NMR analysis and triad sequence distributions of poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

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Abstract. Polyhydroxyalkanoates (PHAs) are considered promising “green” alternatives to synthetic polymers because they are bio-derived, biodegradable and biocompatible. The properties of bacterial PHA copolymers depend on their microstructures, which can be modified with the use of different fermentation processes and feed materials. Thus, it is desirable to have an improved testing method for the determination of PHA microstructures. In this work, a detailed NMR analysis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) microstructure was made. Previously only two of the hydroxyvalerate ¹³C NMR peaks have been assigned at the triad level. In this work, three of the ¹³C hydroxyvalerate peaks and two of the hydroxybutyrate peaks were found to be split into four peaks each due to comonomer sequence effects. Using eight copolymer samples with a wide compositional range, we were able to assign all these peaks to B-centered and V-centered triad sequences. Through curve deconvolution, the triad intensities were determined. These triad sequence intensities can then be analyzed via both the first-order Markovian and two-component Bernoullian models to obtain more in-depth information on copolymer composition and comonomer reactivities.

Keywords: NMR, PHBV, Poly(hydroxyalkanoates), Sequence distribution, Shift reagent, Statistical models

1. Introduction

In recent years there has been continuing interest in agro-based polymers that are biodegradable, eco-friendly, and sustainable as alternatives to synthetic polymers based on petroleum feedstocks [1,2]. The increasing awareness of the problems of single-use plastics and the presence of microplastics in the environment has reinforced the attention towards these materials [3,4]. Among the agro-based polymers, polyhydroxyalkanoates (PHAs) appear attractive [5,6]. These polyesters are derived from bacterial fermentation, typically under nutrient-deficient conditions. They are biodegradable, biocompatible and non-toxic. Currently they are being considered for use in packaging films, food containers, agricultural films and foils, and a range of medical and bioengineering applications [5–8].

The most well-known PHA is poly(β -hydroxybutyrate) (PHB). However, PHB exhibits high stiffness and crystallinity. In order to increase its flexibility and processing capabilities, PHBV, a copolymer of 3-hydroxybutyrate (B) and 3-hydroxyvalerate (V), has been developed [6–8] (Fig. 1). Increasing V content in PHBV reduces the stiffness, melting point, and crystallinity of the resulting polyester. PHAs with longer alkyl chains have also been developed [9–11]. Copolymers of B and higher alkyl hydroxyl esters have also been reported [12,13].

Because the physical properties of PHBV depend on the copolymer composition and comonomer sequence distribution, their analysis has been reported many times in the literature [14–21]. The copolymer composition (amount of B and V units present) can be readily determined by analyzing the NMR spectra in solution [14–16] or in the solid state [17].

Fig. 1. Schematic structure of PHBV and the numbering scheme for the carbons and the protons.

For B and V comonomer sequence distribution, solution ^{13}C NMR is usually used because the ^{13}C peaks show more splitting due to sequence effects. Bluhm et al. [14] and Doi et al. [15] assigned the ester carbons of the ^{13}C spectra to diads (BB, BV, VB, VV), the B2 methylene carbons to diads (BB and BV), and V2 and V4 methylene carbons to V-centered triads (BVB, VVB, BVV, and VVV). In both papers, the sequence distribution of B and V units was found to be approximately random. In addition, Doi et al. [15] and Kamiya et al. [16] also fitted these observed sequence intensities to a first-order Markov statistics, and Kamiya et al. [16] to a mixture model. Later workers [18–21] used the same assignment scheme [14–16], where the BB, BV, VV-diad distribution was extracted from ester peak intensities and the V-centered triad distribution from V4 peak intensities. In addition, the two samples Zagar and Krzan [19] analyzed (and the fractions obtained thereof) showed varying degrees of blockiness in the comonomer distribution. The samples reported by Ghysels et al. [20] were found to be random in comonomer sequence distribution. Ferre-Guell and Winterburn [21] observed better resolved spectra; in addition to V2 and V4 (known to be split to triads), they also reported that V3 and V5 split to two peaks each, which were assigned to diads.

In this work, we have re-examined the ^1H and ^{13}C NMR spectra of eight samples of PHBV with a large compositional range. We were able to achieve good spectral resolution, where four peaks were obtained for B3, B4 and V3 carbons, which were assigned to triad sequences. In other words, instead of the B-diad and V-centered triad analysis as done before, we were able to do the analysis on B-centered *triads* and V-centered triads. These improved assignments (not previously reported) enabled us to provide more precise values for both B-centered and V-centered triad sequence intensities and permitted better fitting of these values to copolymerization models.

2. Materials and methods

2.1. Materials

The PHBV copolymers included a commercial PHBV grade and several non-commercial PHBVs with different hydroxyvalerate content produced by using mixed microbial cultures fed with different industrial by-products such as fermented fruits, dairy waste (cheese whey) and municipal waste water (see Table 1). The commercial grade was ENMATM Y1000P, produced by Tianan Biologic Materials (Ningbo, China) and supplied by Ocenic Resins S.L., Valencia, Spain. The production and purification processes of the non-commercial polymers were described elsewhere [22–24]. Briefly, the PHBV copolyesters were produced using as feedstocks fermented fruits, cheese whey, or waste water. Once the microbial population reached a substantial level, the nutrient composition was changed to force the micro-organisms to synthesize PHBV. By using different cultivation conditions, different PHBV compositions were obtained. For isolation and further purification, the PHBV samples were dissolved in chloroform

at 5 wt % and stirred for 24 h at 50 °C to degrade the non-PHBV cellular material. Each solution was transferred to a centrifugation tube in which distilled water was added at 50 wt %. The tube was shaken manually and centrifuged for 5 min at 4000 rpm. The PHBV suspension was recovered from the bottom of the tube with a pipette and transferred to a beaker, leaving them in the extractor hood until the solvent was completely evaporated. The PHBV sample purity was about 90%.

The expected composition of PHBV in Table 1 was, in the case of the commercial sample, taken from the technical data sheet. In the case of the biowaste-derived PHBVs, they were determined in the biomass prior to purification by gas chromatography (GC) using the method described earlier [25], involving a Varian CP-3800 gas chromatograph equipped with a FID detector and a ZB-WAX plus column (60 m, 0.53 mm internal diameter, 1 µm film thickness, Phenomenex, USA).

Table 1 PHBV samples used in this investigation and composition (V = hydroxyvalerate).

No	Source	Expected V (mol %)	Observed V (mol %)
1	PHBV Commercial	2-3	1
2	Municipal waste water	10	10
3	Fruit Residues	20	15
4	Municipal waste water	20	23
5	Cheese whey	20	15
6	Cheese whey	27	19
7	Cheese whey	40	40
8	Cheese whey	60	60

2.2. NMR analysis

NMR spectra were obtained on an Avance 500 spectrometer from Bruker Instruments (Carlstadt, Germany), using a 5-mm broadband observe probe at 27 °C. Spectra were processed and overlapping peaks deconvoluted using Bruker Topspin 1.3 pl 8 software. Polymer samples were dissolved as 5–20% concentrations in CDCl₃. All chemical shifts were referenced to tetramethylsilane at 0 ppm based on the lock solvent. Standard instrumental conditions were used for ¹H and ¹³C NMR experiments. CDCl₃ was purchased from Cambridge Isotope Laboratories, Andover, MA, USA, and Eu(fod)₃ shift reagent came from Alfa Products, Danvers, MA, USA.

2.3. Model calculations

Fitting of the experimental triad intensities to Markovian statistical models [26–28] and mixture models [29,30] was achieved with QuickBasic programs, as published earlier [26,27]. These were optimized through a simplex algorithm and could accommodate Markovian and Bernoullian reaction probability models and their mixtures. Interested readers may write to the corresponding author for a copy of the program.

3. Results and discussions

In this work, we used several known NMR methodologies, including ¹H and ¹³C NMR, two-dimensional (2D) NMR, lanthanide shift reagent, curve deconvolution, and statistical models, to study the microstructures of PHBV. Eight samples of PHBV were used, including one commercial sample (sample 1), two samples derived from municipal waste water (samples 2 and 4), one sample derived from fruit residues (sample 3) and four samples derived from

cheese whey (samples 5–8) as shown in Table 1. It is interesting to see that the expected biomass composition approximately matches the V determination made in this study, with the exception of the samples with lowest contents of V derived from cheese whey and fruit residues, where the extraction process applied may be affecting the composition. This comparison will be further elaborated in a separate study by the authors.

3.1. NMR analysis of copolymer composition

The 2D HSQC plot for PHBV Sample 8 (V = 60%) is shown in Fig. 2. As expected for microbial PHAs, the spectrum shows only isotactic peaks, indicating stereospecificity during the polymerization process. The ^1H NMR assignments can be made as given in the literature [14,15], with B3 methine at 5.26 ppm and V3 methine at 5.16 ppm, V4 methylene at 1.62 ppm, B4 methyl at 1.27 ppm and V5 methyl at 0.88 ppm. The B2 and V2 methylenes are overlapped at around 2.53 ppm, as shown clearly in Fig. 2, where the two B2 methylenes are shown as non-equivalent and resonating at 2.46 and 2.61 ppm, whereas the V2 methylenes resonate as a cluster near 2.52 ppm. Through the spectral areas of B2, V3, V4, B4 and V5 peaks, the copolymer composition can be determined.

Fig. 2. 2D HSQC Plot of Sample 8 (60% V), peaks labelled according to Fig. 1.

In the NMR literature [31,32], it is common for the two methylene peaks of isotactic vinyl polymers to be non-equivalent. Thus, the observed non-equivalence of the B2 methylenes was not unexpected, but it was not clear-cut that the V2 methylenes were non-equivalent. We added $\text{Eu}(\text{fod})_3$ shift reagent to sample 8 and observed all the ^1H peaks to shift downfield (Fig. S1). Through the HSQC experiment, the ^1H – ^{13}C correlation in the $\text{Eu}(\text{fod})_3$ -shifted spectrum was obtained. From the HSQC data (Fig. S2), it is clear that both B2 and V2 methylenes were non-equivalent. They were also shifted at about the same extent by the shift reagent.

The ^{13}C NMR spectrum of Sample 8 is given in Fig. 3. It can be assigned according to the literature [14,15], and the assignments are noted on the spectrum. The intensities of the peaks V3, B3, B2, V2, V4, B4, and V5 can all be used to determine the copolymer composition. The averages of these values are shown in the last column of Table 1. The compositions obtained from NMR were approximately the same as the expected values (column 2) but some slight deviations were observed, especially for samples 3–6.

Fig. 3. ^{13}C NMR spectra of sample 8 (V = 60%); peaks labelled according to Fig. 1.

3.2. NMR assignments of comonomer sequences

For the determination of comonomer sequences, the detailed ^{13}C spectral assignments are needed. As noted in the Introduction, previous analysis used diad sequences (BB, BV, VB, and VV) from the ester peaks, and V-centered triad sequences from V2 and V4 peaks. In this work, we were able to produce improved resolution of ^{13}C spectra such that both B-centered and V-centered triad sequences could be obtained for all the copolymer samples. For example, the detailed spectral features for all ^{13}C peaks in sample 8 are shown in Fig. 4. (The corresponding expanded spectra for the other seven samples are given in the Supplemental Information.) As reported previously [14–16], V2 and V4 resonances were split into four peaks, corresponding to triad sequences, the ester resonance into peaks that could be assigned to diad sequences, and B2 resonance into two peaks. In addition, in our data we could clearly see B4, B3, and V3 splitting into four peaks. Because we had a large range of copolymer composition, through intensity variations, we could assign all these peaks into the corresponding V-centered and B-centered triads. Through curve deconvolution, we could then determine the intensities of all these sequences.

Fig. 4. Expanded regions of ^{13}C NMR spectrum of sample 8 ($V = 60\%$); black curves are the observed spectra, and red curves are the fitted spectra after curve deconvolution.

It is of interest that in the ester region, the BB diad signal (ca. 169.12 ppm) seems to show further splitting into a doublet. This is most likely due to sensitivity of these peaks to tetrad sequences. Thus, the peak at 169.12 ppm can be assigned to BBBB + BBBV tetrads, and the peak at 169.13 ppm to VBBB + VBBV tetrads. The intensities of the expected tetrad sequences seem to match the expected values.

3.3. Quantitative analysis of NMR data

In the new assignment scheme shown in Fig. 4, the B-centered triads (BBB, BBV, VBB, and VBV) can be obtained from B3 and B4 spectral region, and the V-centered triad (VVV, VVB, BVV, and BVB) can be obtained from V2, V3,

and V4 spectral region. From the areas of these peak intensities, the triad sequence intensities can be obtained. The quantitative triad sequence data for the eight samples of PHA copolymers are summarized in Table 2.

Table 2 Observed triad sequence data (in mole %) for PHBV copolymer samples.

triad	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8
VVV	0.0	0.4	0.6	4.7	1.2	1.9	10.8	30.0
BVV + VVB	0.0	1.8	4.0	8.3	3.5	5.7	17.2	22.2
BVB	1.1	7.5	10.6	9.7	10.4	11.3	12.0	7.8
VBV	0.0	1.8	1.7	3.9	1.4	2.4	7.6	11.0
BBV + VBB	2.0	10.8	24.6	20.3	19.3	26.0	25.0	17.0
BBB	96.9	77.7	58.5	53.1	64.2	52.7	27.4	12.0

An advantage of NMR sequence data is that they can be compared to an appropriate copolymerization model, thereby providing guidance on the nature of the copolymerization. A commonly used model is the terminal copolymerization model (also known as first-order Markovian model) [28,31,32]. These can be characterized by two parameters, P_{BV} and P_{VB} , corresponding to the reaction probability of monomer V reacting with a chain end that terminates in B residue, and the reaction probability of monomer B reacting with a chain end that terminates in V residue, respectively. Moreover, $P_{BB} = 1 - P_{BV}$, and $P_{VV} = 1 - P_{VB}$. The theoretical (model) expressions for the triad intensities are shown in Table 3 (column 2). By varying the P_{BV} and P_{VB} values, the calculated intensities can be fitted to the observed intensities [26,27]. These values, together with reaction probabilities and the product of the reactivity ratios ($r_1 r_2$) are shown in columns 3–11 in Table 3.

Table 3 First order Markovian model expressions and calculated intensities (in mole %) for PHBV samples.

triad	model expressions ^a	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6	sample 7	sample 8
VVV	$kP_{BV}P_{VV}^2$	0	0.1	0.3	4.7	0.3	0.6	10.7	30.0
BVV + VVB	$2kP_{BV}P_{VV}P_{VB}$	0	1.8	4.0	11.6	3.5	5.7	20.1	25.1
BVB	$kP_{VB}^2P_{BV}$	1.0	6.4	11.8	7.1	10.0	12.8	9.3	5.3
VBV	$kP_{BV}^2P_{VB}$	0	0.6	2.3	2.2	1.6	3.0	6.3	8.0
BBV + VBB	$2kP_{VB}P_{BB}P_{BV}$	2.0	13.4	23.1	21.4	20.3	25.2	26.2	19.6
BBB	$kP_{VB}P_{BB}^2$	96.9	77.7	58.5	53.1	64.2	52.7	27.4	12
	Mean deviation	0.02	0.87	0.60	1.45	0.42	0.69	1.35	1.84
	P_{BV}	0.010	0.079	0.165	0.168	0.137	0.193	0.323	0.450
	P_{VB}	0.995	0.876	0.855	0.551	0.852	0.817	0.481	0.295
	$r_1 r_2$	0.45	1.63	0.86	4.03	1.10	0.93	2.25	2.92

$$^a k = (P_{BV} + P_{VB})^{-1}.$$

From the $r_1 r_2$ values, samples 1,3,5 and 6 show roughly random sequence distributions (indicating approximately equal reactivities for B and V), but the two municipal wastewater derived samples (samples 2 and 4) and the two samples with the highest V contents (samples 7 and 8) indicate the presence of blocky structures. It is likely that these samples contain mixtures of distinct polymers. This is entirely possible because PHBVs derived from microbial mixed cultures can lead to molecular architectures that are not totally understood due to the multiplicity of bacterial strains involved in the polymerization process [23].

In order to analyze the NMR data further, the two-component Bernoullian/Bernoullian mixture model [29,30] was attempted. The mixture model has been previously used on PHBV NMR data by Kamiya et al [16]. In this case, the copolymer is assumed to contain two components, each being a random copolymer obeying Bernoullian statistics. This model is characterized by three parameters, w_1 = fraction of component 1, P_{V1} and P_{V2} = Bernoullian probabilities of V addition in components 1 and 2 respectively. Thus, each triad intensity is the sum of the intensities of the corresponding triads for components 1 and 2, weighted by the relative preponderance of each component. The theoretical (model) expressions for this model are given in Table 4 (column 2). By varying these three parameters, the calculated intensities can be fitted to the observed intensities.

Table 4 Two-component (Bernoullian/Bernoullian) mixture model expressions and calculated intensities (in mole %) for selected PHBV samples.

triad	model expressions ^a	sample 2	sample 4	sample 5	sample 6	sample 7	sample 8
VVV	$w_1 P_{V1}^3 + w_2 P_{V2}^3$	0.4	4.7	1.2	1.9	10.8	30.0
BVV + VVB	$2w_1 P_{V1}^2(1 - P_{V1}) + 2w_2 P_{V2}^2(1 - P_{V2})$	1.8	8.3	3.5	6.2	16.3	22.1
BVB	$w_1 P_{V1}(1 - P_{V1})^2 + w_2 P_{V2}(1 - P_{V2})^2$	6.4	9.9	9.8	12.0	12.5	8.3
VBV	$w_1 P_{V1}^2(1 - P_{V1}) + w_2 P_{V2}^2(1 - P_{V2})$	0.9	4.1	1.8	3.1	8.1	11.0
BBV + VBB	$2w_1 P_{V1}(1 - P_{V1})^2 + 2w_2 P_{V2}(1 - P_{V2})^2$	12.8	19.9	19.5	24.1	24.9	16.6
BBB	$w_1(1 - P_{V1})^3 + w_2(1 - P_{V2})^3$	77.7	53.1	64.2	52.7	27.4	12.0
	Mean deviation	0.67	0.16	0.21	0.65	0.34	0.18
	P_{V1}	0.070	0.134	0.130	0.184	0.290	0.352
	w_1	0.958	0.802	0.977	0.973	0.750	0.420
	P_{V2}	0.444	0.610	0.760 ^b	0.757 ^b	0.710	0.786
	w_2	0.042	0.198	0.023	0.027	0.250	0.580

^a w_2 is the fraction of component 2 and is equal to $1 - w_1$.

^b Because of the low values of w_2 in these entries, P_{V2} was allowed to iterate only around 0.7–0.8.

The six samples shown in Table 4 all gave satisfactory fits with observed data. The mean deviation between observed and fitted values is low in each case, indicating the applicability of the two-component model. Since samples 2 and 4 were derived from municipal wastewater microbial cultures, it was not surprising that these two samples contained multiple polymeric components. Samples 5–8 were derived from cheese whey. Samples 7 and 8 clearly showed the presence of two components, each with a distinct P_V value. The NMR data for samples 5 and 6, however, can be fitted to either a single first-order Markovian model (as in Table 3) or a mixture model (as in Table 4). It may be noted that in Table 4 the second component for both samples 5 and 6 contributes only about 2–3% by weight to the overall intensities. Thus, the second component does not contribute significantly in these two samples.

3.4. Future prospects

NMR spectroscopy is among the fields that benefit from advances in physics, electronics, and computer technology. One consistent trend over the years is the development of superconducting magnets with higher and higher magnetic field strengths [33]. An advantage of a higher field is the greater separation of NMR peaks that may be overlapped at lower fields. Thus, some of the triad sequence peaks that are partly overlapped (thereby requiring curve deconvolution to obtain their intensities) may be better separated at higher magnetic fields. Moreover, in our data, we have seen that the ester BB diad resonance shows sensitivity toward tetrad sequences (BBBB, BBBV, VBBB, VBBV). As higher order sequences become resolvable and quantifiable, an even more accurate description of PHBV microstructure may be possible in the future.

4. Conclusions

NMR is a premier technique for the determination of polymer microstructure, but an essential step is to be able to assign the NMR peaks to the microstructures in the polymer in question. In this work, we have made more detailed assignments of the NMR spectra of PHBV copolymers using a range of NMR methodologies. In this way, both B-centered triads and V-centered triad sequence distributions of this copolymer can be obtained. The NMR data have been fitted to statistical models. The same analysis and model fitting can be made with either whole polymers or polymer fractions. As the interest in developing PHA copolymers as commercial products continues, these new NMR findings are expected to be useful in the testing and characterization of the copolymers and a better understanding of copolymerization mechanisms.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

H.N. Cheng: Writing - original draft, Methodology, Software. **Atanu Biswas:** Writing - review & editing, Investigation, Data curation. **Karl Vermillion:** Writing - review & editing, Investigation, Methodology. **Beatriz Melendez-Rodriguez:** Writing - review & editing, Data curation. **Jose Maria Lagaron:** Writing - review & editing, Data curation.

Acknowledgement

The authors would like to thank the Spanish Ministry of Science and Innovation (MICI) through the RTI2018-097249-B-C21 program number and the EU H2020 YPACK (reference number 773872) for funding. B. Melendez would like to thank MICI her FPI fellowship (BES-2016- 077972). A. Biswas acknowledges the receipt of a fellowship from the OECD Co-operative Research Programme: Biological Resource Management for Sustainable Agricultural Systems in 2019. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture; USDA is an equal opportunity provider and employer.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.polymertesting.2020.106754>.

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