Structural study of isolated poly(β-hydroxybutyrate) granules

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Nascent poly(β-hydroxybutyrate) (PHB) granules are found in most bacterial strains and can be isolated as a milky suspension. Their structure was examined by transmission and scanning electron microscopy and has been shown to be made of two distinct components: a solid shell composed of overlapping lamellar crystals and a soft non-crystalline core. It was possible to obtain a ductile deformation of the core in liquid nitrogen even though the glass transition of isotactic PHB approaches 0°C. Finally, glycerol triacetate, a minor component of the granule, was found to be a good solvent for PHB from which metastable solutions can be obtained. Self-seeding of these solutions led to the formation of lamellar crystals comparable to the structure of the shells.

(Keywords: poly(β-hydroxybutyrate); biopolyester; biodegradation; morphology; native; granules; suspension)

INTRODUCTION

Poly(β-hydroxyalkanoates) are a class of chiral biopolyesters produced by many bacterial strains in the form of submicrometre granules that serve as an energy source and carbon reserve[1–3]. Poly(β-hydroxybutyrate) (PHB) is the most ubiquitous of these polyesters. Several different purification procedures are used to isolate the granules from the producing bacteria, e.g. enzymatic breakdown of cell components or hypochlorite treatment of the bacteria containing PHB yield suspensions of native granules[4–6]. Extraction of PHB from dried cells using solvents such as chloroform, tetrachloroethane or propylene carbonate provides a good yield of a crystalline powder isolated by non-solvent precipitation[7–9]. X-ray diffraction[10,11] of oriented fibres has shown that the PHB molecules pack in an orthorhombic unit cell with a 2_1 helical structure and a fibre repeat of 5.96 nm.

Recent ^{13}C high resolution n.m.r. studies of aqueous suspensions of *Alcaligenes eutrophus* cells containing PHB granules showed that it was possible to obtain spectra of the polyester near room temperature[12]. The authors concluded that at least 70% of the PHB molecules in the bacteria were in a mobile state. This finding was important since it showed that the granules do not undergo simultaneous synthesis and crystallization. Furthermore, this mobile state has been shown to be present in isolated granule suspension of poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) even after several months standing at room temperature[13].

Earlier work on fresh enzymatically isolated PHB granules showed that they can be swollen if put in a 25% acetone–water solution[14]. The granules, in this state, revealed a morphology which was composed of laths and fibril-like structures. When put in a 75% acetone–water solution the granules were transformed into apparent lamellar crystals as observed by transmission electron microscopy (TEM). Ellar *et al.* proposed a model where tangential extended chains were growing by apposition to form the observed structures[14]. Freeze fracture studies on bacterial paste containing PHB and on isolated granules by Dunlop and Robards showed that the granules undergo ductile fractures to form long fibril-like structures[15]. They proposed a three-phase model for the granules. The outer phase is a membrane forming an envelope around the entire granule. Underneath this membrane is a coat of material solid enough to be fractured, while the third phase is a core made of soft deformable material. Both of these studies support the model of an extended chain morphology in the nascent granules but lack the essential electron diffraction patterns to demonstrate orientation and crystallinity features of the observed morphologies. The present study relies on scanning electron microscopy (SEM) and TEM as well as electron diffraction to further explore the crystallization pathway of the granules.

EXPERIMENTAL

Production and isolation of PHB granules

A PHB aqueous suspension containing 36% w/w solids was used. The granules were obtained using a fermentation technique developed by Berger *et al.*[16] using

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A. eutrophus DSM 545. The granules were isolated using a process wherein the pH and the ratios of the surfactant and sodium hypochlorite with respect to PHB content were carefully controlled to avoid polyester degradations. The suspension of granules had been stored for 9 months at room temperature prior to the experiments.

Mechanical transformation of the granules

A sample of the initial granules suspension (0.5 ml) was put in absolute ethanol (5 ml). The ethanol suspension was then poured slowly into a mortar containing liquid nitrogen. After 30 s, a pestle was used to grind the frozen suspension. The grinding lasted for 1 h with continuous addition of fresh liquid nitrogen medium. After this period the sample was recovered and kept for later examination. A second sample of the initial granules suspension (0.5 ml) was put in 12 N HCl at 35°C and left to hydrolyse for 4 days. The acidic suspension was then washed and centrifuged three times in water and twice in ethanol. The sediments were resuspended in absolute ethanol (5 ml) and ground as previously described. The ground sample was recovered and kept for later examination.

High temperature deformation of the granules

Two samples were prepared in the following way: drops of the initial aqueous suspension of granules were deposited on glass slides and immediately put in a Mettler hot stage for 1 min. For the first sample the temperature was set at 190°C. For the second sample the temperature was lowered to 150°C prior to the heating cycle. The slides were then removed and the hot granules were smeared using a cold spatula. The resulting ribbons were floated off the slides by immersion in a dilute hydrofluoric acid solution and were kept for later examination.

PHB metastable solutions and single crystal formation

A suspension of 0.01% w/w of the initial PHB suspension in glycerol triacetate (triacetin) was put in an oil bath at 180°C for 1 h and then transferred to another bath kept at 110°C for a further 3 h. The resulting solution remained metastable for several hours. Self-seeding by addition of previously prepared PHB lamellar crystals from more concentrated solutions forced the precipitation of the dissolved molecules. The crystal suspension in triacetin was kept at room temperature for later examination.

Transmission and scanning electron microscopy

All samples were deposited on 200 mesh carbon-coated copper grids for TEM using a Philips EM-400T electron microscope equipped with a Philips cold stage specimen holder and a Gathan anticontaminant device which keeps the sample at −173°C. The instrument was operated at 120 kV. SEM was performed using a Jeol JSM 6100 instrument. The samples were deposited on 200 mesh carbon-coated copper grids and glued to a cylindrical brass sample holder using carbon paint. A 10 Å thick gold coating was deposited on the samples prior to examination.

RESULTS

Attempts to swell the granules in acetone–water solutions were unsuccessful suggesting that the morphology was somewhat different from those of the fresh, enzyme prepared granules of Ellar *et al.*[14]. Grinding at liquid nitrogen temperature provided insight into the initial crystallization process. *Figures 1* and *2* are scanning electron micrographs of an ethanol suspension of granules which had been ground in liquid nitrogen without any further treatment. It appears that the granules consist of two components: a hard shell and a soft core. Instead of fracturing like crystalline or glassy material during the grinding step, the core (*Figure 2*) remained attached to all the fragments of broken shell and it smeared like a soft paste. *Figure 3* is a transmission electron micrograph of the same sample. The image is darker since the thickness of the granules does not allow good transmission. Electron diffraction patterns on the smeared core showed neither orientation nor crystallinity.

Figure 4 is a micrograph of granules that were first hydrolysed and then ground. The acid seems to penetrate the granule by hydrolysing weak sites in the shell as shown by the large shell in the centre of the micrograph, completely emptied of its non-crystalline content. The core is made of material that is much more easily hydrolysed than the shell. This is in keeping with the results reported by Stejny *et al.* which showed that disordered regions of single crystals are attacked more readily by gaseous methylamine[17].

The resistance of the shells to mechanical stress is greatly reduced by the hydrolysis step. *Figure 5* is a

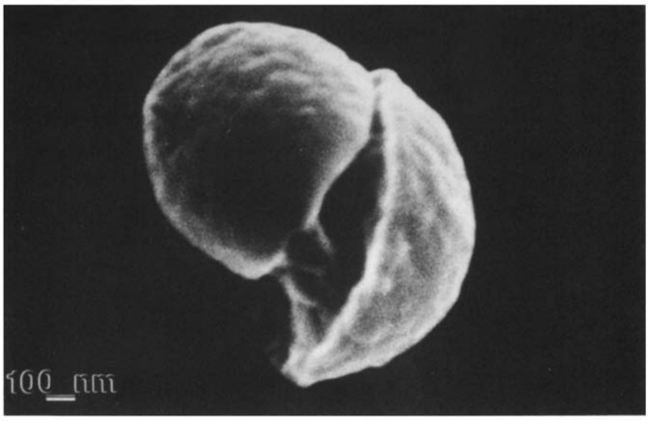

Figure 1 Scanning electron micrograph of a broken shell showing the beginning of deformation. The hard shell is fractured but the core remains inside. The deformation was performed at liquid nitrogen temperature

Figure 2 Scanning electron micrograph showing a highly deformed granule. The smeared core remains attached to the shell fragments of the granule. The deformation conditions are the same as for *Figure 1*

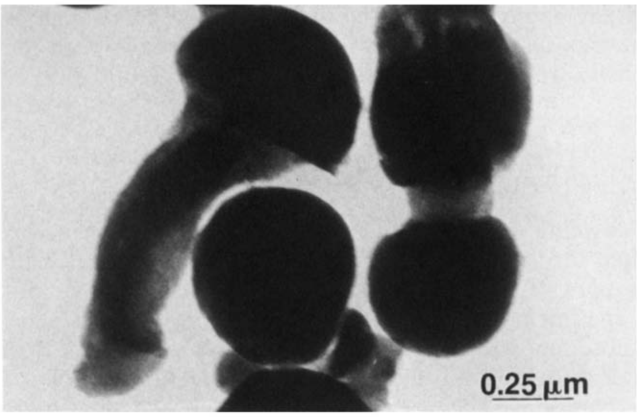

Figure 3 Transmission electron micrograph of a granule deformed in the same conditions as for *Figure 1*. The thickness of the granule is responsible for the darker image

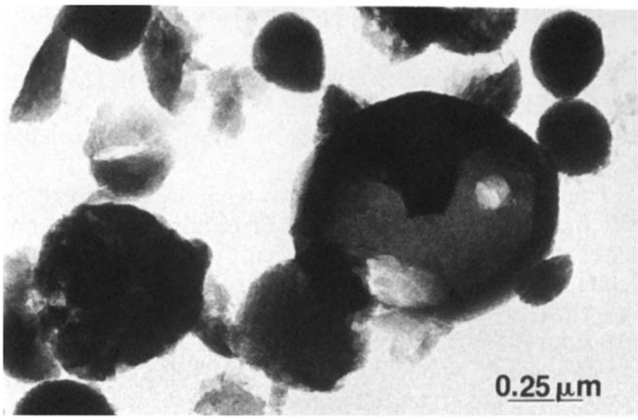

Figure 4 Transmission electron micrograph of shells emptied of their amorphous content by hydrolysis and then ground in liquid nitrogen. The large empty half-shell in the centre is seen from its open end

micrograph of a fragment of a shell that was broken during the grinding operation. Isotactic PHB has a glass transition temperature (T_g) near 0°C and can thus be fractured easily in liquid nitrogen[18]. This fragment was examined by electron diffraction and the pattern has been aligned with the image. It is typical of a PHB lamellar crystal pattern with intense reflections of the (0 2 0), (0 4 0) and (1 1 0) planes[19]. It is interesting to note that the reflections are arcs with an intensity falling off evenly on both sides. This is probably due to the presence of superimposed lamellar crystals in poor registry. The electron diffraction pattern of a thicker fragment is shown in *Figure 6*. The (0 2 0), (0 4 0) and (1 1 0) reflections are arcs but with an uneven distribution of the intensity in a clockwise fashion. This could be a sign of a left-handed rotation of the superimposed single crystals during growth, as expected for a chiral polyester.

The cracks in the shell fragment of *Figure 5* are perpendicular to the *b* axis of the unit cell. The fractures seem to occur between layers of parallel and antiparallel molecules in the unit cells, i.e. parallel to the (0 2 0) and (0 4 0) planes so that they run across the long side of the lamellar crystals. This is in keeping with observations made by Barham *et al.* in a study of fractured single crystals of PHB[18].

The sample which was deformed at 190°C is shown in *Figure 7*. The electron diffraction pattern of the resulting ribbon is typical of lamellar single crystals. It demonstrates that under certain conditions PHB single crystals can be grown from the melt as was shown for other polymer systems[20]. Fragments of a broken shell remained on the smear. This suggests that the lamellar crystals were formed from the core upon cooling. The diffraction pattern is identical to that of *Figure 5* although more crystallographic planes can be identified. It is interesting to note that there was no correlation between the deformation axis and the orientation of the crystallographic planes. The crystalline shell fragments were possibly responsible for seeding the crystallization according to their orientation.

As shown in *Figure 8*, when deformed at 150°C, which is 30°C below the melting point of PHB, the structure of the smeared film is a shish kebab. The spine molecules of those shish kebabs are formed of high molecular weight PHB molecules that were not affected by the temperature but were rather drawn into fibrils when the granules were smeared into a film. On these fibrils, lower molecular weight molecules, in the molten state, formed small crystallites perpendicular to the spine axis but with the same molecular orientation. The electron diffraction pattern of the shish kebabs is typical of a PHB fibre pattern with the *c* axis oriented horizontally on the micrograph[11].

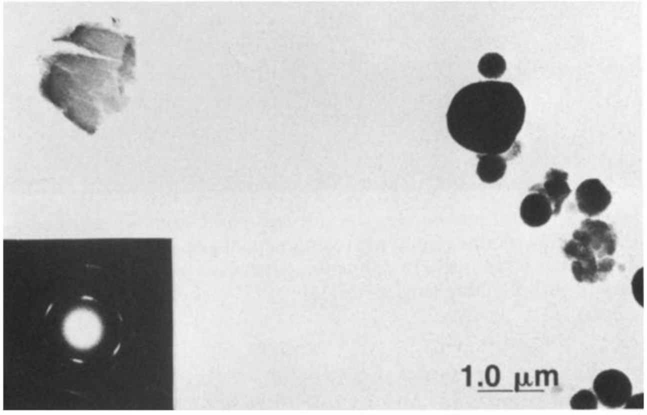

Figure 5 Transmission electron micrograph of a fragment of a shell obtained by grinding a hydrolysed sample. The diffraction pattern is typical of lamellar PHB single crystals

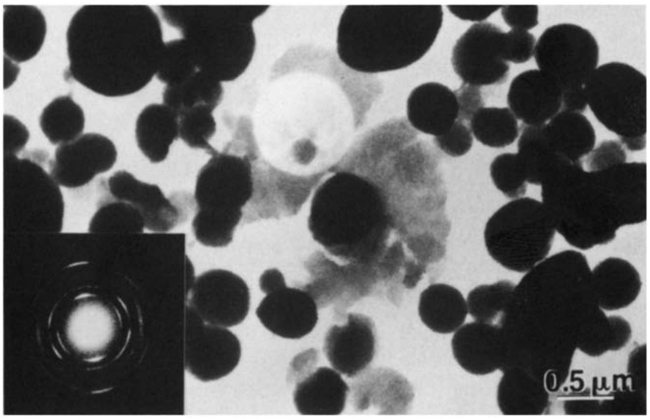

Figure 6 Transmission electron micrograph of fragments of a shell obtained by grinding a hydrolysed sample in liquid nitrogen. The electron diffraction pattern of the left portion of the fragment is similar to that shown in *Figure 5* with the difference that the intensity falls off unevenly along the azimuth

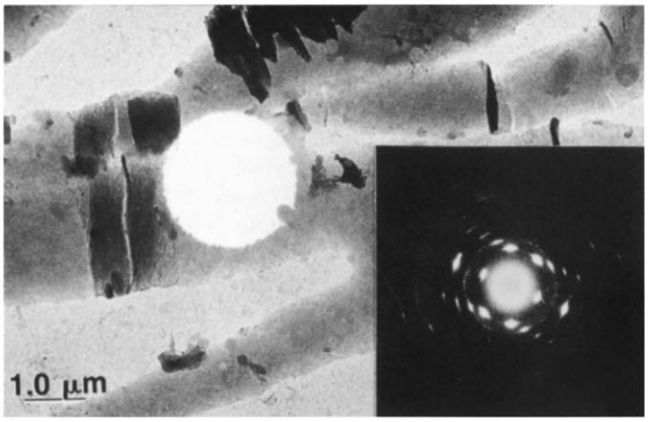

Figure 7 Transmission electron micrograph of granules smeared at 190°C. The resulting ribbon is thin and its diffraction pattern is typical of PHB lamellar single crystals. Fragments of shells are still present on the crystalline film which proves that the lamellar crystals were formed from the molten core upon cooling

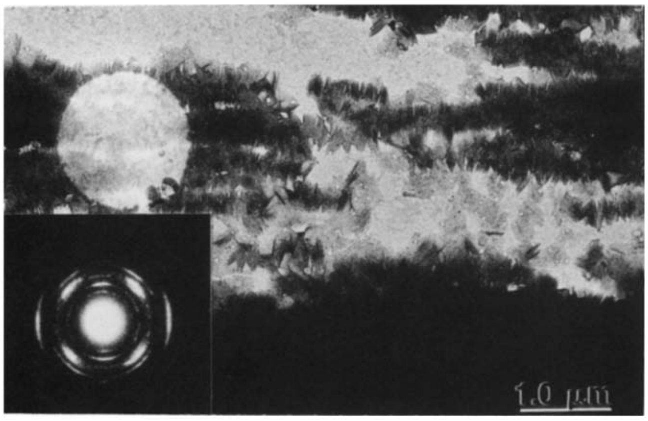

Figure 8 Transmission electron micrograph of granules smeared at 150°C. The resulting film was composed of regions that formed a shish-kebab structure. The diffraction pattern is typical of a PHB fibre pattern with the fibre axis horizontal

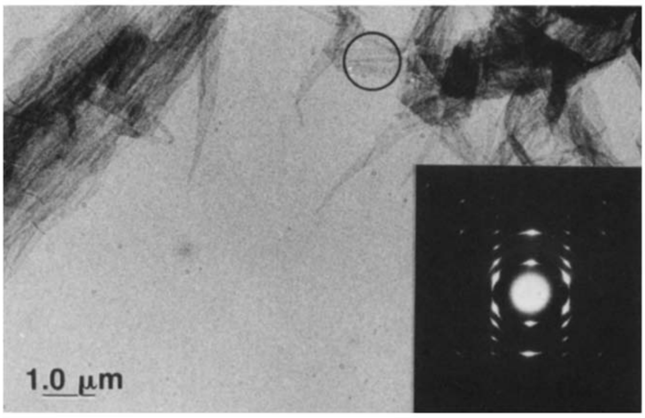

Figure 9 Transmission electron micrograph of PHB single crystals grown from a diluted triacetin solution

The micrograph shown in *Figure 9* was recorded from lamellar crystals of PHB grown from triacetin. This glycerol derivative has been found to be one of the major lipid components with tributyrin and tripropionin representing ~0.47 wt% of the lipid extract from freshly isolated PHB granules[21]. The diffraction pattern shown was taken from the circled region of the micrograph where many crystals are overlapping. The pattern is very similar to *Figure 5* which supports the view that the shells are made of overlapping lamellar crystals.

DISCUSSION

Scanning and transmission electron micrographs show that the granules are composed of a crystalline shell with a core of non-crystalline material. The results of our experiments on the isolated PHB granules closely parallel those reported by Dunlop and Robards in their study of freeze fracture of bacteria containing PHB and of isolated granules[15]. They suggested a membrane–coat–core layering for the granules similar to what we have observed although we have no evidence for the presence of a membrane surrounding the granules. Our inability to record electron diffraction on the core of the granules confirms that it is non-crystalline. The fact that it remained deformable in liquid nitrogen suggests that the core was above its T_g. Hydrolysis of this soft material leaves a solid shell whose crystalline character was confirmed by electron diffraction. The diffraction patterns show that the shells are made of overlapping lamellar crystals of PHB.

There are several literature references concerning the presence of a membrane surrounding PHB granules[15,22–24]. The relation between the observed shell of lamellar PHB crystals and the purported membrane is not clear at this time. It seems likely that the isolation procedure is responsible for the development of surface crystallization which locks the inner soft matter in its non-crystalline state. Subsequent crystallization is slow and leads to a rather disordered crystalline inner organization.

Air-dried PHB granules prepared by the classical hypochlorite method can be resuspended in water and they readily undergo enzymatic degradation by PHB depolymerase, as is also found for solvent cast films of natural and synthetic PHB[13,25].

The model of PHA synthesis proposed by Ellar *et al.* is based on the presence of a lipid–protein membrane from which the PHA molecules would be synthesized thus causing the granules to grow by apposition[14]. This does not preclude the possibility that smaller molecules acting as a plasticizer could be synthesized simultaneously or that water could act as a plasticizing agent. The protection offered by this membrane could then allow the plasticized PHA molecules to remain isolated from the cytoplasm and in a non-crystalline state. Isolation of the granules from the bacteria necessarily exposes the granule surface to a medium different in chemical composition from the cytoplasm. Reorganization of the surface may then occur by simple diffusion of the plasticizing agent out of the outer layers of the granule. The PHB molecules would then rearrange into lamellar crystals which overlap and eventually form a solid shell of a thickness dependent on the extent of the diffusion gradient within the surface. Several published and unpublished results provide evidence of the absence of the crystalline shell for granules still in the bacteria. In our work a pellet of *A. eutrophus* containing ~60% PHB granules was completely dried and annealed and even after such a treatment the typical X-ray diffraction pattern of PHB was absent from the diffractogram. This supports the model of Ellar *et al.* for the presence of a membrane around the granules which prevents surface crystallization[14].

If we now consider the ease with which the amorphous core rearranged into shish kebabs or lamellar crystals after being smeared at temperatures slightly below and/or above the melting point temperature we can deduce that the core molecules are in a metastable state. A small change in the chemical composition of the solution or a rise in temperature of the surrounding medium appears sufficient to induce the surface crystallization of the granules that we observe.

CONCLUSIONS

The results presented show that isolated PHB granules develop a shell made of lamellar crystals. It is not yet clear what keeps the core non-crystalline but the possibility of obtaining a metastable solution of PHB in triacetin is a good indication that such oils could combine with water contained in the granules to form a plasticizing medium. It is unlikely that the small amount of these glycerol derivatives reported by Kawaguchi and Doi[21] is enough to plasticize the entire mass of PHB molecules contained in a single granule. Based on the overall observations, we propose a model of a granule core in which the core is in a metastable gel state due to interactions between high molecular weight PHB molecules and a plasticizing medium.

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REFERENCES

1 Lemoigne, M. *Ann. Inst. Pasteur (Paris)* 1925, **39**, 144
2 Lemoigne, M. *Bull. Soc. Chim. Biol.* 1926, **8**, 770
3 Lemoigne, M. *Ann. Inst. Pasteur (Paris)* 1927, **41**, 148
4 Merrick, J. M. and Doudoroff, M. *J. Bact.* 1964, **88**, 60
5 Ramsay, J. A., Berger, E., Ramsay, B. A. and Chavarie, C. *Biotechnol. Techniques* 1990, **4**, 221
6 Berger, M. *MSc Thesis* Ecole Polytechnique, Montréal, 1990
7 Baptist, J. N. *US Pat. 3044942*, 1962
8 Solvay et Cie. *Eur. Pat. Appl. EP14490*, 1979
9 Lafferty, R. M. and Heinzle, E. *Chem Rundschau* 1977, **30**, 15
10 Alper, R., Lundgren, D. G., Marchessault, R. H. and Côté, W. A. *Biopolymers* 1963, **1**, 545
11 Okamura, K. and Marchessault, R. H. 'Conformation of Biopolymers' (Ed. G. N. Ramachandran), Vol. 2, Academic Press, London, 1967, p. 709
12 Barnard, G. N. and Sanders, J. K. M. *J. Biol. Chem.* 1989, **264**, 3286
13 Marchessault, R. H., Monasterios, C. J., Jesudason, J. J., Ramsay, B., Sarcovan, I., Ramsay, J. A. and Saito, T. *Polym. Degrad. Stab.* in press
14 Ellar, D., Lundgren, D. G., Okamura, K. and Marchessault, R. H. *J. Mol. Biol.* 1968, **35**, 489
15 Dunlop, W. F. and Robards, A. W. *J. Bacteriol.* 1973, **114**, 1271
16 Berger, E., Ramsay, B. A., Ramsay, J. A., Chavarie, C. and Braunegg, G. *Biotechnol. Techniques* 1989, **3**, 227
17 Stejny, J., Otun, E. L. and Keller, A. *Polym. Commun.* 1989, **30**, 302
18 Barham, P. J., Keller, A., Otun, E. L. and Holmes, P. A. *J. Mater. Sci.* 1984, **19**, 2781
19 Revol, J. F., Chanzy, H. D., Deslandes, Y. and Marchessault, R. H. *Polymer* 1989, **30**, 1973
20 Wunderlich, B. 'Macromolecular Physics', Vol. 2, Academic Press, London, 1980, p. 240
21 Kawaguchi, Y. Y. and Doi, Y. *FEMS Microbiol. Lett.* 1990, **70**, 151
22 Lundgren, D. G., Pfister, R. M. and Merrick, J. M. *J. Gen. Microbiol.* 1964, **34**, 441
23 Boatman, E. S. *J. Cell. Biol.* 1964, **20**, 297
24 Wang, W. S. and Lundgren, D. G. *Arch. Mikrobiol.* 1969, **97**, 947
25 Doi, Y., Kanesawa, Y., Kunioka, M. and Saito, T. *Macromolecules* 1990, **23**, 26