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A natural-synthetic hybrid copolymer of polyhydroxyoctanoate-diethylene glycol: biosynthesis and properties

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Abstract

The bacterium *Pseudomonas oleovorans* was utilized to produce a novel natural-synthetic hybrid block copolymer. This hybrid biomaterial consists of the natural hydrophobic polymer polyhydroxyoctanoate (PHO) end-capped with hydrophilic synthetic diethylene glycol (DEG). The addition of the synthetic component, in the form of PEG106, to the bioprocessing stage significantly affected polymer yield and properties compared to its non-hybridized counterpart. Compared to PHO polymer, the PHO–DEG hybrid possessed a significantly higher C8 component and reduced molecular mass. In contrast, thermal and crystalline properties were similar in both polymer types. While the molecular weight ratio of the natural and synthetic blocks is approximately 1000:1, the small hydrophilic component exerted a powerful influence during polymer processing. Under conditions of high humidity, solvent cast films of the PHO–DEG hybrid self-assembled into ordered microporous arrays. Thus, a novel natural-synthetic hybrid comprized of biocompatible components has been produced.

Keywords: Polyhydroxyalkanoate; Diethylene glycol; Microporous films

1. Introduction

Polyhydroxyalkanoates (PHAs) are a family of biopolyesters synthesized by a wide range of microorganisms [1]. Under conditions of nutrient stress with an environmental excess of carbon PHAs are accumulated intracellularly and function primarily as carbon and energy reserves [2,3]. Fig. 1 shows the general structure of β-linked PHAs. The versatility of the PHA biosynthetic pathway in utilising a diverse range of carbon substrates has been explored extensively and utilized to synthesize a wide range of PHAs with over 100 different monomeric components [4]. These novel biopolymers have material properties ranging from rigid and highly crystalline to flexible, rather amorphous and elastomeric [1,2,5].

Poly-β-hydroxybutyrate (PHB) is the most studied member of the PHA family and is a homopolymer comprized of the monomer β-hydroxybutyric acid (HBA). HBA is also one of the ketone bodies produced in mammalian systems suffering starvation or diabetes mellitus, during which it is a vital source of carbon to the brain. Microbial HBA is chemically identical to its mammalian counterpart and is processed by biological systems [6]. As a consequence of its biocompatibility; PHB saw commercialisation in the middle of the twentieth century as a biomaterial for medical implantation devices [7].

PHB, however, has a relatively high crystallinity and is brittle when processed through various melt crystallisation or solvent casting techniques [8–10]. Such properties also influence its degradation behaviour in vivo and its ability to successfully serve as a biomaterial. The physicomechanical properties, and degradation behaviour, of PHB were modified by its copolymerisation with hydroxyvalerate to yield PHB-*co*-HV, leading to its application as an environmentally friendly bioplastic [11,12].

In an effort to improve the physicomechanical properties of PHB, Gross and co-workers manipulated its molar mass through the addition of polyethylene glycol (PEG) during the bioprocessing stage [13–16]. PEG was shown to affect

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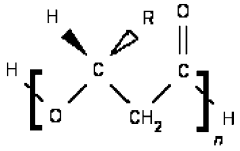

Fig. 1. Chemical structure of β-linked polyhydroxyalkanoates.

cell growth, viability and activity, as well as polymer yield and composition. These parameters were influenced by the molecular weight of the PEG, its concentration and its effects on the PHB producing microbial species [13–18]. In addition, Gross and co-workers report the potential for microbial species to incorporate PEG units at the terminal of the PHB chain, a phenomenon they named 'PEGylation' [13].

The family of PEGs has a wide range of members varying in molecular mass and structure. A number of biocompatible members have important biomedical functions such as organ and tissue preservation [19]. Material surfaces modified by covalent attachment of PEG chains are resistant to cell and protein adsorption and have potential as biomaterials [20,21].

In contrast to crystalline PHB, the flexible and amorphous nature of Polyhydroxyoctanoate (PHO), another member of the PHA family, makes it more suited as a biomaterial [7]. Thus, a biocompatible natural-synthetic hybrid of PHO and diethylene glycol (DEG, PHO–DEG) may possess unique properties for biomedical applications. In this report we demonstrate the biosynthesis of such as hybrid copolymer and illustrate the influence of 'DEGylation' on its material properties.

2. Experimental section

2.1. Materials

Octanoic acid and diethylene glycol (DEG, PEG106) were purchased from Sigma-Aldrich, (Sydney, Australia). All other chemicals were of at least 98% purity and obtained from APS Chemicals, (Seven Hills, Australia).

2.2. Bacterial strains, inoculum preparation and growth conditions

Stock cultures of the bacterium *Pseudomonas oleovorans* (ATCC 29347) were stored at −10 °C from which working cultures were obtained monthly. The strain was maintained through successive subculture onto fresh agar modified E* medium plates containing 20 mM octanoic acid and stored at 4 °C [22]. Modified E* medium consisted of: $(NH_4)H_2PO_4$, 5.94 g/L; K_2HPO_4, 5.8 g/L; KH_2PO_4, 3.7 g/L; 100 mM $MgSO_4$, 20 mL/L and micro-element solution, 1 mL/L. The micro-element solution contained 2.78 g/L $FeSO_4 \cdot 7H_2O$, 1.98 g/L $MnCl_2 \cdot 4H_2O$, 2.81 g/L $CoSO_4 \cdot 7H_2O$, 1.67 g/L $CaCl_2 \cdot 2H_2O$, 0.117 g/L $CuCl_2 \cdot 2H_2O$ and 0.29 g/L $ZnSO_4$ dissolved in 1 M HCl.

P. oleovorans was grown aerobically in a 500 mL baffled Erlenmeyer flask containing 100 mL modified E* medium and 20 mM octanoic acid (30 °C, 200 rpm). This was used as a pre-seed culture and during late growth phase inoculated four seed flasks with the same conditions to that of the pre-seed. Four hundred milliliter of these seed cultures were used as an inoculum for a 5 L fermentor containing 3.6 L of modified E* medium and 20 mM octanoic acid (final operating volume 4L, Braun Biostat, Braun Scientific, Germany). The same protocol was simultaneously carried out with the addition of 2% (v/v) PEG106 to each seed flask. Inoculation of both 5 L fermentors proceeded with similar total cell numbers.

2.3. Polymer extraction

The intracellular polymer was extracted from the lyophilized cell mass through chloroform and purified by repeat methanol precipitation. During fermentation, duplicate volumes (3 mL) were sampled periodically. Viable cell numbers were determined in triplicate using a standard spread plate technique. Cells were harvested by centrifugation (6000 g, 15 min) and washed in sterile fresh medium with no carbon sources. The biomass was lyophilized and the dry cell weight calculated. The supernatants were used to determine concentrations of carbon substrates octanoic acid and PEG106.

2.4. Gas chromatography-mass spectrometry

Polymer concentration was determined after methanolysis of the biomass by gas chromatography-mass spectrometry (Hewlett Packard, PA, USA). The GC was equipped with a BP5 capillary column (SGE, Sydney, Australia) and Helium was used as carrier gas (1.2 mL/min). The oven was maintained at 90 °C for 1 min prior to a rise to 160 °C at a rate of 7 °C/min. The temperature was held for 1 min, and then raised at 20 °C/min to 250 °C, which was subsequently maintained for 5 min. 1.0 μL injections were made with a split ratio of 10:1. The mass spectrometer was autotuned with perfluorotributylamine (PFTBA). Polymer composition was identified and quantitated based on their retention times against known standards and their *m/e* values (103, 74, and 71).

GC-MS was also used to quantitatively monitor the concentrations of octanoic acid and PEG106 in supernatant samples and washings after a clean-up stage. In addition, the aqueous phase from the methanolysis protocol was also examined to determine the presence and concentration of intracellular PEG. Sample pHs were adjusted below the pKa for octanoic acid (4.89) so that all octanoate molecules were protonated prior to addition to Supleclean LC-18 SPE tubes

(Supelco, Sydney, Australia). Undesired salts from the medium were thus removed before elution of analytes with methanol.

Octanoic acid and PEG106 were quantitatively monitored by GC-MS using a BP21 (SGE) capillary column. The temperature profile was as follows: 85 °C rising at 10 °C/min to 160 °C, 5 °C/min to 180 °C then 10 °C/min to a final temperature of 220 °C. 1.0 μL injections were made with a split ratio of 10:1.

2.5. Polymer characterisation

Confirmation of hybrid synthesis through PEGylation was conducted using ^{1}H and 2D NMR spectroscopy (^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC). Polymer samples were dissolved in deuterated chloroform (ca. 4 mg/mL) and examined using a Bruker DMX600, operating at 600.13 MHz for ^{1}H and 150.92 MHz for ^{13}C. ^{1}H spectra were recorded with a pulse width of 4.5 μs (45° pulse), a spectral width of 6.6 kHz, an acquisition time of 2.5 s, a relaxation delay of 6 s with 64–256 scans for required signal-to-noise and referenced internally to chloroform (7.26 ppm with respect to tetramethylsilane).

GPC analyses of polymers were performed using an LC-10ATVP Shimadzu solvent delivery system combined with a SIL-10ADVP Shimadzu auto-injector possessing a stepwise injection control motor (accuracy of ± 1 μL), a column set consisting of a PL 5.0 μm bead size guard column and a set of 3–5.0 μm PL linear columns (10^3, 10^4, 10^5 Å) kept at a constant 40 °C inside a CTO-10AC VP Shimadzu Column Oven and an RID-10A Shimadzu Refractive Index Detector. Tetrahydrofuran (THF) was utilized as the continuous phase at a flow rate of 1 mL/min. A calibration curve using polystyrene standards of low polydispersity (<1.1) was determined.

Thermal properties of the polymers were investigated using DSC (Perkin–Elmer Model DSC-7). 7 mg samples were heated at the rate of 20 °C/min from −80 to 127 °C [23]. The data obtained were used to calculate the glass transition temperatures (T_g), melting point (T_m) and fusion enthalpies (ΔH_f). Means of 3 samples were determined. X-ray diffraction analysis of solvent cast films was performed using a Siemens Diffractometer D5000 generator operating at 30 kV and 30 mA. Monocrom K_α radiation ($\lambda = 1.5418$ Å) was used.

2.6. Microporous film fabrication

Thin films of PHO and PHO–DEG were fabricated through complete evaporation of chloroform under clean conditions. Microporous films of PHO–DEG were fabricated under similar conditions with the addition of humid air flow (85% rH, 5 mL/min airflow). Films were examined using scanning electron microscopy.

3. Results and discussion

3.1. PHO and PHO–DEG hybrid biosynthesis

Fermentation of *P. oleovorans* in modified E* medium with 20 mM octanoic acid produced a maximum dry cell concentration of approximately 2.3 ± 0.05 g/L. In contrast, the addition of 2% (v/v) PEG106 to the medium significantly reduced the cell mass (1.9 ± 0.05 g/L, Fig. 2(a)). More importantly for polymer synthesis, cell viability monitored as colony-forming units (Log No./mL), was also affected by the PEG106 addition. In the presence of PEG106 cell viability substantially decreased from $1.93 \pm 0.02 \times 10^{10}$/mL to $2.37 \pm 0.02 \times 10^{9}$/mL (Fig. 2(b)). These results contrast with the work of Ashby et al. [17] and Kim [18] who reported negligible changes in cell masses and viability in the presence of PEG200 and PEG400.

As a consequence of the reduction in cell mass and viability, polymer yield was significantly reduced (Fig. 2(c)). In the fermentation for the production of PHO, GC-MS analysis of the washed, methanolysed biomass showed a polymer yield of approximately 0.38 ± 0.003 g/L. In the production of the PHO–DEG hybrid this yield was reduced by 34% (0.25 ± 0.008 g/L). Despite the reduction in cell and polymer masses during fermentation of *P. oleovorans* in the presence of PEG106, the utilisation of octanoic acid substrate was similar to that of the cells in its absence (Fig. 2(d)). Thus, efficiency of the system was also reduced.

PEG106 was quantitatively monitored in the medium using GC-MS. Fig. 3 clearly shows a decrease in PEG106 concentration from 20 to 13.5 ± 0.05 g/L within the first 8 h of the bioprocess. This period coincided with the almost complete utilization of octanoic acid and maximum hybrid yield (Fig. 2(c)). The decrease in PEG106 was not due to hydrolytic degradation as an uninoculated control showed negligible decrease over a similar duration (Fig. 3). Furthermore, GC-MS analysis of the cell washings demonstrated that PEG106 was also associated with the outer cell surface. The concentration of DEG associated with the cell mass increased from approximately 0.04 ± 0.05 g/L at the initiation of fermentation to 0.55 ± 0.05 g/L after 6 h incubation before reducing (Fig. 3). PEG106 was also detected in the aqueous fraction of the methanolysed biomass. Thus, PEG106 was adsorbed onto the cell surface and penetrated into the cell.

3.2. Polymer characterisation

In addition to affecting the growth and viability of *P. oleovorans*, the addition of PEG106 to the medium also had a significant influence on composition of the PHA. *P. oleovorans* utilized the octanoic acid to synthesize a PHA with an alkyl substituent of medium chain length (mclPHA). This mclPHA possessed three monomeric components with the major constituent hydroxyoctanoate (HO-C8, 84.5%) and relatively minor components hydroxyhexanoate

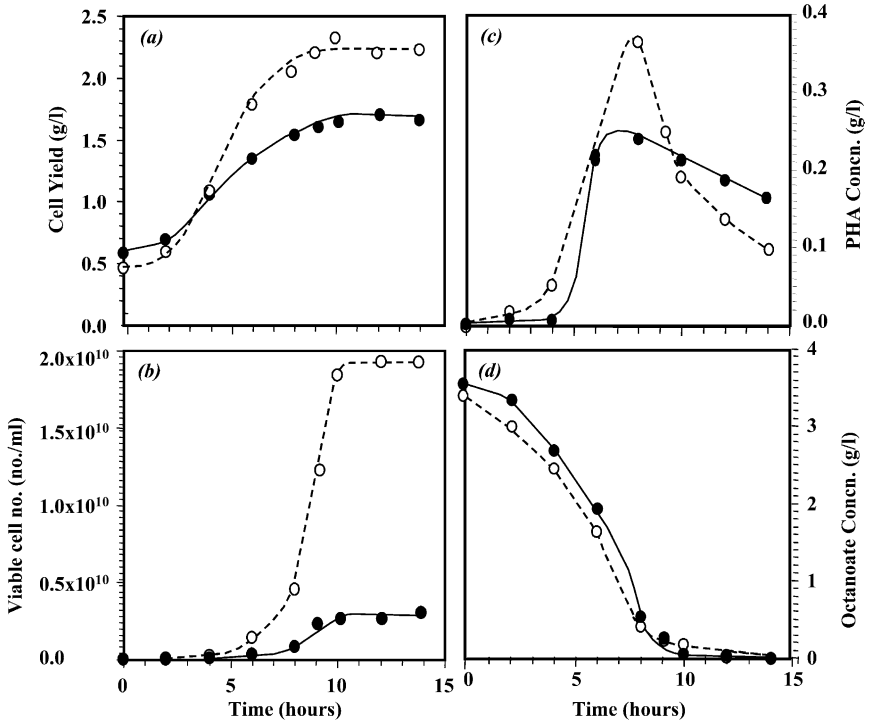

Fig. 2. Comparison of fermentations of *P. oleovorans* with 20 mM octanoic acid in the absence (○) and presence of 2% (v/v) PEG106 (●): (a) dry cell concentration in g/L, (b) viable cells in Log No./mL, (c) PHA concentration in g/L, (d) octanoic acid concentration in medium (g/L).

(HX-C6, 11.2%) and hydroxydecanoate (HD-C10, 4.3%). The presence of intracellular PEG106 as determined by GC-MS, resulted in a noticeable shift towards the C8 component (95%, Table 1). These results contradict those regarding mclPHA production in *P. oleovorans* cultivated with slightly higher molecular weight PEG200 [15,18].

Purified polymer samples from the PEG106 modulated fermentation were examined using ^{1}H NMR spectroscopy (Fig. 4). The spectrum shows a strong methine signal at 5.2 ppm and the signal for the main chain methylene protons adjacent to the chiral carbon between 2.4 and 2.6 ppm. The

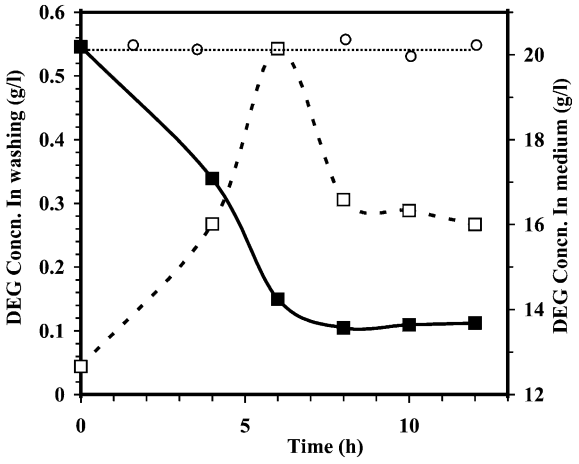

Fig. 3. Concentration of PEG106 in uninoculated fermentation medium (○) and inoculated with *P. oleovorans* (■); concentration of PEG106 removed from cell mass by washing in sterile media (□).

methylene protons of the side chains (1.2 and 1.4 ppm) and the terminal methyl groups in the side chains (0.9 ppm) are also readily observed (Fig. 4). These signals are consistent with the overlap of peaks anticipated for the mclPHA as determined by GC-MS.

The ^{1}H NMR spectrum also showed signals at 3.6, 3.7 and 3.74 ppm, corresponding to protons of PEG without internal repeating unit, i.e. DEG (PEG106). A signal at 4.2 ppm correlating to a covalent bond between DEG and PHO was also readily observed, strongly suggesting the termination of the mclPHA chains with DEG units. This contrasts with cultivations of *P. oleovorans* in the presence of PEG200 and PEG400 which failed to show covalent bonding of PEG units, i.e. no PEGylation was observed. Assuming Bernoullian characteristics, the relative intensity of peaks for protons 'B:D:C' was 1:1:1, indicating that the hybrid sample contained no unbound PEG106 (Fig. 4).

Confirmation of the 'DEGylation' of PHO chains was sought from 2D NMR correlation experiments. ^{1}H–^{1}H COSY and ^{1}H–^{13}C HSQC experiments confirmed the covalent linkage between terminal units of mclPHA and DEG. The HSQC spectrum (Fig. 5(a)) confirms the link to ^{13}C sites of the DEG moiety, while the COSY spectrum (Fig. 5(b)) clearly demonstrates that the four sets of peaks in the 3.6–4.3 ppm region are linked together as two ethylene units.

A number of authors have reported reductions in the molecular masses of PHAs with microorganisms cultivated in the presence of various PEGs [13–18]. Consistent with these reports, PHO–DEG synthesized by *P. oleovorans* also

Table 1
Composition and molar masses of PHAs produced by *P. oleovorans* in the presence and absence of 2% (v/v) PEG106

PHA sample	Composition (mol%)			M_w	M_n	PDI	T_g (°C)	T_m (°C)	ΔH_f (J/g)
	C6	C8	C10	($\times 10^3$)[a]					
PHO	11.2	84.5	4.3	235	142	1.65	−36	55	24
PHO–DEG	4.3	95.0	0.7	100	77	1.30	−36	55	31

DEG was added to microbial growth medium prior to inoculation.
[a] Molar masses were determined by GPC.

showed significant reductions in its molecular weight, number and polydispersity (M_w, M_n, PDI), when compared to PHO produced by *P. oleovorans* in the absence of PEG106 (Table 1). Thus, *P. oleovorans* was utilized to produce a natural-synthetic hybrid copolymer of PHO–DEG, which had a noticeably different composition of monomeric units and a significantly reduced molecular mass when compared to its non-hybridized counterpart, PHO.

Despite the change in composition and molecular mass, PHO–DEG showed negligible difference in its thermal properties when compared to PHO (Table 1). The melting point (T_m) and glass transition temperature (T_g) remained the same, around 55 and −36 °C, respectively. A slight difference in the fusion enthalpy (ΔH_f) may have been related to the presence of a sharp peak around −1 °C, (Fig. 6). This peak is consistent with the T_m for water and suggests that the hydrophilic nature of the hybrid may have adsorbed water more readily than PHO. This is supported by the work of Lee et al. [24], reporting an increase in mclPHA hydrophilicity with hydroxylation in the side chain. The

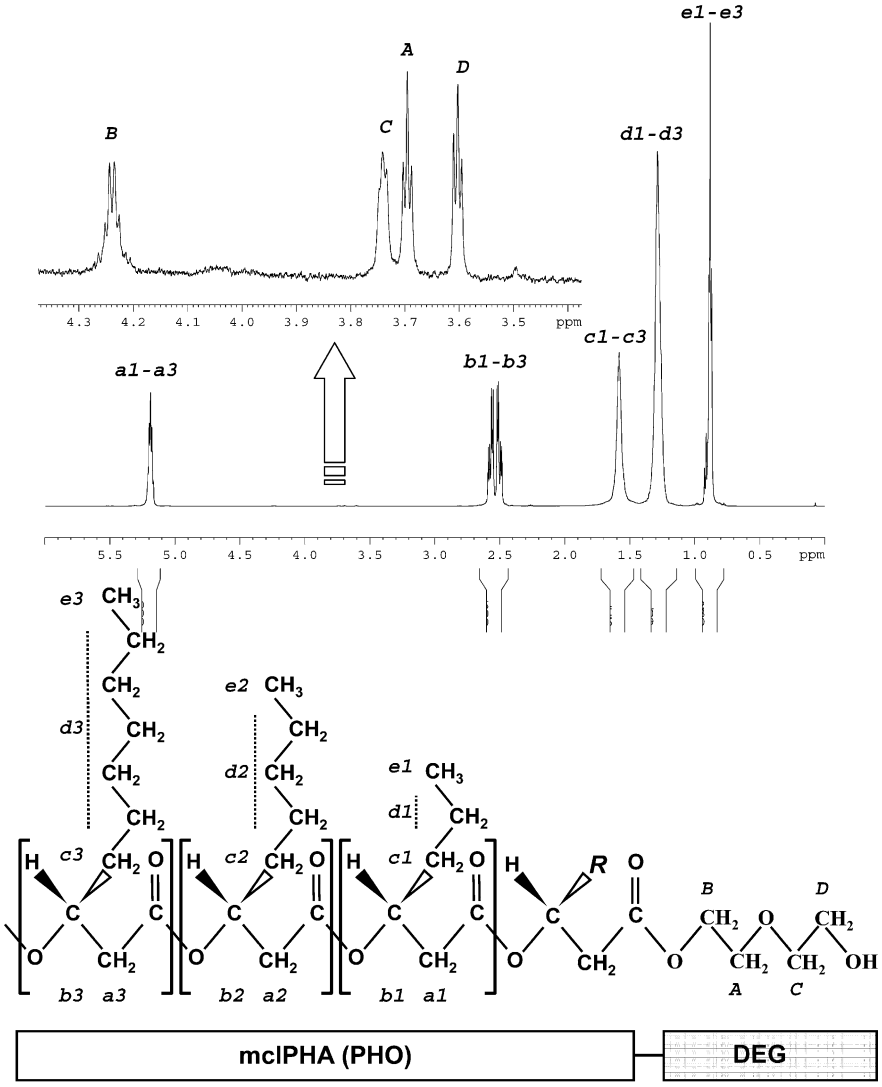

Fig. 4. 600 MHz ^{1}H NMR spectra of mclPHA purified from DEG modulated fermentation of *P. oleovorans*.

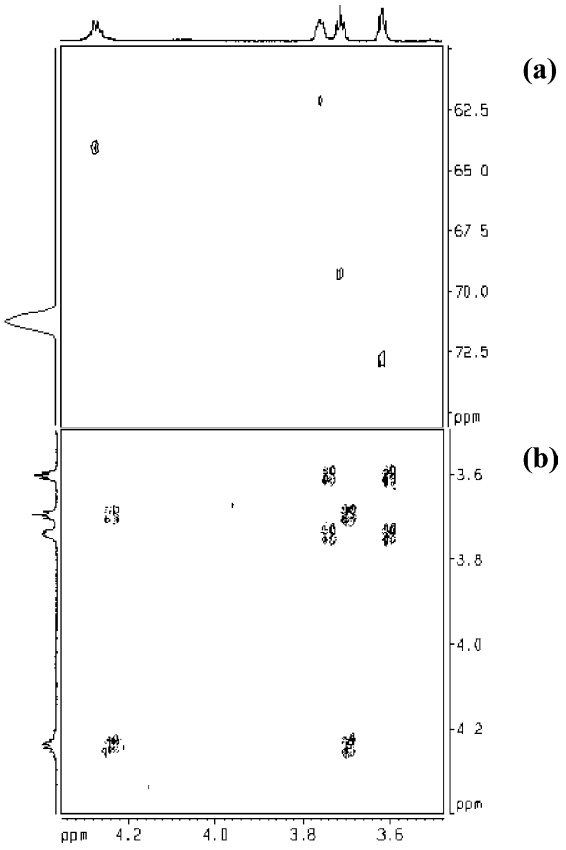

Fig. 5. 600 MHz NMR spectroscopy of PHO–DEG hybrid, (a) ^{1}H–^{13}C HSQC spectrum showing the expansion of the region 3.5–4.3 ppm and 60–75 ppm (b) 600 MHz ^{1}H–^{1}H COSY spectrum showing the expansion of the region 3.5–4.3 ppm.

PHO–DEG hybrid also showed a similar X-ray diffractometry pattern as that obtained for PHO, (Fig. 7). Interplanar *d*-spacings were similar in both cases, except that those for the hybrid were found to be slightly larger (Table 2). These diffraction maxima are consistent with those previously reported for PHO as produced by *P. oleovorans* using sodium octanoate as carbon source, with a noticeable exception; in the study reported here the PHO

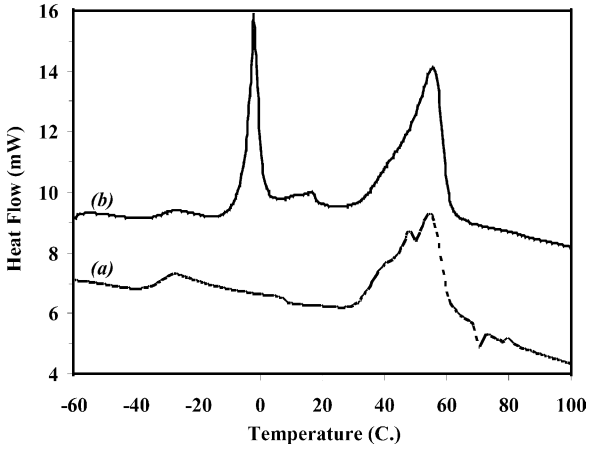

Fig. 6. DSC thermographs of hydrated PHO–DEG hybrid (a) and PHO (b).

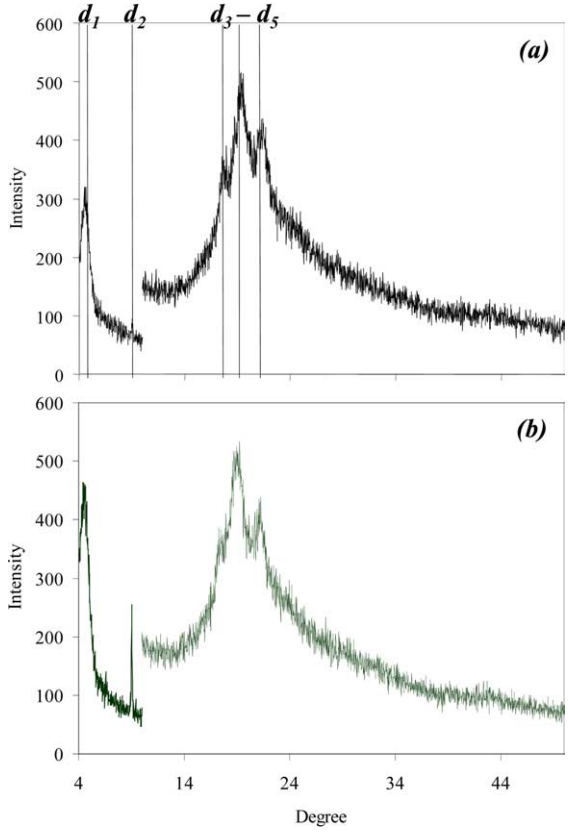

Fig. 7. X-Ray diffraction spectra for solvent cast films of PHO (a) and PHO–DEG hybrid (b).

exhibited a diffraction maximum at 9.82 Å, although its intensity was weak. In contrast, the PHO–DEG hybrid showed a strong diffraction maximum at 9.86 Å (Table 2 and Fig. 7) [23].

3.3. Fabrication of microporous films

The slight differences in crystalline structure between PHO and PHO–DEG, as determined by X-ray diffractometry, were not readily observable using electron microscopy. Fig. 8(a) shows the surface morphology of PHO film cast through evaporation of chloroform with only slight differences to a film of PHO–DEG produced in the same fashion (Fig. 8(b)). However, in addition to PHA composition, the physicomechanical properties of PHA based devices can be affected by their processing [10]. In an effort to investigate whether the relatively small hydrophilic component of the hybrid could be utilized to manipulate morphology and hence physicomechanical properties, solvent cast films of PHO and PHO–DEG were fabricated under conditions of high humidity air flow. The presence of water in the form of condensed 'breath figures' on the solvent surface may have supported the orientation of the hydrophilic DEG component [25]. As a consequence of this process, the solvent cast hybrid film displayed an ordered

Table 2
X-ray diffraction maxima for PHO and PHO–DEG produced by *P. oleovorans*

PHA sample	Diffraction maxima (A)				
	*d*1	*d*2	*d*3	*d*4	*d*5
PHO	19.13	9.82	5.01	4.57	4.17
PHO–DEG	19.72	9.86	5.09	4.68	4.21
PHO[a]	18.40	–	5.01	4.54	4.09

[a] Data from Ref. [23].

microporous appearance (Fig. 8(c) and (d)). Nishikawa et al. [26] have reported the fabrication of microporous films from amphiphillic polymers that were used as a matrix for the immobilisation of biomolecules. Furthermore, the authors report that these films were found to increase the adhesion of bovine aorta endothelial cells compared to their corresponding non-porous counterparts. For biocompatible polymeric materials, the tissue–polymer interface, where cellular adhesion occurs is of paramount importance, with biocompatibility dependent on surface morphology [27–29]. Thus, microporous films fabricated from a hybrid of biocompatible natural and synthetic components has enormous technological potential.

4. Conclusions

We have successfully utilized *P. oleovorans* to bioengineer a natural-synthetic hybrid copolymer consisting of the mclPHA, PHO, terminated with units of DEG. Compared to the microbial production of PHO, the presence of DEG (PEG106) in the fermentation reduced polymer yield and efficiency of the bioprocess. Furthermore, in addition to DEGylation of the PHO chain, composition of the PHO in the hybrid was also modified. The PHO–DEG hybrid exhibited similar thermal and crystalline properties to PHO, but showed a molecular weight less than 50% that of the non-hybridized PHO. While the molecular weight ratio of

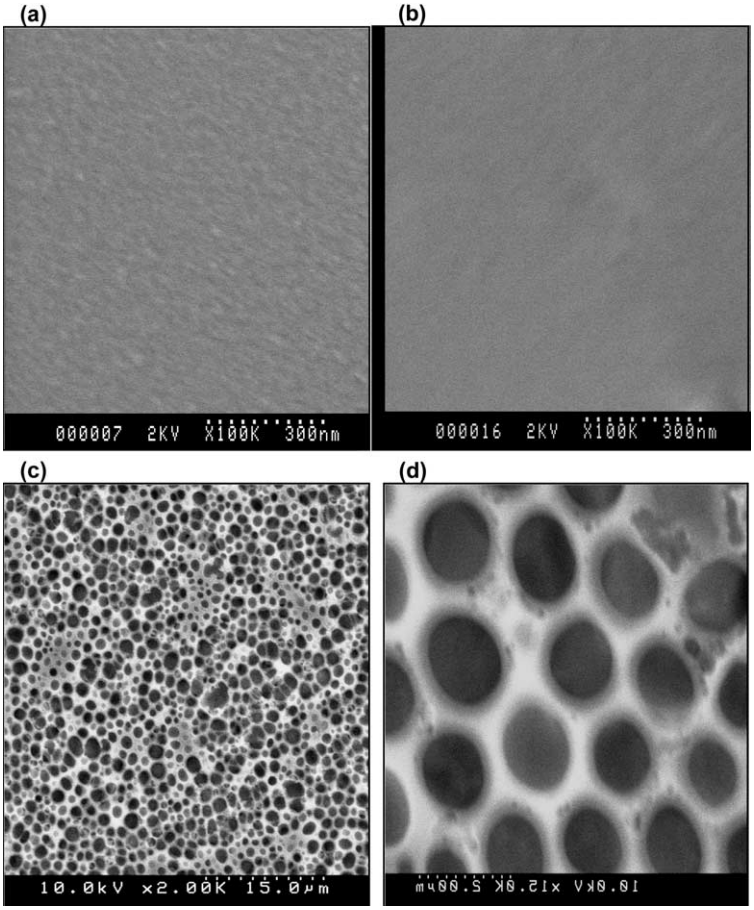

Fig. 8. Scanning electron micrographs of solvent cast films of PHO (a), PHO–DEG (b) and SEM of microporous films of PHO–DEG fabricated under humid conditions (c) and (d).

the natural PHO and synthetic DEG blocks is approximately 1000:1, the small hydrophilic component exerted a powerful influence during polymer processing. Under conditions of high humidity, solvent cast films of the PHO–DEG hybrid self-assembled into ordered microporous arrays. These microporous films, by virtue of their biocompatible composition and final structure, have the potential to serve a range of biomedical and biotechnological applications.

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