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Notes

Fluorescence of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in Solution and Its Dependence on P(HB-*co*-HV) Composition

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Introduction

A wide variety of poly(D-(−)-β-hydroxyalkanoates:) homopolymers and copolymers (PHA) have been isolated from different environmental microbial and nonmicrobial sources.[1,2] Copolymers of β-hydroxybutyrate and β-hydroxyvalerate, P(HB-*co*-HV), have also been produced commercially by *Alcaligenes eutrophus* from propionic acid and glucose by ICI (now by Zeneca, U.K.) under the trade name Biopol.[3] Analytical methods to determine the presence of PHB or PHA in microbial cultures have originally been based on gas-chromatographic analysis,[4] and more recently, the composition of comonomers in poly(D-(−)-β-hydroxyalkanoates) has been investigated by using NMR[5,6] or mass spectrometry.[7] With these latter methods it has also been possible to resolve dyad fractions[6−8] and high level sequences.[7] All these results underline that the monomer sequence distribution in copolymers of P(HB-*co*-HV) follows the random statistics of Bernoulli.[8]

While studying solution properties of PHB, we have observed the presence of fluorescence bands which have not previously been reported in the literature. We have also noticed that the fluorescence properties were a function of the monomer composition in the P(HB-*co*-HV) samples. These fluorescence properties can provide a simple alternative means to determine the monomer composition in P(HB-*co*-HV) samples. It is worth noting that this method is relatively more accessible and easily carried out compared to GC and NMR techniques. Another interesting observation is that chloroform, the most commonly used solvent for PHAs, is unable to manifest fluorescence properties in samples of P(HB-*co*-HV). These polymers are fluorescent in dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and trifluoroethanol (TFE), among other solvents. To the best of our knowledge, such a fluorescence process in poly(hydroxyalkanoates) has not yet been reported and deserves further spectroscopic analysis.

Experimental Section

Polymer Samples. Commercial samples of PHB and P(HB-*co*-HV) were generously provided by Dr. J. F. Stageman, ICI (Billingham, U.K.), and purified by acetone precipitation from the chloroform solution. Two copolymers with HV content greater than 40% were a kind gift from Prof. Y. Doi (Kyoto, Japan) and were used as received; the solid state properties of these samples have already been characterized. The HV content in the copolymers has been measured using ^{1}H-NMR (Table 1).

Table 1. Composition and Molecular Weight of PHB and P(HB-*co*-HV) Copolymers

sample	% HV^a	% HV^b	$10^{-5}M_w{}^a$	$10^{-5}M_w{}^b$
G08	0.0	0.0	5.40	3.40
P019	4.4	4.2	6.90	
P011	16.3	16.3	5.10	2.72
P031	19.7	18.6	4.24	
HV55	55			
HV76	76	73	1.75	

[a] Data obtained by ICI for samples G08, P019, P011, and P031 and by Prof. Y. Doi for samples HV55 and HV76. [b] Data determined in this work.

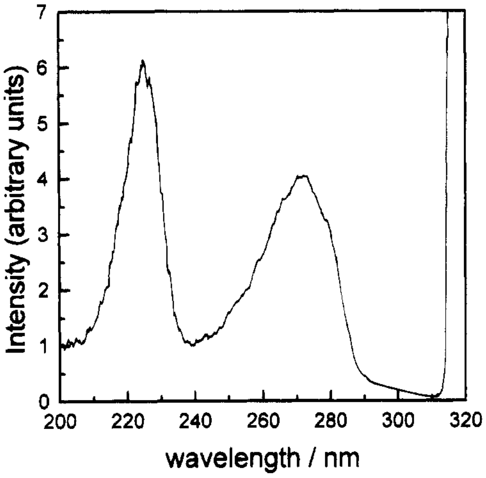

Figure 1. Excitation spectrum of a PHB solution in TFE (0.3 g L^{-1}) obtained with an emission wavelength at 320 nm.

Instrumentation. A JASCO FP-770 spectrofluorometer with a thermostated cell at 25 °C has been used to obtain the fluorescence data in the excitation mode. The experimental setup (e.g., slits of 3 nm) and other conditions (e.g., sensitivity and response time) were selected to optimize the response of the instrument and, in particular, to provide a reproducible signal-to-noise ratio under different concentrations.

Results and Discussion

The excitation spectrum of a dilute solution of PHB (0.3 g L^{-1}) in TFE at 25 °C shows two well-defined fluorescent bands centered at 220 and 270 nm (Figure 1). The choice of TFE as the solvent in this study was mainly dictated by its transparency in UV light. Similar results were also obtained in DMF and DMSO, but not in chloroform solution. However, only the spectral results in TFE could be unambiguously interpreted in terms of an intrinsic property of the copolymer chemical composition, since at 25 °C in other solvents investigated, such as DMF and DMSO, the polymer formed aggregates.[9] At high concentrations these aggregates went through a gel phase which was considered to cause interference with the fluorescence phenomenon. The characterization of the gel phase has also been carried out with several experimental approaches aiming at elucidating the kinetics and the thermodynamics of this process.[9] The extrinsic fluorescence in all these solvents tends to prove that the phenomenon was derived from a property of the polymer itself and not from the gel phase.

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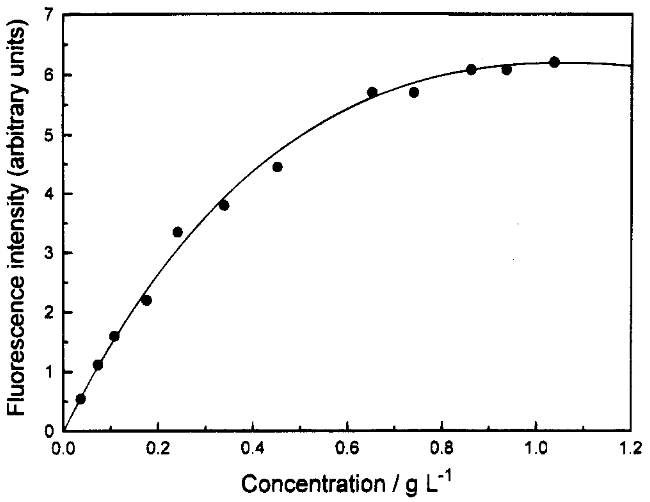

Figure 2. Intensity of fluorescence at 220 nm as a function of concentration for the copolymer with 4.2% HV (sample P019). The curve is calculated by taking into account the self-absorption.

Several experiments of fluorescence at high and low polymer concentrations in TFE (from 10 g L^{-1} down to 0.05 g L^{-1}) were performed. The spectra were recorded in the excitation mode between 320 and 200 nm by setting the emission wavelength at 320 nm. Both the homopolymer and copolymer solutions at high concentrations (10 g L^{-1} in TFE) showed a nonsymmetric high-intensity band at about 270 nm together with a very low-intensity band at about 230 nm. The relative intensity of the two bands is a function of the polymer concentration. When the concentration was decreased to 0.3 g L^{-1}, the intensity of the band at 230 nm, normalized by the concentration, increased much more than that of the band at 270 nm, and in addition, the band at 230 nm showed an apparent detectable hypsochromic shift down to 220 nm. This band at about 220 nm has been assigned to the permitted $\pi \rightarrow \pi^*$ electronic transition of the carbonyl group, while that in the 270 nm spectral range has been assigned to the forbidden $n \rightarrow \pi^*$ transition of the same group.[10] The intensity of this latter band is larger than that expected for a forbidden transition. The hypothesis has to be made that some specific interactions cause an orbital symmetry variation in the electronic transition, giving the transition partially permitted. These interactions may involve the ester group either with the solvent or with another ester group.

The nonlinear dependence of fluorescence intensity as a function of the chromophore concentration is mainly due to the well-known so-called "self-absorption" effect (Figure 2). The experimental intensity can be therefore corrected by this absorption to give a linear dependence with the concentration. The different extinctions of the polyester solution at the two wavelengths brings about a more effective deviation from the linearity for the band at 220 nm. Therefore, at a high polymer concentration the forbidden band appears more intense only because it is less screened by the solution absorption.

As a practical conclusion of the facts reported above, the range of concentration of 0.1–0.3 g L^{-1} of polymer in TFE has been usefully selected for the analytical determination of copolymer composition (typically 0.3 g L^{-1}). The experimental data of fluorescence are reported in Figure 3 as relative intensity, $I_{\mathrm{P(HB\text{-}co\text{-}HB)}}/I_{\mathrm{PHB}}$, *versus* the HV content of the polymer, where $I_{\mathrm{P(HB\text{-}co\text{-}HB)}}$ is the fluorescence intensity of the copolymer solution and I_{PHB} is that of the PHB solution at the same concentration (0.3 g L^{-1}). The higher fluorescence intensity of PHB with respect to all other P(HB-*co*-HV) samples is evident. However, the experimental points do not fit a linear dependence but rather the theoretical F_{BB} *versus* composition curve, where F_{BB} is the fraction of HB–HB dyads in a mere statistical copolymer P(HB-*co*-HV). When the sequence of monomers in the chain is given on a statistical basis and therefore the copolymers are described by the Bernoulli statistics, the frequency of dyads is given by:

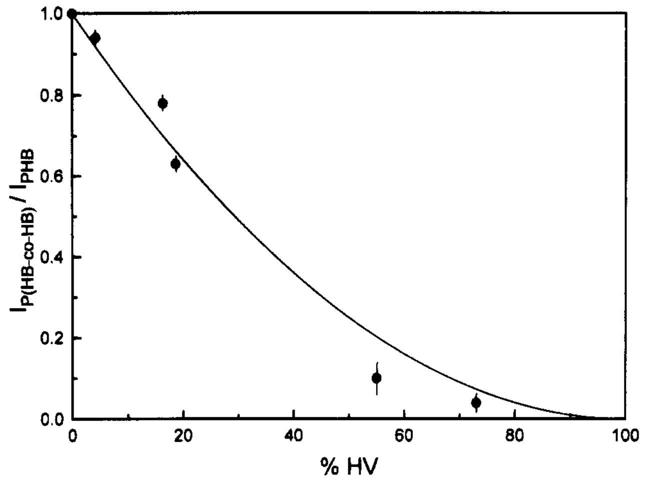

Figure 3. Relative fluorescence intensity $I_{\mathrm{P(HB\text{-}co\text{-}HB)}}/I_{\mathrm{PHB}}$ as a function of % HV of P(HB-*co*-HV) polymers. The error bar is based on the ascertained purity of the samples. The theoretical F_{BB} vs F_{V} curve for statistical copolymers (eq 1a) is also drawn.

$$F_{\mathrm{BB}} = (1 - F_{\mathrm{V}})^2$$

$$F_{\mathrm{BV}} = F_{\mathrm{VB}} = F_{\mathrm{V}}(1 - F_{\mathrm{V}})$$

$$F_{\mathrm{VV}} = F_{\mathrm{V}}^{\,2}$$

where F_{B} and F_{V} are the β-HB and β-HV molar fractions in the copolymers.

It is necessary not only to emphasize the potential of this analytical method but also to discuss the source of possible experimental errors. Firstly, the molecular weight of PHB samples did not affect the fluorescence results, in the range of M_{r} between 1×10^5 and 6×10^5 (Table 1). However, particular consideration for routine quantitative analysis focuses on the determination of the absolute polymer concentration in dilute solution, and at first instance the quality of the data may appear subject to improvement. However, the simplicity of the method and possibility of a direct routine application on small scale microbial cultures is considered of high value, provided that a well-defined protocol is followed.

Although the main purpose of this note is to discuss the phenomenological aspects of the fluorescence of P(HB-*co*-HV), it is worth noting that the homopolymer PHB itself is not fluorescent in chloroform. This absence of fluorescence can arise from the quenching effect of heavy atoms of the solvent. Alternatively, PHB appears to be fluorescent in solvents where chain stiffness and/or aggregation is thought to be increased, as detected, for example, by solution viscosity. It can be postulated that some peculiar interactions of the PHB carbonyl group, either directly with the solvent or indirectly by solvent mediation, play a major role in determining the extent of fluorescence. A specific adduct perturbing the carbonyl group would then be formed within the TFE solutions of PHB, but not within the carbonyl group of PHV or hydroxyvalerate units of P(HB-*co*-HV). Alternatively, dyads of HB units could be responsible for excimer formation either with an intrachain or interchain mechanism. In all cases, the results clearly show a decrease of fluorescence intensity with an increase of HV content in the copolymers, and therefore it must be recognized that the ethyl group (in the β-HV units) has a direct quenching role (e.g., due to steric hindrance or to a change in local conformation), being the perturbation exerted on both the preceding and the following carbonyl group in the chain to the presence of ethyl groups.

Conclusion

The most important original aspects of this paper concern the fluorescence properties exhibited by PHB in solution. Other relevant results of our investigations show the dependence of the fluorescence on the dyad composition of P(HB-*co*-HV). This fact provides a new, simple method to determine copolymer composition. The complex molecular interpretation of these phenomena is presently being investigated.

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