	0.084
27.1%	0.729	0.271	0.616	0.113	0.109	0.162	0.115	0.042	0.040	0.074
12.0%	0.880	0.120	0.768	0.112	0.102	0.018	0.004	0.014	0.013	0.089
mixture	0.811	0.189	0.677	0.134	0.101	0.088	0.0635	0.023	0.024	0.0785

[a] Subscripts B and V represent butyrate and valerate monomer units, respectively. [b] Determined from ^{1}H NMR spectra.

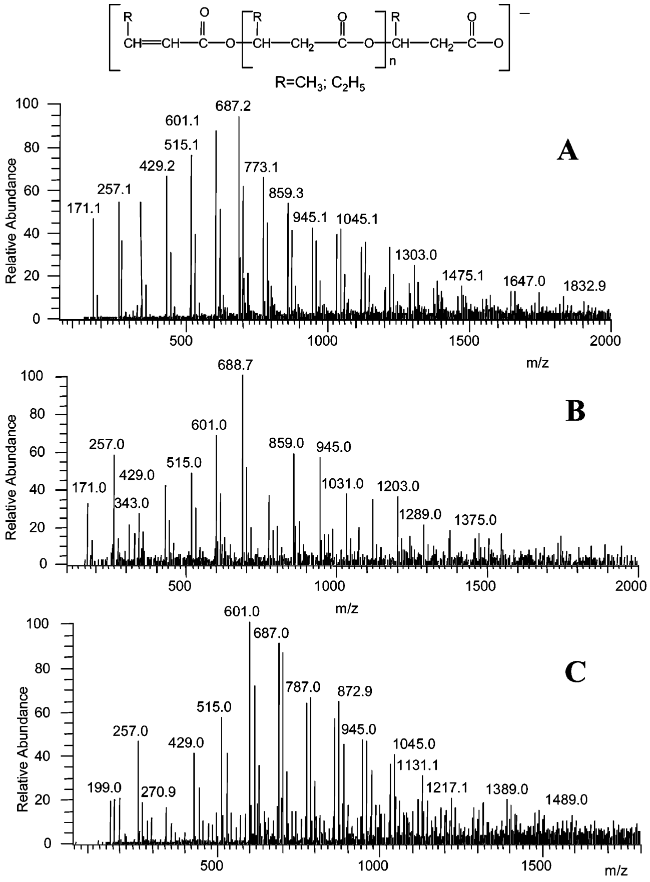


Figure 1. ESI-MS spectra (negative ion-mode) of PHBV oligomers of partially depolymerized: (A) 12.0, (B) 12.9, and (C) 27.1 mol % PHBV samples.

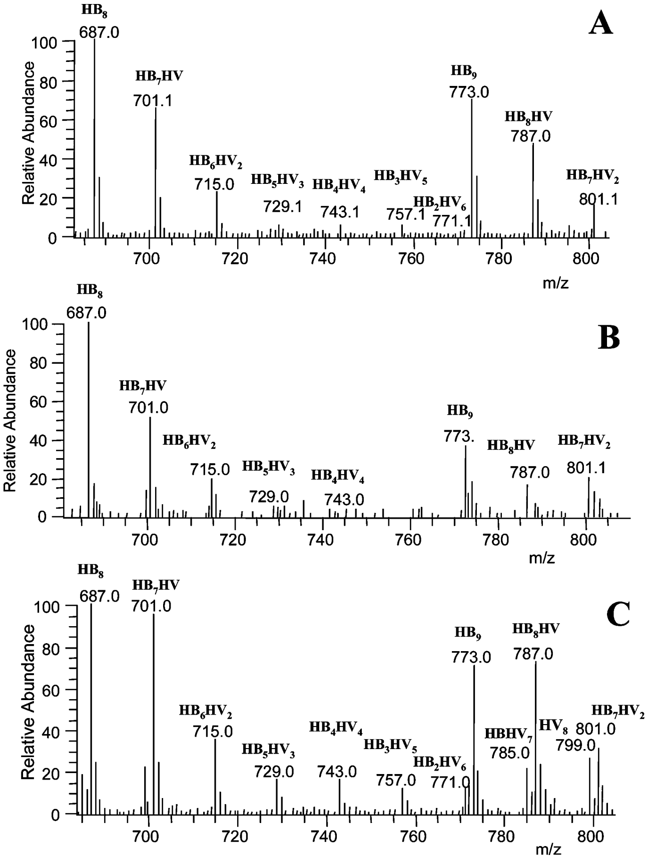


Figure 2. Expanded ESI-MS spectra (negative-ion mode) in the mass range 660 < m/z < 820 Da for PHBV oligomers of: (A) 12.0, (B) 12.9, and (C) 27.1 mol % PHBV samples.

occurring during the partial saponification of ester linkages: $\delta = 1.9$ ppm (doublet of crotonate CH_3 protons), 5.8 ppm (multiplet), and $\delta = 6.9$ ppm (multiplet) (see the scheme in Figure 1).[44]

Figure 1 represents the general chemical structure of oligomers, and the distribution of singly charged negative $[M - H]^-$ ions. $[M - H]^-$ anions correspond to the individual oligomer chains composed of HB and HV units and terminated by unsaturated and carboxylic end groups. The ions in each mass spectrum were grouped into numerous clusters due to differences in the degree of oligomerization and composition of oligomers.

The mass difference in the experimental m/z values between the highest intensity peaks in neighboring groups of peaks was equal to 86 Da corresponding to the molar mass of the HB unit (Figure 1). The mass difference between the neighboring peaks within a group of peaks was equal to 14 Da, which corresponds to the mass difference between HV and HB units (Figure 2). The obtained ESI-mass spectra of the oligomers allowed the identification of their structure up to the 15-mer level.

Figure 2 shows the expanded ESI-mass spectra of PHBV oligomers in the mass range (m/z) between 680 and 820 together with the chemical assignment of the peaks. This mass range consisted of the signals corresponding to the oligomer chains of the 8-mer cluster and a few signals of the 9-mer cluster. A detailed comparison of the expanded mass spectra of investigated PHBV samples indicated that the distribution of signal intensities belonging to the 8-mer cluster (m/z: 687 = HB$_8$; 701 = HB$_7$HV; 715 = HB$_6$HV$_2$; 729 = HB$_5$HV$_3$; 743 = HB$_4$-HV$_4$; 757 = HB$_3$HV$_5$; 771 = HB$_2$HV$_6$; 785 = HB$_1$HV$_7$; 799 = HV$_8$) differ due to the differences in sample composition and comonomer sequence distribution. For all three PHBV copolymers the intensity of the signals in the 8-mer oligomer cluster decreased with increasing HV content. In the case of 12.0 and 12.9 mol % PHBV samples (Figure 2a,b), the decrease is smoother and to lower values than in the case of the 27.1 mol % HV copolymer (Figure 2c). This sample showed more intense signals for HV rich oligomers since the relative amount of HV units was higher.

The sequence distribution in the copolymer chains was determined from the relative peak intensities of the detected anions in the mass spectra. The estimated values were compared to the theoretical ones calculated according to the Bernoullian chain statistic for copolymers of similar composition, but randomly distributed comonomers in the copolymer chains.[42,43] The theoretical peak intensities were then compared with those acquired experimentally. The agreement between the observed and calculated distributions was expressed by means of the Hamilton agreement factor (AF) defined by

$$AF = [\Sigma(I_{exp} - I_{calc})^2/\Sigma I_{exp}^{\ 2}]^{1/2} \qquad (8)$$

where I_{exp} and I_{calc} are the normalized experimental and calculated abundances of partially degraded copolyesters.[42,44] The AFs were calculated for the specific cluster of oligomers (from 2-mer up to 15-mer) as a function of oligomer composition. The obtained results for all three PHBV copolymers are presented in Figure 3.

The AF calculated for each specific oligomer cluster of 12.0 mol % PHBV sample shows a minimum in the composition range from 12 to 13 mol % HV units, which is similar to the original PHBV copolyester. These results indicated that the 12.0 mol % PHBV copolyester has randomly distributed comonomer units, which is in agreement with the sequence distribution

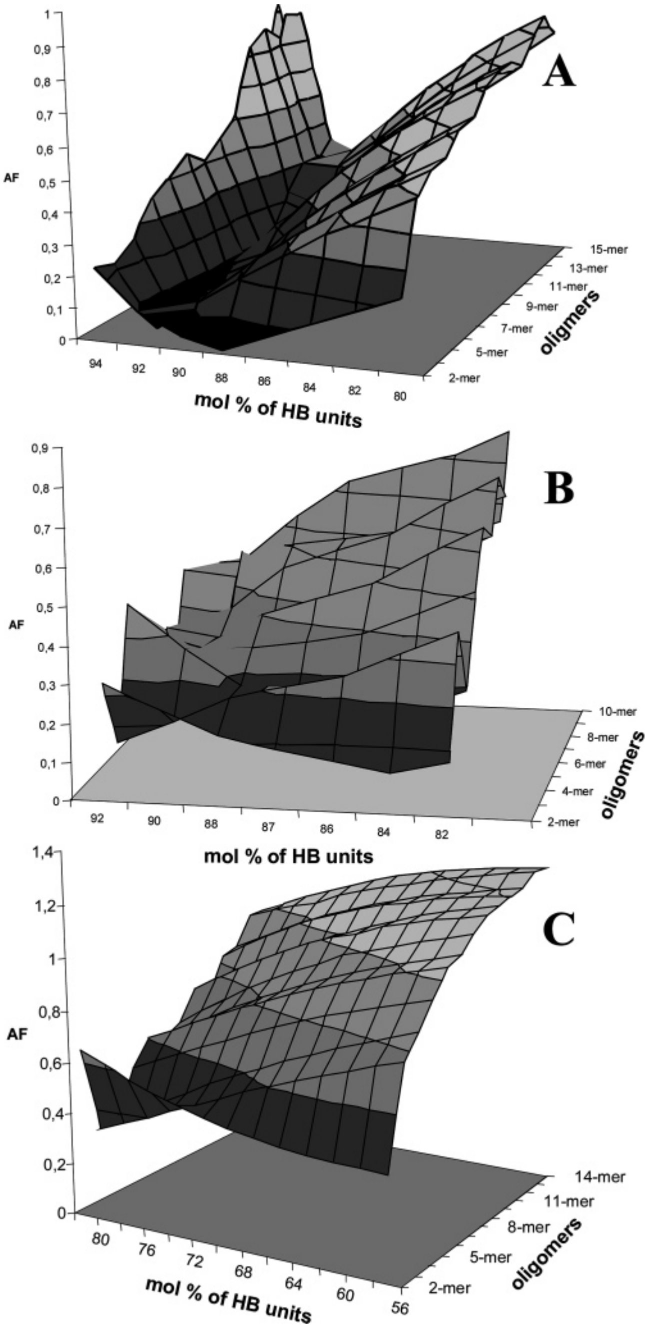


Figure 3. Calculated agreement factors (AF) as a function of copolymer composition for PHBV oligomers of: (A) 12.0, (B) 12.9, and (C) 27.1 mol % PHBV samples.

determined by ^{13}C NMR spectroscopy on the dyad and triad level. The calculated AF for 12.9 and 27.1 mol % PHBV oligomers as a function of oligomer composition gave no well-defined minimum; instead, the AF systematically increased with increasing oligomer molecular mass. From these results we inferred that 12.9 and 27.1 mol % PHBV samples are not pure random copolyesters, but rather mixtures of random copolymers of different comonomer unit composition. The differences between the experimental and theoretical oligomer distributions (calculated based on the Bernoulli chain statistic) that are expressed by AF factors (Figure 3c) are larger for 27.1 mol % PHBV sample than for 12.9 mol % PHBV sample. These results are consistent with NMR results, which indicate that with higher HV content in the copolymer the deviation from random copolymer distribution increases. In general, it may be concluded that the obtained results on sequence distribution determination in PHBV copolyesters by ESI-MS are in good agreement with those obtained by ^{13}C NMR measurements.

To verify the random distribution of comonomer units in individual oligomer chains of the 12.0 mol % PHBV sample a multistage MS experiment (MS^2) was performed for the parent ion $m/z = 715$ (HV$_2$HB$_4$) selected from the 8-mer cluster in the mass spectrum (Figure 3a). A MS^2 spectrum of this molecular ion showed fragment anions grouped in clusters containing two fragment anions in the first step and three in the following steps, all having the same degree of oligomerization but a different content of HB and HV units (Figure 4). The fragment anions were produced by the loss of crotonic acid (86 Da) or 2-pentenoic acid (100 Da), respectively (see fragmentation pathway in Figure 4). The proposed fragmentation pathway for a molecular anion $m/z = 715$ was consistent with the obtained MS^2 mass spectrum. The results from the MS^2 experiment thus confirmed that a 12.0 mol % PHBV sample has a random distribution of HB and HV comonomer units in the copolyester chains.

3.3. Thermal Characterization of PHBV Copolymers. The influence of comonomer unit composition on the thermal properties of PHBV copolyesters was investigated by DSC measurements. The melting curves were obtained on PHBV samples, which were subject to the same thermal history (see section 2.5. in the Experimental Section). To avoid recrystallization during heating, a heating rate of 100 °C min^{-1} was used.[52] The DSC melting curve (Figure 5A) of 12.0 mol % PHBV copolyester showed a single melting peak at 130 °C, which is in agreement with randomly distributed comonomer units. This melting point corresponds well with the previously reported data on melting points versus HV mole fraction of the sample for a random copolymer.[11]

The DSC melting curves of the 12.9 and 27.1 mol % PHBV copolyesters indicated complex multiple melting (Figure 5B,C). The 12.9 mol % PHBV copolyester showed three melting peaks at 160 and 144 and a broad peak of low intensity at 112 °C (Figure 5B), whereas the 27.1 mol % PHBV copolyester shows peaks at 134 and 93 °C (Figure 5C). Such melting behavior revealed that as-produced PHBV copolymers are really blends of many component copolymers with widely different comonomer-unit composition. Namely, when the difference in the comonomer unit content between the components of the blend is high, the individual components crystallize separately.[16,20]

3.4. Mixture of PHBV Copolymers of Different HV Composition and CCD. A 1:1 by weight mixture of the random PHBV copolyester with 12.0 mol % and the PHBV copolyester with 27.1 mol % HV comonomer units consisted of 18.9 mol % HV units and gave determined parameters *D* and *R* of 4.4 and 0.67, respectively (Tables 2 and 3). These values are between the values determined for the constituent copolyesters. The composition and sequence distribution of the mixture were also determined by ESI-MS analysis according to the procedure described previously.[46,53] The mixture showed a systematic increase in minimal AF. In addition, the AF values estimated for specific oligomers were not located at the same composition (Figure 6).

In DSC measurements, the mixture showed a less complex melting behavior compared to the constituent copolymer PHBV 27.1 mol % (Figure 5D). The reason for this could be that 12.0 mol % random copolymer cocrystallized with the major component of 27.1 mol % copolymer, which, according to its melting point, has a similar composition as 12.0 mol % copolymer. The melting point of the minor component of 27.1 mol % copolymer (93 °C) is missing in the thermogram of the

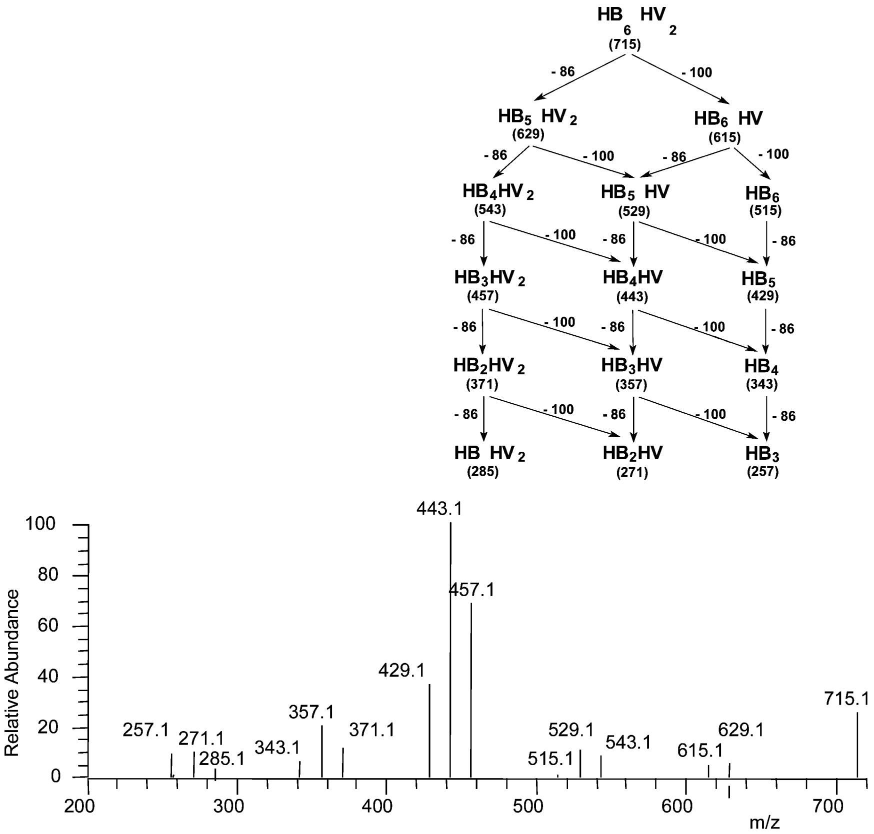


Figure 4. Sequential fragmentation spectrum (MS^2) obtained for molecular ion HB_6HV_2 at $m/z = 715$ selected from 8-mer oligomer cluster of a 12.0 mol % PHBV sample with the proposed fragmentation pathway.

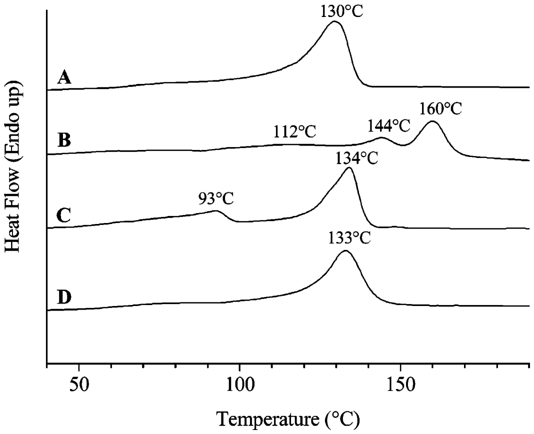


Figure 5. DSC melting curves (first heating scan) at heating rate of 100 °C min^{-1} for: (A) 12.0, (B) 12.9, and (C) 27.1 mol % PHBV copolymers and (D) 1:1 by weight mixture of 12.0 and 27.1 mol % PHBV copolymers.

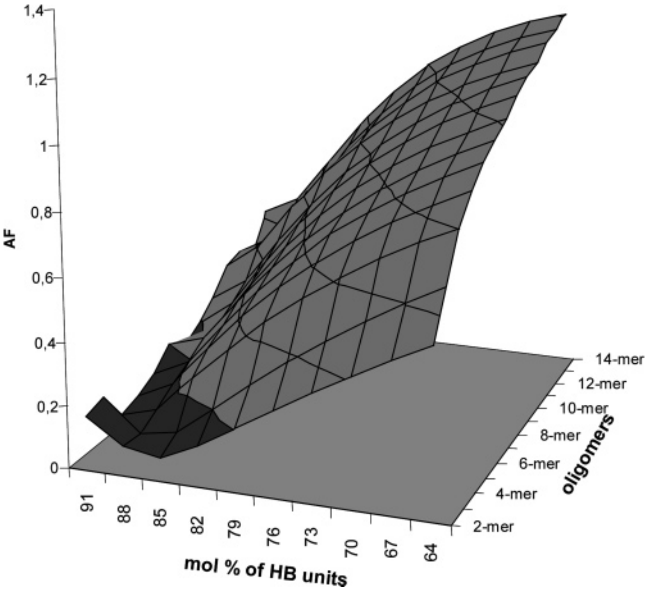


Figure 6. Calculated agreement factors (AF) as a function of copolymer composition for 1:1 by weight mixture of 12.0 and 27.1 mol % PHBV copolymers.

mixture, implying that the relative amount of this component is too small to be detected or too broadly diffused to crystallize. As a consequence, the DSC melting trace of the mixture showed only one peak. Therefore, in this case, it is difficult to estimate the complexity of the composition from the melting behavior by itself.

4. Conclusions

This study compares the results obtained on sequence distribution of comonomer units in microbial PHBV copolyesters obtained by ^{13}C NMR spectroscopy based on dyad and triad analysis and the ESI-MS^n technique. For the first time, it was shown that both methods give comparable results. In the case of PHBV copolymers with uniform composition distribution and randomly distributed comonomer units, both techniques give a clear indication of the sequence distribution. On the other hand, in the case of PHBV copolymers with nonuniform comonomer composition distribution (broad or with multiple distribution centers), the parameters D and R as well as the AFs depend not only on sequence distribution but also on composition distribution of comonomers. To obtain the exact sequence distribution in such PHBV copolymers, the samples have to be fractionated according to composition prior to determination of sequence distribution.

Analysis of the effect of microstructure on copolyester melting behavior indicated that random PHBV showed a single melting peak, whereas PHBV samples with complex composition distribution showed multiple melting peaks in their DSC thermograms. DSC trace of 1:1 by weight mixture of the random PHBV copolyester with 12.0 mol % and the PHBV copolyester with 27.1 mol % HV comonomer units surprisingly showed only one melting peak indicating that the complexity of PHBV composition is difficult to infer solely from the copolymer melting behavior.

Acknowledgment. The authors gratefully acknowledge financial support from the EC 5th RTD (Project WHEYPOL, 5FP G5Rd-CT-2001-00591), the Slovenian Research Agency (Program P2-0145), and the Polish Ministry of Scientific Research and Information Technology (Projects EUREKA3064 and PBZ-KBN-3/070/*T*09/2002). The authors are indebted to Prof. Janusz Kasperczyk for helpful discussions and Izabela Goebel, M.Sc., for the preparation of PHBV oligomers.

Appendix

$$R = L_{\mathrm{B}}^{\mathrm{R}}/L_{\mathrm{B}}^{\mathrm{E}} = L_{\mathrm{V}}^{\mathrm{R}}/L_{\mathrm{V}}^{\mathrm{E}} \tag{2}$$

where $L_{\mathrm{V}}^{\mathrm{E}}$ and $L_{\mathrm{B}}^{\mathrm{E}}$ are the experimental number average lengths of HV and HB blocks in the copolymers and can be calculated by the following equations:

$$L_{\mathrm{V}}^{\mathrm{E}} = (F_{\mathrm{VVV}} + F_{\mathrm{VVB}} + F_{\mathrm{BVV}} + F_{\mathrm{BVB}})/(F_{\mathrm{BVB}} + F_{\mathrm{VVB}}) \tag{3}$$

$$L_{\mathrm{B}}^{\mathrm{E}} = (F_{\mathrm{BBB}} + F_{\mathrm{VBB}} + F_{\mathrm{BBV}} + F_{\mathrm{VBV}})/(F_{\mathrm{VBV}} + F_{\mathrm{VBB}}) \tag{4}$$

where F_{XYZ} indicates the relative molar fraction of XYZ triad sequence. $L_{\mathrm{V}}^{\mathrm{R}}$ and $L_{\mathrm{B}}^{\mathrm{R}}$ are the number average lengths of HV and HB blocks of statistically random distribution of HB and HV units in the copolymer. They are defined by equations

$$L_{\mathrm{B}}^{\mathrm{R}} = k + 1 \tag{5}$$

$$L_{\mathrm{V}}^{\mathrm{R}} = (k + 1)/k \tag{6}$$

where k is defined as the ratio between the concentration of HB and HV units in the copolymer chain

$$k = [\mathrm{HB}]/[\mathrm{HV}] \tag{7}$$

$$[\mathrm{HB}] = k/(k + 1) \text{ and } [\mathrm{HV}] = 1/(k + 1) \tag{8}$$

References and Notes

(1) Koller, M.; Bona, R.; Braunegg, G.; Hermann, C.; Horvat, P.; Kroutil, M.; Martinz, J.; Neto, J.; Pereira, L.; Varila, P. *Biomacromolecules* **2005**, *6*, 561.
(2) Sudesh, K.; Abe, H.; Doi, Y. *Prog. Polym. Sci.* **2000**, *25*, 1503.
(3) Doi, Y. *Microbial Polyesters*; VCH: New York, 1990.
(4) Inoue, Y.; Yoshie, N. *Prog. Polym. Sci.* **1992**, *17*, 571.
(5) Ishihara, Y.; Shimizu, H.; Shioya, *S. J. Ferment. Bioeng.* **1996**, *81*, 422.
(6) Bluhm, T. L.; Hamer, G. K.; Marchessault, R. H.; Fyfe, C. A.; Veregin, R. P. *Macromolecules* **1986**, *19*, 2871.
(7) Bloembergen, S.; Holden, D. A.; Hamer G. K.; Bluhm, T. L.; Marchessault, R. H. *Macromolecules* **1986**, *19*, 2865.
(8) Bloembergen, S.; Holden, D. A.; Bluhm, T. L.; Hamer G. K.; Marchessault, R. H. *Macromolecules* **1989**, *22*, 1663.
(9) Doi, Y.; Kunioka, M.; Tamaki, A.; Nakamura, Y.; Soga, K. *Makromol. Chem.* **1988**, *189*, 1077.
(10) Kunioka, M.; Tamaki, A.; Doi, Y. *Macromolecules* **1989**, *22*, 694.
(11) Inoue, Y.; Kamiya, N.; Yamamoto, Y.; Chûjô, R.; Doi, Y. *Macromolecules* **1989**, *22*, 3800.
(12) Wang, Y.; Yamada, S.; Asakawa, N.; Yamane, T.; Yoshie, N.; Inoue, Y. *Biomacromolecules* **2001**, *2*, 1315.
(13) Yoshie, N.; Saito, M.; Inoue, Y. *Macromolecules* **2001**, *34*, 8953.
(14) Yamada, S.; Wang, Y.; Asakawa, N.; Yoshie, N.; Inoue, Y. *Macromolecules* **2001**, *34*, 4659.
(15) Mitomo, H.; Morishita, N.; Doi, Y. *Polymer* **1995**, *36*, 2573.
(16) Organ, S. J. *Polymer* **1994**, *35*, 86.
(17) Pearce, R. P.; Marchessault, R. H. *Macromolecules* **1994**, *27*, 3869.
(18) Saito, M.; Inoue, Y.; Yoshie, N. *Polymer* **2001**, *42*, 5573.
(19) Yoshie, N.; Fujiwara, M.; Ohmori, M.; Inoue, Y. *Polymer* **2001**, *42*, 8557.
(20) Feng, L.; Yoshie, N.; Asakawa, N.; Inoue, Y. *Macromol. Biosci.* **2004**, *4*, 186.
(21) Yoshie, N.; Menju, H.; Sato, H.; Inoue, Y. *Polym. J.* **1996**, *28*, 45.
(22) Yoshie, N.; Menju, H.; Sato, H.; Inoue, Y. *Macromolecules* **1995**, *28*, 6516.
(23) Kamiya, N.; Sakurai, M.; Inoue, Doi, Y. *Macromolecules* **1991**, *24*, 2178.
(24) Bauer, H.; Owen, A. *J. Colloid Polym. Sci.* **1988**, *266*, 241.
(25) Scandola, M.; Ceccorulli, G.; Doi, Y. *Int. J. Biol. Macromol.* **1990**, *12*, 112.
(26) Mitomo, H.; Barham, P. J.; Keller, A. *Polym. J.* **1987**, *19*, 1241.
(27) Doi, Y.; Kanesawa, Y.; Kunioka, M.; Saito, T. *Macromolecules* **1990**, *23*, 26.
(28) Kamiya, N.; Yamamoto, Y.; Inoue, Y.; Chûjô, R.; Doi, Y. *Macromolecules* **1989**, *22*, 1676.
(29) Yoshie, N.; Fujiwara, M.; Ohmori, M.; Inoue, Y. *Polymer* **2001**, *42*, 8557.
(30) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 2860.
(31) Mitomo, H.; Morishita, N.; Doi, Y. *Macromolecules* **1993**, *26*, 5809.
(32) Organ, S. J.; Barham, P. J. *Polymer* **1993**, *34*, 459.
(33) Feng, L.; Wang, Y.; Inagawa, Y.; Kasuya, K.; Saito, T.; Doi, Y.; Inoue, Y. *Polym. Degrad. Stab.* **2004**, *84*, 95.
(34) Cao, A.; Arai, Y.; Yoshie, N.; Kasuya, K.-I.; Doi, Y.; Inoue, Y. *Polymer* **1999**, *40*, 6821.
(35) Randall, J. C. *Polymer Sequence Determination by Carbon−13 NMR Method*; Academic Press: New York, 1977.
(36) Koenig, J. L. *Chemical Microstructure of Polymer Chains*; John Wiley: New York, 1980.
(37) Herbert, I. R. In *Statistical Analysis of Copolymer Sequence Distribution from NMR Spectroscopy of Polymers*; Ibbett, R. N., Ed.; Blackie Academic & Professional: Glasgow, 1993.
(38) Bovey, F. A. *High-Resolution NMR of Macromolecules*; Academic Press: New York, 1972.
(39) Kasperczyk, J.; Bero, M. *Macromol. Chem.* **1993**, *194*, 913.
(40) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehous, C. M. *Mass Spectrom. Rev.* **1990**, *9*, 37.
(41) Tanaka, K.; Waki, H.; Ido, Y.; Akida, S.; Yashida, Y.; Yashida, T. *Rapid Commun., Mass Spectrom.* **1988**, *2*, 151.
(42) Montaudo, G. *Trends Polym. Sci.* **1996**, *4*, 81.
(43) Montaudo, G., Lattimer, R. P., Eds.*; Mass Spectrometry of Polymers*; CRC Press: Boca Raton, FL, 2002; p 30.
(44) Adamus, G.; Sikorska, W.; Kowalczuk, M.; Montaudo, M.; Scandola, M. *Macromolecules* **2000**, *33*, 5797.
(45) Kowalczuk, M.; Adamus, G. In *Biorelated Polymers: Sustainable Polymer Science and Technology*; Chiellini, E., et al., Eds.; Kluwer Academic/Plenum Publishers: New York, 2001; p 341.
(46) Adamus, G.; Sikorska, W.; Kowalczuk, M.; Noda, I.; Satkowski, M. M. *Rapid Commun., Mass Spectrom.* **2003**, *17*, 2260.
(47) Adamus, G.; Montaudo, S. M.; Montaudo, G.; Kowalczuk, M. *Rapid Commun., Mass Spectrom.* **2004**, *18*, 1436.
(48) Arnould, M. A.; Wesdemiotis, C.; Geiger, R. J.; Park, M. E.; Buehner, R. W.; Vanderorst, D. *Prog. Org. Coat.* **2002**, *45*, 305.
(49) Kowalczuk, M.; Adamus, G. *Polym. Preprints* **2005**, *46* (1), 280.
(50) Montaudo, M. S. *Mass Spectrom. Rev.* **2002**, *21*, 108.
(51) Žagar, E.; Kržan, A. *Biomacromolecules* **2004**, *5*, 628.
(52) Pijpers, T. F. J.; Mathot, V. B. F.; Goderis, B.; Scherrenberg, R. L.; Vegte, E. W. *Macromolecules* **2002**, *35*, 3601.
(53) Ballistreri, A.; Garozzo, D.; Giuffrida, M.; Montaudo, G.; Montaudo, M. S. *Macromolecules* **1991**, *24*, 1231.

BM060201G