Eur Biophys J (2008) 37:711–715
DOI 10.1007/s00249-008-0333-9

ORIGINAL PAPER

In vivo deuteration strategies for neutron scattering analysis of bacterial polyhydroxyoctanoate

Robert A. Russell · Peter J. Holden · Karyn L. Wilde · Christopher J. Garvey · Kerie M. Hammerton · L. John R. Foster

Received: 11 October 2007 / Revised: 7 April 2008 / Accepted: 11 April 2008 / Published online: 15 May 2008

Abstract The cultivation of microorganisms on deuterated substrates has allowed us to control deuterium incorporation into biopolymer systems which is important for characterisation using neutron scattering techniques. Bacterial polyhydroxyoctanoate (PHO) is a polyester formed within inclusions inside bacterial cells and was deuterated in vivo under various conditions to characterise the formation of these inclusions by neutron scattering. Manipulation of deuterated media during microbial growth and PHO production phases resulted in polymer with partial or complete substitution of hydrogen by deuterium, as shown by gas chromatography. Sequential feeding of hydrogenated and deuterated forms of the same precursor was used to demonstrate that neutron scattering analysis could be used to differentiate between chemically similar phases in these polymer inclusions.

Keywords Biodeuteration · Polyhydroxyoctanoate · PHO · USANS · Core/shell model

Advanced neutron scattering and complementary techniques to study biological systems. Contributions from the meetings, "Neutrons in Biology", STFC Rutherford Appleton Laboratory, Didcot, UK, 11–13 July and "Proteins At Work 2007", Perugia, Italy, 28–30 May 2007.

R. A. Russell (✉) · P. J. Holden · K. L. Wilde · C. J. Garvey · K. M. Hammerton
ANSTO Institute for Environmental Research, PMB 1, Menai, Sydney, NSW 2234, Australia
e-mail: robert.russell@ansto.gov.au

L. J. R. Foster
Bio/Polymer Research Group, School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia

Introduction

Biopolymers such as polyhydroxyoctanoate (PHO) are becoming increasingly attractive as biodegradable alternatives to conventional plastics, as well as for biomedical applications (Van de Velde and Kiekens 2002; Chen and Wu 2005). Studies of polyhydroxyalkanoate (PHA) inclusions with chemically different phases have provided insights into their formation, however biodeuteration provides a unique opportunity to study the phase morphology of a single class of PHA. Sequential addition of precursors was hypothesised to produce a core/shell arrangement of polymer inclusions as previously described for addition of different classes of precursors (Curley et al. 1996; Kelley and Srienc 1999; Kim et al. 1991). Substituting deuterium in place of hydrogen in a material affects the way neutrons interact with the material (King 1999), so the ability to selectively deuterate PHO makes it well-suited to neutron scattering analysis. In addition, the analysis can occur hydrated at ambient conditions and is non-destructive to these biological systems, allowing samples to be analysed as close as possible to their native state. Small Angle Neutron Scattering (SANS) analysis of inclusions undertaken by our group has demonstrated different scattering properties for variously deuterated PHO inclusions (Russell et al. 2007), and the effect of deuteration on the properties of biopolymer films has also been investigated (Foster et al. 2006). This paper describes a biodeuteration strategy for a polymer system that can be applied to other biomolecules in preparation for neutron scattering analysis.

Material and methods

Production of hydrogenated and deuterated PHO

Fed-batch cultivations were undertaken in 2L or 5L vessels with a Biostat B bioreactor (B. Braun Biotech International, Germany) at 30°C. *Pseudomonas oleovorans* ATCC 29347 was grown on Modified E medium (Vogel and Bonner 1956) with 20 mM octanoic acid (Sigma, Australia) as sole carbon source in the batch phase, and cell growth was monitored spectrophotometrically at A_{660nm} and by dissolved oxygen (DO) profile. When the carbon source was depleted, impeller speed was reduced from 600 to 200 rpm, creating suitable conditions for PHO production via oxygen limitation. The fed phase was initiated by adding PHO precursor in aliquots to maintain DO below 20%.

Biodeuteration of PHO was achieved by addition of perdeuterated octanoic acid (Isotec: Sigma Aldrich, USA), alone or in combination with hydrogenated octanoic acid. For sequential PHO production, hydrogenated precursor was added (1.68 mM) at commencement of the fed phase. When a rise in DO indicated depletion of the precursor, perdeuterated precursor was added (6.31 mM), and a final deuterated aliquot (0.42 mM) was added just prior to harvest to prevent any enzymatic degradation of PHO. Samples were taken throughout cultivation for GC-MS analysis, and PHO polymer film was produced by solvent extraction from lyophilised biomass (modified from Nomura et al. 2004). PHO inclusions were isolated from bacterial cells as described previously (Russell et al. 2006) prior to neutron scattering analysis.

PHO monomer analysis

Biomass samples were lyophilised then refluxed in acidified methanol at 110°C to form methyl esters for analysis by Gas Chromatography-Mass Spectrometry (GC-MS). Mixtures were cooled then water added to separate the polyester-containing chloroform fraction. The derivatised samples were analysed using a Varian GC 3800 coupled to a Varian 1200 MS (Varian, Australia). One microlitre aliquots from the chloroform phase were injected into a Varian 3800 GC equipped with a Vf5-ms capillary column (30 m/0.25 mm i.d./0.25 μm film, Varian, Australia) and helium carrier gas at 1.2 ml min^{-1}. The column was held at 40°C for 3 min then ramped to 250°C (20°C min^{-1}) and held for 1.5 min. The ionising energy for electron impact MS was 70 eV. Mass fragments from the chromatograph were compared to NIST libraries for identification.

Density measurements

Polymer film pieces were accurately weighed into a solvent resistant vial and dissolved in a known amount of high purity chloroform (ReagentPlus, Sigma, Australia) to approximately 1% w/v. Solutions and solvent were injected through a 0.2 μm PTFE syringe filter (Sartorius, Australia) into a DMA 5000 density meter (Anton Parr, Austria) and density measurements were calculated from volume of polymer displacement in the solvent.

USANS analysis

Experiments were undertaken using Ultra Small Angle Neutron Scattering (USANS) at NIST Centre for Neutron Research (Gaithersburg, USA) to investigate core/shell phase morphology of inclusions (Barker et al. 2005). Inclusions were suspended in D_2O (AECL, Canada) and filtered through a 0.45-μm syringe filter (Sartorius, Australia) to minimise aggregations and produce a more uniform inclusion size range. Dynamic light scattering analysis (DynaPro Titan, Wyatt Technology Corporation, USA) was used to determine size and degree of polydispersity of the inclusion preparation. Samples were analysed as fresh as possible to minimise degradation, which necessitated diluting from a 100% (nominal) D_2O suspension to 80, 60 and 40% with H_2O. Samples were analysed in 5 mm thick quartz cuvettes (2 mm for 40% D_2O suspension) for 15 h to obtain sufficient count statistics over the desired q range. USANS data was reduced and modelled using Igor macros (Kline 2006).

Results

Growth of *P. oleovorans* in a fed-batch fashion allowed control of deuterium incorporation during cell and polymer production phases. GC-MS analysis of PHO samples revealed variations from the spectra of the normal hydrogenated PHO monomer, which has an indicative ion peak at m/z 103 (Russell et al. 2007). This ion fragment originates from the carbon backbone (rather than the side chain) and contains three hydrogen atoms that may be substituted by deuterium. Addition of hydrogenated precursor to the growth medium containing deuterated solvent (D_2O) produced a fragment with a dominant m/z 104 peak (Fig. 1) corresponding to a single deuterium substitution in the main carbon chain. Perdeuterated PHO, indicated by the m/z 106 peak resulting from three deuterium atoms replacing hydrogen in the monomer, was obtained from growth in fully deuterated medium in the batch and fed phases.

Density measurements of purified polymer in chloroform showed some increase in the deuterated form of PHO over the hydrogenated form (Table 1), which resulted in large differences in neutron scattering length densities (SLD). The SLD ρ, was calculated from Eq. 1, where δ is the bulk density of the molecule in solvent, N_A is Avogadro's

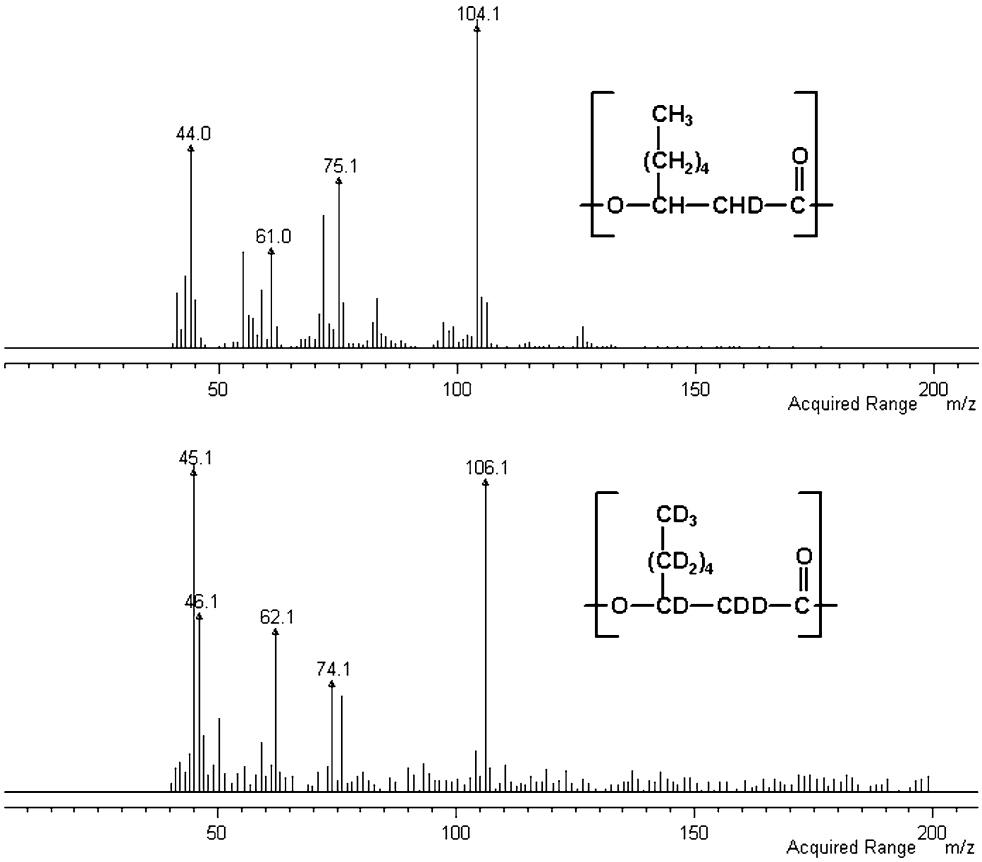

Fig. 1 Mass spectrum of partially (*upper*) and fully (*lower*) deuterated PHO fragments

Table 1 Effect of biodeuteration on physical and neutron scattering properties of PHO

PHO monomer composition	Growth regime	Density (g cm^{-3})	SLD ($\times 10^9 cm^{-2}$)	Theoretical contrast match point (% D_2O)
$C_8H_{14}O_2$	H_2O, hydrogenated precursor	1.04	5.54	16
$C_8D_{13}HO_2$	H_2O, deuterated precursor	1.11	63.6	100
$C_8D_{6.5}H_{7.5}O_2$	H_2O, co-fed H- and D-precursors	Not tested	35.9[a]	60

[a] Assumed average density of hydrogenated and deuterated PHO

number, M is the molecular weight and b is the coherent neutron scattering length of the nucleus of i atoms (King 1999).

$$\rho = \delta N_A / M \sum_i b_i \quad (1)$$

The SLD of hydrogenated PHO corresponded to 16% D_2O, while the deuterated form was at the upper limit of the contrast series (ca. 100% D_2O). An equal mix of the two, which has previously been achieved in our group by co-feeding equal amounts of precursors, has a theoretical SLD equal to 60% D_2O (Table 1).

Polymer precursors were added in appropriate quantities during batch-fed growth to obtain inclusions with hypothesised core/shell of approximately equal thickness. Average inclusion radius measured by dynamic light scattering was 166 nm, with a polydispersity of 12%. The proportion of hydrogenated and deuterated PHO in these inclusions was calculated from areas under GC-MS peaks to be 13 and 87%, respectively, with a standard deviation of 4.4 (data not shown). The proposed core and shell thickness were determined from these volume fractions in a representative sphere of 166 nm radius according to volume of a sphere radius (Eq. 2).

$$V = 4/3\pi r^3 \quad (2)$$

Allowing for an outer boundary layer consisting of lipid monolayer and embedded proteins (Sudesh et al. 2000) estimated at 5 nm thickness, the volume of combined PHO phases with 161 nm radius was 1.75×10^7 nm^3, so the volume of the proposed hydrogenated core was 13% of this, i.e. 2.27×10^6 nm^3. Similarly, the proposed core radius was 82 nm, and by subtraction the proposed deuterated shell would be 79 nm thick. These length scales

corresponded to a q-range around the lower limit of the BT5 instrument (Barker et al. 2005).

Ultra Small Angle Neutron Scattering curves were plotted together after normalising for the volume fraction of particles, calculated from dilution of the original 100% D_2O suspension (Fig. 2). I(0) values were estimated from the plateau region of each curve and plotted for each D_2O concentration in this limited contrast series (Fig. 3). Failure of I(q) to reach 0 was reasonable, due to boundary layer components of the inclusions providing different SLD (Roe 2000). A contrast match point ca. 80% D_2O was observed, which agrees with the theoretical match point of the PHO inclusions. Using SLD values of hydrogenated and deuterated PHO monomers shown in Table 1, the theoretical SLD of 13% hydrogenated and 87% deuterated PHO

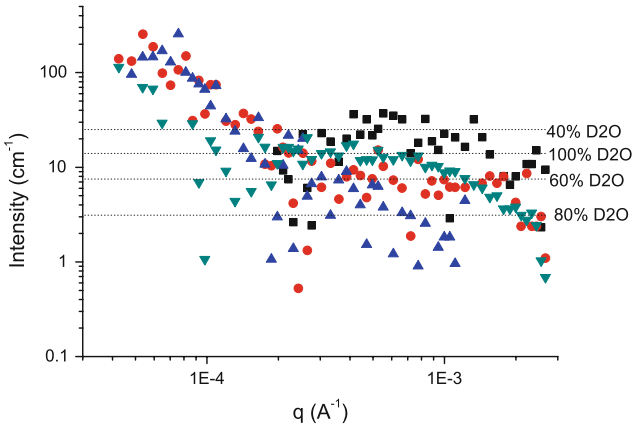

Fig. 2 Ultra Small Angle Neutron Scattering slit-smeared scattering data of PHO inclusions from *P. oleovorans* sequentially fed hydrogenated followed by deuterated precursor, corrected for empty cell scattering and sample dilution. *Dashed lines* indicate plateau regions for I(0) estimation of *dark filled square* indicates 40% D_2O, *dark filled circle* indicates 60% D_2O, *dark filled triangle* indicates 80% D_2O, *dark filled inverted triangle* indicates 100% D_2O

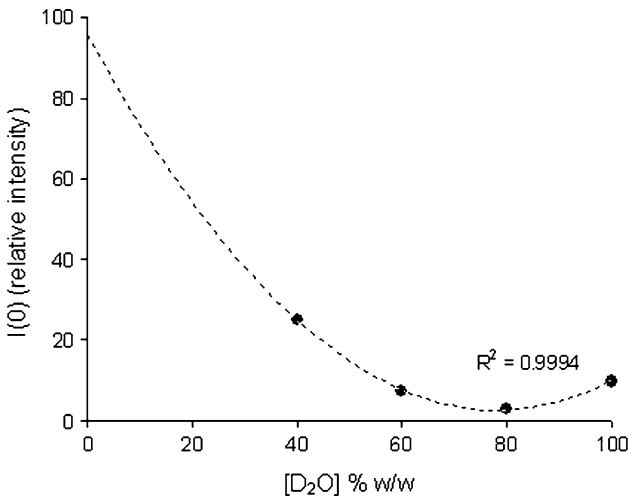

Fig. 3 Extrapolated I(0) values of PHO inclusions over a limited series of D_2O concentrations

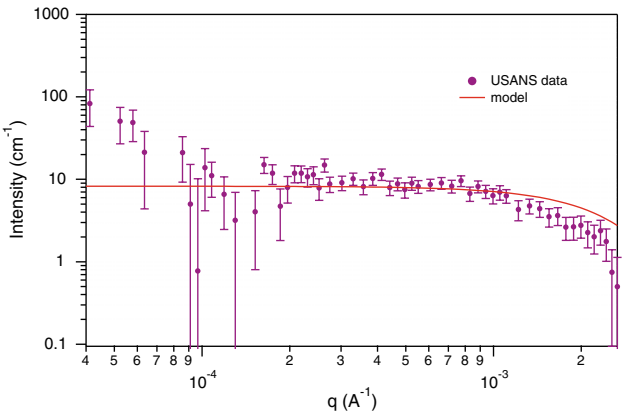

Fig. 4 Ultra Small Angle Neutron Scattering from inclusions in 100% D_2O (slit-smeared data) and core/shell model (*solid line*) with the following parameters: scale 4e-06, core radius 82 nm, shell thickness 79 nm, core SLD $5.54 \times 10^9 cm^{-2}$, shell SLD $6.3 \times 10^{10} cm^{-2}$, solvent SLD $6.38 \times 10^{10} cm^{-2}$, background 0.1 cm^{-1}

was $5.6 \times 10^{10} cm^{-2}$, while the SLD of 80% D_2O was $5.0 \times 10^{10} cm^{-2}$.

Despite the complexity of these inclusions, an attempt was made at modelling core/shell spheres to determine whether the USANS data fits this proposed structure. The low scattered intensity from the samples meant only the undiluted 100% D_2O sample gave suitable data that could be fit to a model (Fig. 4). The SLD parameters were set at theoretical values given in Table 1 (D_2O solvent was $6.38 \times 10^{10} cm^{-2}$) and the scale parameter was varied to match intensity of USANS data (*y*-axis). A better fit in the higher q region could be obtained by increasing the core and shell thicknesses in proportion to a combined radius of 242 nm (not shown) however the filtration step and light scattering data do not allow for inclusions of this size in this experiment.

Discussion

Previous work by our group has demonstrated partial deuteration of PHO and its application in neutron scattering experiments (Foster et al. 2006; Russell et al. 2007). Addition of deuterated precursor (perdeuterated octanoic acid) resulted in deuteration of the side chain as well as two of the three hydrogen atoms in the main carbon chain.

In this work we have demonstrated perdeuteration of the PHO monomer as well as a single deuterium substitution in the main carbon chain. The β-oxidation pathway is used by pseudomonad bacteria to convert fatty acids to PHAs (Sudesh et al. 2000), and the deuteration of enoyl-CoA from D_2O solvent in this process is the step most likely responsible for the single deuterium substitution at the C2 atom.

The SLD of a molecule is highly dependant on deuterium content, as the coherent scattering lengths of hydrogen

and deuterium are quite different (-3.741×10^{-15}m and $+ 6.671 \times 10^{-15}$m, respectively), and this can be useful in constructing SANS contrast variation experiments (King 1999). The match points obtained for hydrogenated and deuterated PHO traverse almost the entire range of D_2O:H_2O solvent mixtures, and co-feeding or sequential feeding produces SLDs that lie between these extremes. In addition, the ability to selectively substitute 1/14 or 13/14 hydrogen atoms may prove useful when blending or mixing biopolymers of similar SLD. Initial SANS experiments on polymer inclusions undertaken at ANSTO have shown good agreement between theoretical match points of variously deuterated PHO monomers and experimental match points of PHO inclusions in D_2O:H_2O mixtures (Russell et al. 2007). In this study, the minimum scattered intensity observed at 80% D_2O agreed with the GC-MS data and indicated that the proportion of hydrogenated and deuterated PHO would support a core/shell inclusion structure with length scales suitable for USANS measurement. The core/shell thickness derived from dynamic light scattering and GC-MS data described above gave a reasonable agreement between USANS data and core/shell model, supporting the proposed phase structure within inclusions of ca. 166 nm diameter.

A clearer picture of phase morphology might be obtained with less polydispersity in the sample and a greater volume fraction of inclusions to improve the signal/noise of scattered intensity. It may be possible that mixing of hydrogenated and deuterated PHO occurred at the boundary of the phases, which would affect the fit of the core/shell model to the scattering data. This data, however, goes some way to supporting a core/shell structure of inclusions formed sequentially in vivo from hydrogenated and deuterated precursors that are otherwise chemically identical. The different patterns of deuterium incorporation achieved in our group illustrate the potential for biodeuteration in the characterisation of PHO polymers by neutron scattering, and can be applied to a variety of biomolecules where the precursor is available in deuterated form.

Conclusions

This study described a biodeuteration strategy for characterisation of a bacterial polyester, PHO, by neutron scattering and complementary techniques. Selective deuterium substitution in the PHO monomer was demonstrated by GC-MS, and USANS data of PHO inclusions with hypothesised hydrogenated core/deuterated shell of similar thicknesses indicated a contrast match point ca. 80% D_2O. The scattering data made a reasonable fit to a core/shell model, however the complexity of inclusions and limitations of sample heterogeneity made interpretation of scattering data difficult. Biodeuteration is nevertheless important for neutron scattering analysis of biomolecules in solution as well as biopolymers in the bulk phase.

Acknowledgments This work utilised facilities supported in part by the National Science Foundation under Agreement No. DMR-0454672. We acknowledge the support of the National Institute of Standards and Technology, U.S. Department of Commerce, in providing the neutron research facilities used in this work. The assistance of Man-Ho Kim (Korea Institute of Science and Technology) for USANS data analysis, Andrew Whitten (Bragg, ANSTO) for density measurements and reviewers for their helpful comments is gratefully acknowledged.

References

Barker JG, Glinka CJ, Moyer JJ, Kim M-H, Drews A, Agamalian RM (2005) Design and performance of a thermal-neutron double-crystal diffractometer for USANS at NIST. J Appl Crystallogr 38:1004–1011

Chen G-C, Wu Q (2005) The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials 26:6565–6578

Curley JM, Lenz RW, Fuller RC (1996) Sequential production of two different polyesters in the inclusion bodies of *Pseudomonas oleovorans*. Int J Biol Macromol 19:29–34

Foster LJR, Russell RA, Sanguanchaipaiwong V, Stone DJM, Hook JM, Holden PJ (2006) Biosynthesis and characterisation of deuterated polyhydroxyoctanoate. Biomacromolecules 7:1344–1349

Kelley AS, Srienc F (1999) Production of two phase polyhydroxyalkanoic acid granules in *Ralstonia eutropha*. Int J Biol Macromol 25:61–67

Kim YB, Lenz RW, Fuller RC (1991) Preparation and characterization of poly(β-hydroxyalkanoates) obtained from *Pseudomonas oleovorans* grown with mixtures of 5-phenylvaleric acid and *n*-alkanoic acids. Macromolecules 24:5256–5260

King SM (1999) Small-angle neutron scattering. In: Pethrick RA, Dawkins JV (eds) Modern techniques for polymer characterisation. Wiley, New York, pp 171–226

Kline SR (2006) Reduction and analysis of SANS and USANS data using Igor Pro. J Appl Cryst 39(6):895–900

Nomura CT, Tagichi K, Taguchi S, Doi Y (2004) Coexpression of genetically engineered 3-ketoacyl-ACPSynthase III (fabH) and polyhydroxyalkanoate Synthase (phaC) genes leads to short-chain-length-medium-chain-length polyhydroxyalkanoate copolymer production from glucose in *Escherichia coli* JM109. Appl Environ Microbiol 70:999–1007

Roe R-J (2000) Methods of x-ray and neutron scattering in polymer science. Oxford University Press, New York, Oxford

Russell RA, Holden PJ, Garvey CJ, Wilde KL, Hammerton KM, Foster LJR (2006) Investigation of the phase morphology of bacterial PHA inclusion bodies by contrast variation SANS. Physica B 385–386:859–891

Russell RA, Holden PJ, Wilde KL, Hammerton KM, Foster LJR (2007) Production and use of deuterated polyhydroxyoctanoate in structural studies of PHO inclusions. J Biotechnol 132:303–305

Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 25:1503–1555

Van de Velde K, Kiekens P (2002) Biopolymers: overview of several properties and consequences on their applications. Polymer Test 21:433–442

Vogel HJ, Bonner DM (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties. J Biol Chem 218:97–106