Phase separation within artificial granules from a blend of polyhydroxybutyrate and polyhydroxyoctanoate: biological implications

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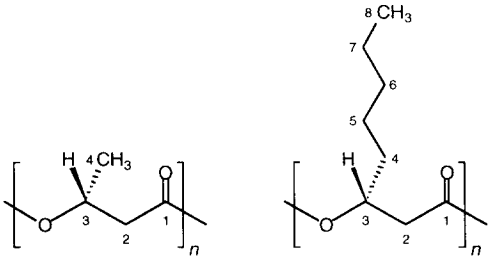
A recently developed *in vitro* procedure for preparing artificial granules of microbial polyesters has been applied to a blend of poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxyoctanoate) (PHO). Density gradient analysis showed that these granules formed a single, uniform population; n.m.r. showed that the polymers retained their amorphous elastomeric state; and thermal analysis indicated that the polymers formed separate phases within these granules. These results contrast strongly with the observation that a recombinant bacterial strain simultaneously expressing PHB and PHO stores the two polymers in separate cellular granules. The implications of the *in vitro* findings for granule formation *in vivo* are discussed.

(Keywords: poly(3-hydroxybutyrate); poly(3-hydroxyoctanoate); artificial granules)

INTRODUCTION

Poly(3-hydroxyalkanoate)s (PHAs) are biologically produced polyesters that have attracted great interest as biodegradable thermoplastics[1,2]. The most widespread of the PHAs in nature—and for many decades the only one known—is poly(3-hydroxybutyrate) (PHB). PHAs are produced *in vivo* in the form of insoluble inclusion bodies or 'granules'. In *Alcaligenes eutrophus*, typical cells contain 8–12 granules averaging 0.25 μm in diameter[3]. The physical state of the polymer granules, which are amorphous *in vivo* but crystallize readily on isolation, has been much debated[4]; it was recently proposed that the amorphous state is the result of the slow nucleation kinetics that often govern the behaviour of small, independent particles[5,6]. In support of this new model, we reported the preparation of artificial PHA granules that are essentially indistinguishable from native granules in size and physical state[7,8]. The artificial granules, in which a detergent or phospholipid layer has been substituted for the native protein/lipid coat, remain amorphous for long periods in aqueous suspension but crystallize rapidly upon disturbance of the protective surface layer.

Although the material properties of PHB are generally similar to polyethylene and polypropylene[9], the polymer degrades thermally at temperatures just above the melting point (180°C), and the crystalline solid undergoes gradual embrittlement[10]. Significant research efforts have thus been dedicated to developing new PHAs for a variety of applications. Some of the difficulties associated with the commercial use of pure PHB homopolymer have been overcome through the inclusion of comonomers such as 3-hydroxyvalerate (HV)[3] or 4-hydroxybutyrate[11], which can be achieved by simple manipulation of the carbon diet in the chemolithotrophic bacterium *A. eutrophus*. The PHB-HV copolymer has been commercially produced by Zeneca Bio Products under the trade name BIOPOL. In the early 1980s it was discovered that *Pseudomonas* bacteria produce a polymer structurally similar to PHB but of lower crystallinity, poly(3-hydroxyoctanoate) (PHO)[12]. Much progress has been made since that time in developing new PHAs from a variety of microorganisms, and the library of known PHA comonomers now includes more than 40 different hydroxyacids.



PHB PHO

In addition, a number of efforts have been made to develop blends of PHB with other, mostly synthetic polymers. PHB has been reported to be wholly or partially miscible with poly(ethylene oxide), poly(vinyl acetate), poly(vinyl chloride), poly(vinylidene fluoride), poly(methyl methacylate) and some esters of cellulose[13,14]. As few if any of these polymers are biodegradable, however, their potential usefulness in widening the applications of PHB is limited. A more subtle approach was taken by Steinbüchel and co-workers, who cloned the PHB biosynthetic genes from the industrial organism *A. eutrophus* into the PHO-producer *Pseudomonas oleovorans*[15]. Rather than elaborating a hydroxybutyrate-hydroxyoctanoate copolymer as might have been hoped,

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however, the recombinant organisms, which contained at least two different active polymerases, produced a blend of PHB and PHO homopolymers. Interestingly, it was found that the two polyesters were stored in separate granules within the producing cells[16].

The ability to prepare biomimetic artificial granules of defined composition provides a new and useful tool for investigating the origins of a number of PHA granule phenomena that have been reported over the years. In the current work, we report the preparation of artificial granules from a blend of PHB and PHO. In contrast to the situation *in vivo*, we find that in the artificial granule case the two polymers coexist within individual granules, evidently within separate phases or compartments. The implications of these findings for granule formation *in vivo* are discussed.

EXPERIMENTAL

General

Aqueously purified PHB ($M_w = 690\,000$; $M_w/M_n = 2.9$; 99.9% pure)[17] was obtained from Zeneca Bio Products (Billingham, UK). PHO, a copolymer comprising 95% 3-hydroxyoctanoate and 5% 3-hydroxyhexanoate, was received from Dr A. Steinbüchel, Göttingen, Germany. Sodium dodecyl sulfate (SDS), cetyl trimethylammonium bromide (CTAB) and Nycodenz [5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N*,*N*′-bis(2,3-dihydroxypropyl)isophthalimide] were obtained from Sigma. Sonications were performed at a frequency of 20 kHz using a Heat Systems-Ultrasonics W-375 fitted with a 0.5 in (13 mm) horn. Particle size measurements were made using a Malvern MS 20 Master Sizer. ^{13}C n.m.r. spectra and wide-angle X-ray scattering patterns were obtained as described previously[8].

Preparation of artificial granules

Chloroform solutions (1.5–10 ml) containing (a) 5% (wt/v) PHB, (b) 5% (wt/v) PHO, and (c) 2.5% (wt/v) PHB, 2.5% (wt/v) PHO were prepared. Twenty volumes of aqueous SDS (50 mM) were layered onto the chloroform polymer solutions and the two layers were emulsified by ultrasonication (power output 200 W, 1–3 min duration), using an ice bath to control sample heating. The emulsions were heated at 75°C in open, magnetically stirred vessels for 90 min to remove the organic solvent. The resulting granule suspensions were stored at room temperature. The granule suspensions used for light scattering studies were prepared as above but 5 mM CTAB was utilized as the detergent.

Nycodenz density gradients

Artificial granule suspensions from above were dialysed against sufficient water to reduce the detergent concentration to ~1.5 mM; the granules were then reconcentrated to ~3 mg ml^{-1} by centrifugal ultrafiltration using Amicon Centriprep 100 concentrators (W. R. Grace, Beverley, MA). The samples (2.5 ml) were then applied to the tops of step gradients prepared from 10, 20, 30 and 40% (wt/v) Nycodenz stock solutions (2.5 ml each). The sealed tubes were placed in a horizontal orientation for 45 min to linearize the gradients and then centrifuged at 110 000 *g* in a Beckman 70.1 Ti fixed-angle rotor for 2 h at 20°C. Gradient profiles and the densities of particle-containing fractions were determined by refractometry using an Abbé refractometer (Bellingham and Stanley) and the literature formula[18].

Thermal analysis

D.s.c. was performed using a Mettler DSC 30 equipped with a Mettler TC11 TA processor. All samples for glass transition measurements were heated from −130 to 50°C at a rate of 20°C min^{-1}. PHB/PHO mixed polymer granules were collected by centrifugation (33 000 *g*, 60 min, 20°C) and measurements were performed directly on the wet pellet (~50% solids). The bulk samples (PHB, PHO, PHB/PHO solvent-cast film), which were partially crystalline, were heated to the melt (2 min at 200°C or 120°C as appropriate) and then rapidly quenched to −130°C prior to glass transition temperature (T_g) determination.

RESULTS AND DISCUSSION

Preparation of artificial granules

Artificial granules were prepared essentially as described previously[8]. Chloroform solutions of PHB, PHO, or a combination of both polymers (total polymer concentration, 5% wt/v) were emulsified ultrasonically with 20 vol. of aqueous surfactant (50 mM SDS or 5 mM CTAB). Chloroform was removed from the emulsions by heating to yield the artificial granule suspensions. Granules were concentrated by centrifugation (33 000 *g*) or centrifugal ultrafiltration as appropriate.

Density and composition of artificial granules

Granules prepared from PHB, PHO, and a combination of the two polymers were analysed by density gradient centrifugation (*Figure 1*). Linear gradients prepared from 0 to 40% Nycodenz (density range 1.01–1.22 g ml^{-1}) were used. Artificial granules prepared from PHB alone (*Figure 1a*) exhibited a density of 1.18 g ml^{-1}, a value close to that of pure amorphous PHB quenched from the melt[19], as previously reported[8]. Likewise granules prepared from PHO (*Figure 1b*) exhibited a density of 1.03 g ml^{-1}, similar to the literature value for the pure polymer[20]. When granules were prepared from a 50:50 blend of PHB and PHO, a single density band of intermediate density (1.10 g ml^{-1}) was observed (*Figure 1c*), indicating the presence of both polymers within individual granules. Finally, a control experiment was performed to exclude the possibility of aggregation between separate PHB and PHO granules (either during preparation or during development of the gradients), which might also have given rise to a single density band. Artificial granules were generated by mixing separately prepared PHO and PHB emulsions and then removing the chloroform from the mixed emulsion by heating. These granules did in fact show two distinct PHO and PHB bands when examined in a Nycodenz gradient (*Figure 1d*).

Artificial granule size and physical state

It was previously shown by a variety of techniques that artificial granules prepared from PHB using the emulsion route described are very similar in size to native PHB storage granules from *A. eutrophus*. Light scattering measurements conducted on artificial granules prepared from a PHB/PHO 50:50 blend (*Figure 2*) confirm that they have essentially the same size distribution as the

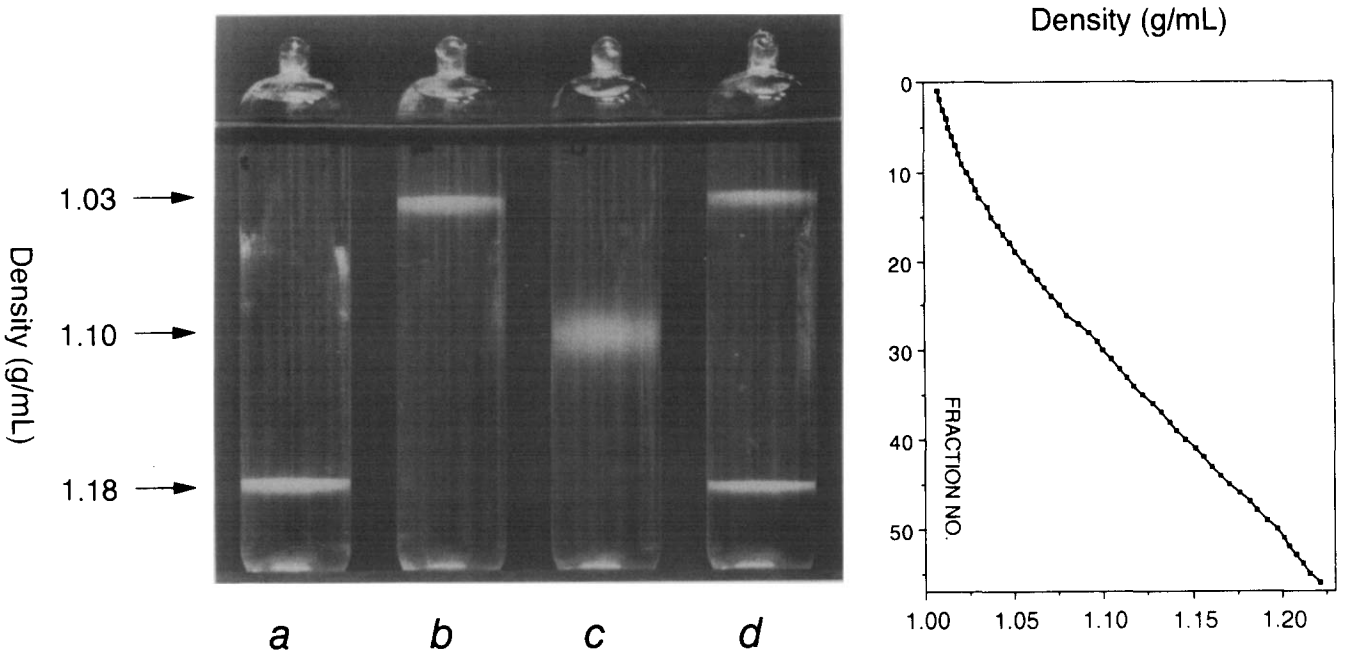

Figure 1 Nycodenz density gradients (0–40% wt/v) of: (a) PHB artificial granules; (b) PHO artificial granules; (c) artificial granules from a 50:50 combination of PHB and PHO; (d) artificial granules from separately prepared PHB and PHO emulsions. Graph on the right shows a typical gradient profile

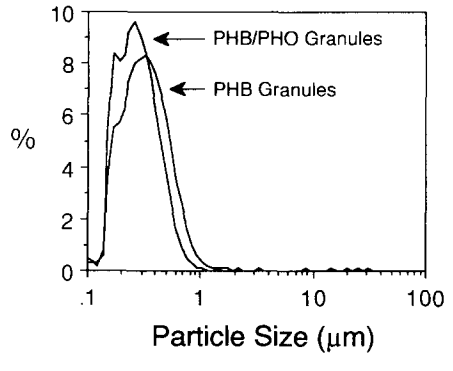

Figure 2 Particle size volume distributions for PHB artificial granules and artificial granules from a 50:50 combination of PHB and PHO, as determined by laser light scattering

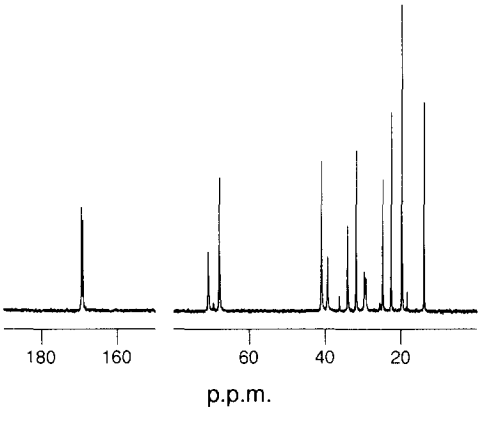

Figure 3 100 MHz solution-state ^{13}C n.m.r. spectrum of an aqueous suspension of artificial granules prepared from 50:50 PHB/PHO, at 70°C. Peak assignments as follows: δ (ppm) 169.5 ($C\text{-}1_{PHB}$), 169.1 ($C\text{-}1_{PHO}$), 70.9 ($C\text{-}3_{PHO}$), 68.0 ($C\text{-}3_{PHB}$), 41.0 ($C\text{-}2_{PHB}$), 39.3 ($C\text{-}2_{PHO}$), 34.0 ($C\text{-}4_{PHO}$), 31.8 ($C\text{-}6_{PHO}$), 24.8 ($C\text{-}5_{PHO}$), 22.6 ($C\text{-}7_{PHO}$), 19.8 ($C\text{-}4_{PHB}$), 13.9 ($C\text{-}8_{PHO}$). Minor peaks are from the detergent (SDS) and a small fraction of 3-hydroxyhexanoate comonomers present in the PHO. Three thousand transients were acquired, and an exponential line-broadening factor of 2.5 Hz was applied prior to Fourier transformation

PHB artificial granules that were studied previously. The median diameter of the PHB/PHO granules of $\sim 0.3\ \mu m$ also appeared to be similar to that of native PHO granules from *P. oleovorans*[12] and of the PHO and PHB native granules from the *Pseudomonas* recombinant expressing both polymers[16].

^{13}C n.m.r. spectroscopy was also used to examine a suspension of artificial granules prepared from a 50:50 blend of PHB and PHO. Elastomeric polymers display a sharp solution-state ^{13}C n.m.r. spectrum at temperatures above T_g, while crystalline solids are invisible. N.m.r. was first used to demonstrate that PHB *in vivo* is amorphous[21] and was later used to show that artificial and native PHB granules have the same physical state and mobility properties[22]. Here, a ^{13}C n.m.r. spectrum at 70°C (above the PHO melting point but well below that of PHB) of the mixed PHB/PHO granules reveals approximately equivalent amounts of the two polymers, both in a highly mobile state (*Figure 3*).

Thermal analysis of PHB/PHO granules

It has been reported previously that PHB is incompatible with PHB-*co*-18%HV at high levels of the copolymer[23], and it was also found that a solvent-cast film and PHB and PHO exhibited two independent melting endotherms[16], suggesting incompatibility in this case as well. D.s.c. thermal analysis of PHB/PHO mixed polymer artificial granules was undertaken to determine whether phase separation had occurred even on the relatively small scale of the granules. It was found that a solvent-cast film of 50:50 PHB/PHO, which was melted and quenched, exhibited two independent glass transitions at −36°C and 5°C (*Figure 4c*), similar to those seen in the two polymers separately (*Figures 4a* and *b*). No

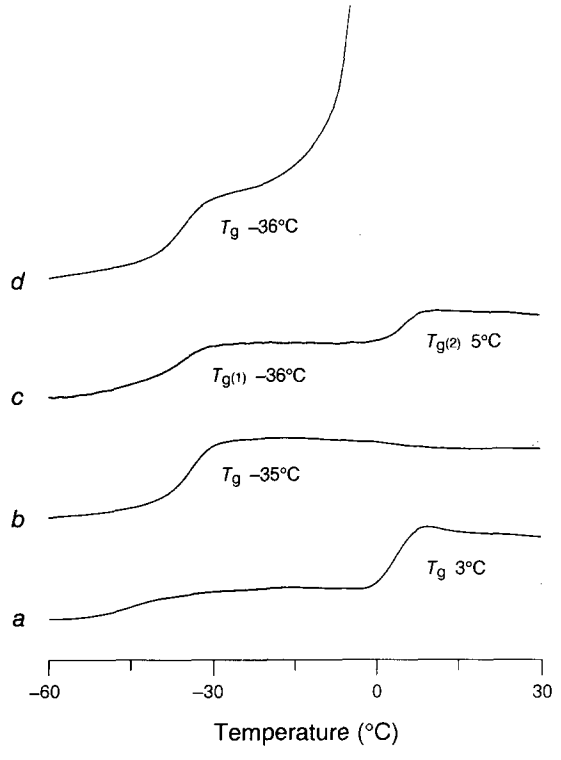

Figure 4 D.s.c. thermograms for: (a) melt-quenched PHB homopolymer; (b) melt-quenched PHO; (c) a melt-quenched film containing 50:50 PHB/PHO; (d) an aqueous slurry of artificial granules prepared from 50:50 PHB/PHO

intermediate glass transition was observed. When an aqueous slurry (~50% solids) of PHB/PHO mixed polymer artificial granules was examined, the PHO glass transition was distinctly visible and its position was unshifted at −36°C (the PHB glass transition was obscured by the large melting endotherm of the residual water).

DISCUSSION

Density gradient analysis of artificial granules prepared from a 50:50 blend of PHB and PHO shows unequivocally that they form a single population having a narrow density distribution. The density observed for these mixed polymer granules was essentially the average of the known densities of PHO and amorphous PHB. The amorphous state of the PHB within the granules was further confirmed by ^{13}C n.m.r. spectroscopy, which showed amorphous PHB and PHO in similar amounts. The results of thermal analysis demonstrate that PHB and PHO are incompatible polymers, showing two independent, unshifted glass transitions after quenching from the melt. The observation of an unshifted PHO glass transition at −36°C in artificial granules from a PHB/PHO blend suggests that the polymers exhibit nanoscale phase separation within individual granules. The preparation of similar biphasic polymer microspheres from synthetic polymers has recently been reported; in that case engulfment of one polymer by another incompatible polymer occurred spontaneously on solvent removal[24].

These results show that PHB and PHO, in spite of their incompatibility and substantially differing densities, are capable of coexisting within submicrometre-size particles in aqueous suspension. Both polymers are initially present together in chloroform solution; upon removal of the solvent from emulsified droplets of this solution, the two polymers are forced together in single 'granules'. Strong centrifugal forces, in excess of 100 000 *g*, did not result in the scission of the non-crystalline granules into their constituent polymers. These findings suggest that if mixed polymer granules were to form *in vivo*, they would be stable and would retain the biologically competent amorphous state. The observation of separate populations of pure PHB and PHO granules *in vivo* thus reflects a specific biological property of granule formation, rather than a physical property of the two polyesters involved.

It has recently been suggested that the formation and growth of polyester storage granules in bacterial cells resemble a chemical emulsion polymerization[25]. In particular, it was argued that in the first stages of granule formation, soluble PHA polymerase molecules acquire surfactant-like properties as the result of covalent attachment to newly synthesized hydrophobic PHA. These 'surfactant' molecules self-associate to form micellar structures, which are then enlarged through further polymer biosynthesis to form full-sized granules. If this model is accurate, then the production of two granule populations *in vivo* in the recombinants is likely to be the direct result of the nanoscale polymer incompatibility observed in the current work. In the cytoplasm of the recombinant pseudomonads, *A. eutrophus* PHA polymerase enzymes with covalently attached PHB will tend to self-associate with each other but not with the host PHA polymerases, which are specific for medium-chain length hydroxyacids and will carry covalently attached PHO. The composition of future granules is determined in this early stage.

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