

LEUCOCYTES SHOW IMPROVEMENT GROWTH ON PHA POLYMER SURFACE

MOHAMAD A HASSAN¹, AMRO A AMARA^{1,2}, ASHRAF T ABUELHAMD³
AND BAKRY M HAROUN³

¹Protein Research Department, Genetic Engineering and Biotechnology Research Institute,
Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt

²Pharmaceutics Department, Faculty of Pharmacy at King Saud University (KSU) Riyadh, Kingdom of Saudi Arabia

³Plant and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt

ABSTRACT

Polyhydroxyalkanoate (PHA) from one fermentation process shows diverse physical properties when extracted using different methods. *Pseudomonas aeruginosa* strain has been previously isolated from the Egyptian ecosystem was cultivated on olive oil as a carbon source under PHA accumulation conditions. PHA was extracted using four different extraction methods and the polymer give different biological properties. Leucocytes grown in different rate on each preparation. RBCs haemolysis test was used to determine the polymers toxicity. PHA isolated directly with chloroform give the highest leucocytes number (19.4×10^4 Cells/48 hr) and the lowest Haemolytic index (2.28). Bioassays used in this study are recommended for evaluating the *in vitro* polymer biocompatibility aiming to *in vivo* application or as a cell line-supporting matrix.

Keywords: Leucocytes; Polyhydroxyalkanoates; *Pseudomonas aeruginosa*

INTRODUCTION

The history of PHAs including PHB started with the solvent used in their extraction. Lemoigne was the first to report that the bacterial granules components were not ether soluble, as in lipid, later, he concluded that PHB is the major constituent of these granules (Lemoigne, 1923, 1925, 1926 and 1927). Wallen and Rohwedder (1974) gave the first indication that the polymer discovered by Lemoigne may contain proportions of 3-hydroxyacids other than 3HB (Wallen and Rohwedder, 1974), De Smet *et al* (1983) characterized PHA_{MCL} in *P. oleovorans* during growth on octane (De Smet *et al.*, 1983). Huisman *et al.* (1989) confirmed that PHA_{MCL} accumulation was the common feature of fluorescent pseudomonads. PHAs are also produced by gram-positive, gram-negative and phototrophic bacteria as well as archaea (Huisman *et al.*, 1989; Fernandez-Castillo *et al.*, 1986; Anderson and Dawes, 1990; Steinbüchel, 1991; Shabeb *et al.*, 2006; Amara, 2008).

The potential hope for PHAs usage was as packaging materials. Meanwhile there is a clear shift and increasing interest in using PHAs in medicinal applications (Amara, 2008). Over the recent years, PHAs were used to develop many devices and material useful for clinical purposes (Amara, 2008; Kunze *et al.*, 2002; Cheng *et al.*, 2006; Misra *et al.*, 2006; Valappil *et al.*, 2006; Van der Walle *et al.*, 2001). Tesema *et al.*, (2004) and Malm *et al.* (1994) implanted PHB nonwoven patches as transannular patches into the right ventricular outflow tract and pulmonary artery in 13 weanling sheep (Tesema *et al.*, 2004; Malm *et al.*, 1994). The reaction of cells and tissues to PHA

depends not only on the chemical composition of the material but also on the degree of its purity and the methods of processing (Malm *et al.*, 1992).

Authors observed early the differences in PHA with the same chemical formula when extracted using different methods. The methods for PHA extraction is a critical steps can be affected significantly on the polymer structure, its chemical, physical and biological properties. Péaud and Kepes (1952) were the first to develop a direct extraction method with chloroform (Péaud and Kepes 1952). They comment that this method gives polymer with high viscosity. Lundgran *et al.* (1965) were reported that only cells that extracted with chloroform could yield high molecular weight PHB (Lundgran *et al.*, 1965). They also concluded that the polymer molecular weight could be varying depending upon bacterial growth conditions, growth stage and the chemical extraction procedures (Lundgran *et al.*, 1965).

Macrophages, which are one type of phagocytes, are able to produce Interleukin-1. One extra advantage of phagocytes that they are able to kill pathogens which are a helpful criterion during cells cultivations. Leucocytes can be easily separated from human blood without requirement of any ethical instructions. They contain the complete genomic DNA (Brooks, 2008). The future will show extended applications for different type of phagocytes. In this study, Human phagocytes were used to test PHA as a supporting and enhancing growth matrix. The extraction of the polymer show different leucocytes number and Haemolytic index. The PHA prove to be bio-available for culturing Human Leucocytes.

Corresponding author: Amro A Amara, Asst. Prof. in Pharmaceutics Department, Faculty of Pharmacy, King Saud University, Kingdom of Saudi Arabia, e-mail: amroamara@web.de

MATERIA AND METHODS

Chemicals used in this study were purchased from Fluka Biochemika (Buchs, Switzerland), Amersham Pharmacia Biotech (Uppsala, Sweden), Riedel-deHaen (Germany), WINLAB (U.K.), Sigma chemicals co. (St.Louis, Mo., USA), Acros (New Jersey, USA), PARK (Northampton, U.K.), Fischer Scientific (U.K.), Scharlau Chemie S.A. (Barcelona, Spain).

PHA used in this study was produced in our Lab., routinely using *Pseudomonas aeruginosa* strain isolated from the Egyptian ecosystem and stored in house as stock culture using -80 deep-freezer (REVCO) for research purposes. The strain is able to produce PHA_{MCL} from olive oil. Five gram of the dry cells containing PHA_{MCL} were used for each preparation. The four different methods were as the follow:

Method 1: Five g of the dry cells was incubated in glass bottle containing 50 ml chloroform and allowed to be extracted overnight at 30°C in shaker incubator at 250 rpm. The chloroform which contains the polymer then filtrated and the polymer isolated using 4-volume cold methanol, re-filtrated and dried.

Method 2: Five g of the dry cells was incubated in glass bottle containing 50 ml chloroform and 300 ml 5% sodium hypochlorite. The mixture left at 37°C for 90 min. The mixture centrifuged and the chloroform phase removed, filtrated and the polymer isolated using 4-volume cold methanol, re-filtrated and dried.

Method 3: Five g of the dry cells was incubated in glass bottle containing 100 ml of 0.4% sodium hypochlorite solution for 1h at 37°C. The suspended granules collected by centrifugation and incubated with chloroform overnight, filtrated and the polymer isolated using 4-volume cold methanol, re-filtrated and dried.

Method 4: Five g of the dry cells was incubated in glass bottle containing 50 ml 10% SDS at 50°C for 20 min then centrifuged and suspended in 50 ml chloroform overnight, filtrated and the polymer isolated using 4-volume cold methanol, re-filtrated and dried.

Haemolytic potential

Blood samples were collected from the vein of adult Human donor. Blood samples were taken and distributed to 5 ml/tube which contain EDTA (0.1 ml of 10% disodium EDTA solution) (Mohri *et al.*, 2007). The haemolysis tests were performed with modification as described in American Society for Testing and Materials (ASTM) (ASTM F 756-00, 2000)³⁸ and dos Santos *et al.*, 2005. 5 mg of PHA extracted from the above described methods were put each in 15 ml sterilize falcon tubes. 1 ml of human venous blood was added to each sample and

maintained at 37°C for 3 hours. Positive and negative controls were prepared by adding the same amount of blood to 7 ml of sterilized bidistilled water and PBS, respectively. Each tube was gently inverted twice each 30 minutes to maintain contact of the blood with the PHA. After incubation, each falcon was centrifuged at 2000 rpm for 15 minutes at 4°C using cooling system eppendorf centrifuge (5810 R, Germany). The optical densities (OD) of the haemoglobin released as a result of RBCs haemolysis in the supernatant was measured at 540 nm using spectrophotometer (PerkinElmer, Lambda EZ 201, USA). The percentage of haemolysis was calculated as follows:

$$\text{Haemolysis \%} = \frac{[(\text{OD}_{\text{sample}} - \text{OD}_{\text{negative control}}) / (\text{OD}_{\text{positive control}} - \text{OD}_{\text{negative control}})] * 100}$$

According to ASTM F 756-00 (2000) materials can be classified to three groups. Materials with percentage of haemolysis over 5% are considered haemolytic; with 5% and 2% slightly haemolytic and below 2% non-haemolytic.

Isolation of leucocytes

Leucocytes isolation was performed according to Klevezas *et al.* (2000) with some modifications. The blood samples were incubated for 10 minutes on ice in presence of 5 volume of freshly prepared NH₄Cl lysis buffer (37 mg tetrasodium EDTA salt, 8.9 g NH₄Cl, and 1 g KHCO₃ per liter of distilled H₂O pH 7.4). After incubation for 15 min the blood samples were centrifuged at 1600 rpm and 4°C for 10 minutes. The leucocytes were precipitated as white pellet (Klevezas *et al.*, 2000).

The pellet which contain the leucocytes cells was suspended in RPMI-1640 supplemented medium (RPMI media with 25 mM HEPES buffer and L-Glutamine) with 10% fatal calf serum. The cells was counted using haemocytometer. 10 µl of suspended cells was taken, and mixed with 10 µl of trypan blue. The cells were counted by light microscope (Olympus Optical Co., 3040-ADU, Japan). The dead cells appear as blue cells while live cells appear highly transparent.

Leucocytes cultivation in presence of PHA according to Pielka *et al.* (2003)

PHA produced using *P. aeruginosa* have been purified using four different extraction methods as described above. Different PHAs were sterilized by embedding them in sterilized absolute ethanol. Ethanol was sterilized using 22µm syringe filter followed by exposing it to UV using Laminar Flow (toTelstar Co., Bio-II-A, Class II, Spain) for 15 minutes under aseptic condition. The sterilize PHAs then dried and were put into wells (CELLSTAR® Cell Culture Products) containing 1 ml of RPMI medium supplemented with serum free of cells for 2 days. After 2 days, the medium is examined for the presence of any kind of contaminant by scanning the plate using phase contrast microscope (Olympus Optical Co., 1X70, Japan). Then 1

ml of lymphocyte cells containing 2×10^6 cells per ml was added to give final number equal to 2×10^3 cells/well. The plate was incubated at 37°C , 5% CO_2 and 89% humidity using CO_2 incubator (New Brunswick Scientific Co., CO28IR, England) for 48 hours. The cells number were counted after 48 hours using Phase contrast microscope (Olympus Optical Co., 3040-ADU, Japan) (Pielka *et al.*, 2003).

RESULTS AND DISCUSSION

Cultivation of different cell lines on different biomacromolecules including PHAs is a subject of investigation aiming to study either their biocompatibility or the best cells growth conditions. The researches dealing with leucocytes cultivation show increasing interest among different scientific groups. Many scientific points still open. The main feature of leucocytes that they contain the genomic DNA and they are able to replicate and maintain *in vitro* growth (Brooks, 2008).

PHAs which are proved biocompatible show an increasing interest in the recent years. The interest in the use of PHAs in medicinal applications is mainly due to their biological properties. Different criteria control the chemical, physical and biological properties of PHAs. In this study, leucocytes have been used to evaluate PHA polymer extracted using four methods. The best result was from

polymer extracted using direct chloroform extraction which show also minimum haemolytic activity as summarized in table 1. The Haemolysis of all the extracted PHA was between 2-5%, which consider as a safe haemolysis percentage (ATSM). Cytotoxicity of the different samples of PHA was applicable on normal leucocytes isolated from human blood using simple protocols according to Klevezas *et al.* (2000) as above (Klevezas *et al.*, 2000). The growth of leucocytes cells according to Pielka *et al.* (2003) indicated that the PHA has the ability to enhance the growth of Leucocytes cells when comparing with negative control as shown in Table 1 (Pielka *et al.* 2003). PHA which extracted by direct chloroform has show the best result (19.4×10^5 cells/well). The PHA culturing on leucocytes was examined using phase contrast microscope after 24 and 48 hours. The cells attached to PHA and grown on its surfaces as in fig. 1. The image of leucocytes which grown and attached to PHA show a clear increase in the cell number in all samples. The high cell number was with PHAs extracted by direct chloroform. Interestingly the cells also were small which an indicator for their viability. Finally we concluded the possibility of using PHA for leucocytes cultivation.

In conclusion PHA extracted using chloroform gives the best result with leucocytes cultivation. Haemolysis test is recommended to investigate the polymer cytotoxicity. The number of cells attached in the polymer surface indicates

Table 1: Cells number and Haemolytic index of various polymer preparation.

Treatment	Cells no/48 hr	Morphological change	% of increase	Haemolytic index
Method 1	19.4×10^4	No	46.97	2.28
Method 2	17.2×10^4	No	30.30	2.84
Method 3	15.3×10^4	No	15.91	3.02
Method 4	16.5×10^4	No	25.00	2.60
Without PHA	13.2×10^4	No	0.00	2.30

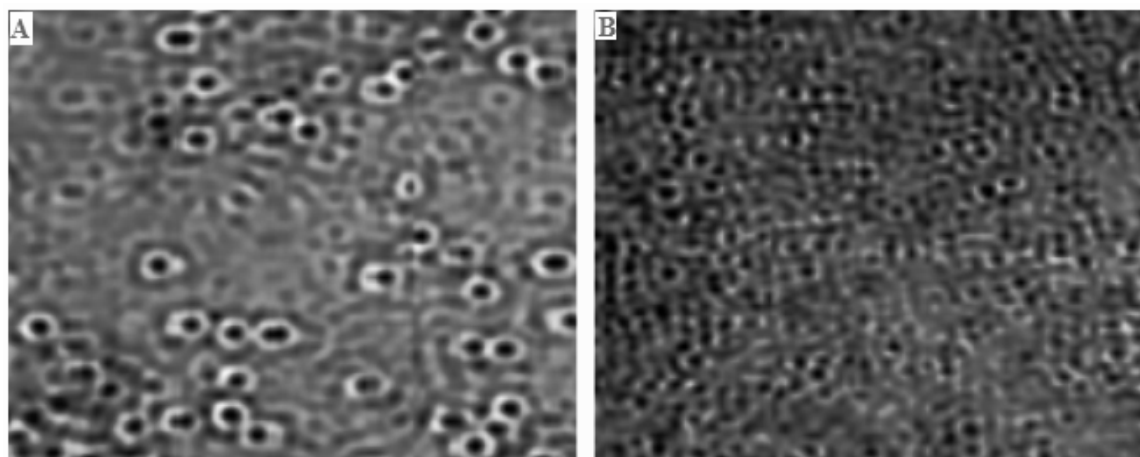


Fig. 1: Leucocytes grown on polystyrene [A] and Polyhydroxyalkanoates extracted using chloroform [B].

the biocompatibility of PHA. Using bioassays used in this study are important to evaluate the biocompatibility of the polymers.

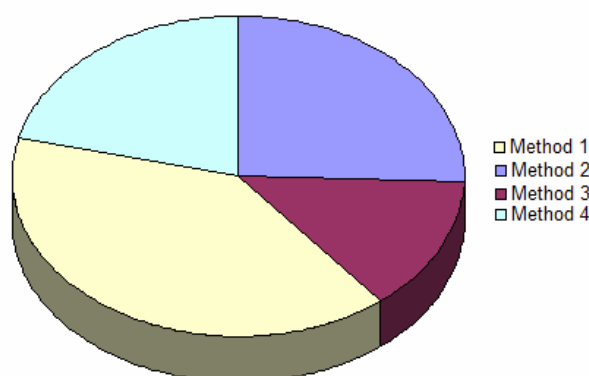


Fig. 2: PHA with different extraction methods give different Leucocytes number when used as sporting matrix

ACKNOWLEDGMENT

This work was supported in part by Mubarak City for Scientific Research and Technology Applications as well as a grant provided to Hassan M. A. during his M.Sc. study by Academy of Scientific Research and Technology, Egypt. We acknowledge the partial support provided to us by ASTF organization.

REFERENCES

- Amara AA (2008). Polyhydroxyalkanoates: from Basic Research and Molecular Biology to Application. *IIUM Eng. J.*, **9**(1): 37-73.
- American Society for Testing and Materials, (2000). ASTM F 756-00: Standard Practices for Assessment of Haemolytic Properties of Materials, Philadelphia, pp 159-167
- Anderson J and Dawes EA (1990). Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.*, **54**(4): 450-472,
- Brooks, ML (2008). Exploring Medical Language: A Student-Directed Approach, 7th Edition. MosbyElsevier, St. Louis, Missouri, USA, pp.398
- Cheng S, Chen GQ, Leski M, Zou B, Wang Y and Wu Q, (2006). The effect of D, L-beta-hydroxybutyric acid on cell death and proliferation in L929 cells. *Biomaterials*, **27**(20): 3758-3765.
- De Smet, MJ, Eggink G, Witholt BB, Kingma J and Wynberg H (1983). Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. *J. Biotechnol.*, **154**: 870-878.
- Fernandez-Castillo R, Rodrigues-Valera F, Gonzalez-Ramos J and Ruiz-Berraquero F (1986). Accumulation of poly(β -hydroxybutyrate) by halobacteria. *Appl. Environ. Microbiol.*, **51**: 214-216.
- Huisman GW, de Leeuw O, Eggink G, and Witholt B (1989). Synthesis of poly-hydroxyalkanoates is a common feature of fluorescent pseudomonads. *Appl. Environ. Microbiol.*, **55**: 1949-1954.
- Klevezas DSL, Martínez-Vázquez IO, Cuevas-Hernández B and Martínez-Soriano JP (2000). Antifreeze solution improves DNA recovery by preserving the integrity of pathogen-infected blood and other tissues. *Clin. Diagn. Lab. Immunol.*, **7**: 945-946.
- Kunze C, Freier T, Kramer S and Schmitz KP (2002). Anti-inflammatory prodrugs as plasticizers for biodegradable implant materials based on poly(3-hydroxybutyrate). *J. Mater. Sci. Mater. Med.*, **13**(11): 1051-1055.
- Lemoigne M (1923). Production d'acide β -oxybutyrique par certaines bactéries du groupe du *Bacillus subtilis*. *CR. Hebd. Seances Acad. Sci.*, **176**: 1761.
- Lemoigne M (1925). Etudes sur l'autolyse microbienne acidification par formation d'acide β -oxybutyrique. *Ann. Inst. Pasteur.*, **39**: 144.
- Lemoigne M (1926). Produits de l'hydratation et de polymérisation de l'acide β -oxybutyrique. *Bull. Soc. Chim. Biol. (Paris)*, **8**: 770-782.
- Lemoigne M (1927). Etudes sur l'autolyse microbienne. Origine de l'acide β -oxybutyrique formée par autolyse. *Ann. Inst. Pasteur (Paris)*, **39**: 144
- Lundgren DG, Alper R, Schnaitman C and Marchessault, RH (1965). Characterization of Poly-Beta-Hydroxybutyrate Extracted from Different Bacteria. *J. Bacteriol.*, **89**: 245-251.
- Malm T, Bowald S, Bylock A, Saldeen T and Busch C (1992). Regeneration of pericardial tissue on absorbable polymer patches implanted into the pericardial sac. An immunohistochemical, ultrastructural and biochemical study in the sheep. *Scand. J. Thorac. Cardiovasc. Surg.*, **26**(1): 15-21.
- Malm T, Bowald S, Karacagil S, Bylock A. and Busch C (1992). A new biodegradable patch for closure of atrial septal defect. An experimental study, *Scand. J. Thorac. Cardiovasc. Surg.*, **26**(1): 9-14.
- Misra SK, Valappil SP, Roy I and Boccaccini AR (2006). Polyhydroxyalkanoate (PHA)/inorganic phase composites for tissue engineering applications. *Biomacromolecules*, **7**(8): 2249-2258.
- Mohri M, Shakeri H and Lotfollah ZS (2007). Effects of common anticoagulants (heparin, citrate and EDTA) on routine plasma biochemistry of cattle. *Comp. Clin. Pathol.*, **16** : 207-209.
- Péaud Lenoël, C, and Kepes A (1952). On the molecular structure of the poly-,hydroxybutyrate lipids. *Bull. Soc. Chim. Biol.*, **34**: 563-575.
- Pielka S, Plauch D, Staniszewska-kus J, Solski L, Szosland L, Czarny A and Zaczyńska E (2003). Wound healing acceleration by a textile dressing containing

- dibutrylchitin and chitin. *Fibers and textile in eastern Europe*, **11**(2): 41.
- Shabeb MS, Younis M, Amara AA and Shoreit A (2005). Production of PHB-co-PHV from Compost Bioremediation by Purple non-sulfur bacteria. *Egyptian. J. Biotechnol.*, **19**: 467-478.
- Steinbüchel A. (1991). Polyhydroxyalkanoic acids. In: Byrom D (ed) "Biomaterials: novel materials from biological sources". Stockton, New York, pp.124-213.
- Tesema Y, Raghavan D. and Stubbs J, (2004). Bone cell viability on collagen immobilized poly (3-hydroxybutyrate-co-3-hydroxyvalerate) membrane: effect of surface chemistry. *J. Appl. Polym. Sci.*, **93**: 2445-2453.
- Valappil SP, Misra SK, Boccaccini AR and Roy I (2006). Biomedical applications of polyhydroxyalkanoates: an overview of animal testing and in vivo responses. *Expert. Rev. Med. Devices*, **3**(6): 853-868.
- van der Walle GA, de Koning GJ, Weusthuis RA and Eggink G(2001). Properties, modifications and applications of biopolyesters. *Adv. Biochem. Eng. Biotechnol.*, **71**: 263-291.
- Wallen LL and Rohwedder WK (1974). Poly- β -hydroxyalkanoate from activated sludge. *Environ. Sci. Technol.*, **8**: 576-579
- Wang YW, Yang F, Wu Q, Cheng YC, Yu PH, Chen J and Chen GQ (2005). Effect of composition of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) on growth of fibroblast and osteoblast. *Biomaterials*, **26**(7): 755-761.