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CHARACTERIZATION AND CYTOTOXICITY OF POLYHYDROXYALKANOATE MICROPARTICLES AS ADJUVANT MATRIX FOR THE IMMOBILIZATION OF *Pasteurella multocida* WHOLE-CELL VACCINE

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Abstract: Polyhydroxyalkanoate (PHA) is a biopolymer synthesized by some microorganisms as a carbon and energy storage material under limited nutrient sources, but in excess carbon supply. The PHA is known for its biocompatibility and biodegradability, with amenable physical properties, which can be tailor made into useful material for medical applications such as heart valve, pericardial patches, vascular graft and drug delivery system. Despite the advancement of PHA incorporation as a drug carrier, there is still limited understanding on the potential application of PHA polymer as adjuvant in delivering vaccine or inactivated whole-cell vaccine. This study explored the application of PHA microparticle as adjuvant in treating *Pasteurella* pneumonia, a bovine respiratory disease. The PHA was synthesized via bacterial fermentation and was fabricated into microparticle through solvent evaporation method. Optimizations in terms of the type of organic solvent used, the concentrations of polymer, and surfactant were carried out. Later, formalin-inactivated *Pasteurella multocida* B:2 cells were incorporated to the PHA microparticle using solvent evaporation technique. Characterization of PHA microparticles suggested sizes, ranging from 300 to 600 nm, with zeta potential values of -0.07 to 0.03 mV and polydispersity index of 0.28 to 0.41. The PHA-whole-cell adjuvant matrix were heterogenous in shape, with poly(3-hydroxybutyrate) [P(3HB] forming smoother and more spherical shape as compared to poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)]. The PHA-whole-cell matrix did not exhibit cytotoxicity on rat skeletal muscle (L6) cells. After 72 hours treatment, more than 84% cell viability was observed at 100 mg/mL concentration.

Keywords: Polyhydroxyalkanoate, *Pasteurella multocida*, pneumonia pasteurellosis, adjuvant, whole-cell vaccine.

Introduction

Biopolymers have wide applications in medical field. One of the common biopolymer is PHA, a microbial-derived polymer, known for its biocompatibility, biodegradability and thermoplastics properties (Doi, 1990). PHA is utilized by a wide variety of microorganisms as intracellular carbon and energy reserve material under the condition of limited nutrient sources, but in high supply of carbon (Sudesh *et al*., 2000). Poly (3-hydroxybutyrate) is the most common type of PHA homopolymer found in natural environment (Madison & Huisman, 1999).

The presence of secondary monomer such as 3-hydroxyvalerate (3HV) and 4-hydroxybutyrate (4HB) in P(3HB) backbone is the primary reason PHA being considered as a promising material in medical or pharmaceutical fields (Zinn *et al*., 2001). Both 3-hydroxybutyric acid and 4-hydroxybutyric acid have been identified as normal constituents of human blood, and therefore show good biocompatibility *in vivo* (Bhubalan *et al*., 2011). PHA can also be hydrolyzed by eukaryotic lipase (Zhao *et al*., 2002).

The physical properties of PHA and its copolymers, ranging from high crystallinity thermoplasts to elastomers which enable their versatile applications in the medical industry (Sudesh *et al*., 2000). Several PHA-based blends have been tested and developed into medical devices such as heart valve and pericardial patches (Bhubalan *et al*., 2011) and formulated as microsphere and nanoparticle in drug delivery system (Salman *et al*., 2003). However, despite PHA microparticle being seen as a promising vehicle, most published study has only reported on the delivery of analgesic and anti-inflammatory drugs (Murueva *et al*., 2013).

In this study, the application of PHA as an adjuvant material was investigated. An adjuvant is a useful additive, responsible for enhancing immune responses in association with the antigens (Gary & Gary, 2007). Biosynthesis of P(3HB) and P(3HB-*co*-4HB) copolymer were attained through the cultivation of isolated bacterial strains and four different types of polymer composition were formulated into microparticle emulsion. The different PHA-whole-cell adjuvant matrix containing *P. multocida* cells were analysed for the microparticle sizes and distributions and the *in vitro* cytotoxicity on L6 rat skeletal muscle cells was investigated.

Materials and Methods

Bacterial strain

The strain used for the biosynthesis of P(3HB-*co*-4HB), *Cupriavidus* sp. USMAA1020 (DSM 19416) was isolated from a sludge collected from Lake Kulim, Malaysia (Amirul *et al*., 2008) and *Bacillus megaterium* UMTKB-1 (KF991583) for the biosynthesis of P(3HB) was isolated from marine sponge, *Callyspongia* sp. (Bhubalan *et al*., 2013). The strain used for vaccine preparation was *P. multocida* B:2. All strains from the exponential growth phase were stored in -20°C in 20% glycerol (v/v) for maintenance purposes.

Biosynthesis of P(3HB) and P(3HB-co-4HB)

Biosynthesis of P(3HB-*co*-4HB) and P(3HB) were carried out using one stage cultivation method. *Cupriavidus* sp. USMAA1020 and *B. megaterium* UMTKB-1 were first pre-cultured in a nutrient rich media (10 g of peptone, 10 g of beef extract and 2 g of yeast in 1 liter of distilled water) at 30°C for 12 and 16 hours, respectively. Later, the pre-cultures were separately transferred into mineral salts medium (MSM) for PHA accumulation. MSM was supplemented with 1 ml/L $MgSO_4.7H_2O$ and 1 ml/L of trace element. To promote the biosynthesis of P(3HB-*co*-27% 4HB) in *Cupriavidus* sp. USMAA1020, filter sterilized 1,4-butanediol and 1,6-hexandiol at the concentration of 0.345 wt% was added into MSM separately. For P(3HB) production, *B. megaterium* UMTKB-1 was supplemented with glycerol at 20 g/L. All cells were harvested by centrifugation (10,000 rpm) at 4 °C and subjected to lyophilization.

Extraction of PHA Crude Polymer

The P(3HB) and P(3HB-*co*-4HB) was extracted from freeze-dried cells. Freeze dried cells were stirred in 200 mL of chloroform for 48 hours. Then, the extract was filtered using Whatman filter paper No.1 and concentrated to a volume of 15 to 20 mL using rotary evaporator (Eyela, Japan). Concentrated solution was then poured drop wise into 150 mL of stirred, chilled methanol to precipitate out the dissolved PHA. The precipitated PHA was filtered using 0.45 µm PTFE syringe filter (Sartorius, Germany) and left to dry overnight at room temperature.

Determination of PHA Content and Monomer Composition

The PHA content and composition from lyophilized cells were determined by Gas Chromatography (GC) 2010 (Shimadzu, Japan) analysis based on the same standard method (Braunegg *et al*., 1978), equipped with Superco SPB-1(L x I.D. 30 m x 0.25 mm) (Sigma, USA) column and *AOC*-20*i Auto Injector* (Shimadzu, Japan).

Preparation of PHA Microparticles and PHA-whole-cell Matrix

The PHA microparticles were formulated using solvent evaporation technique (Murueva *et al*., 2013). The fabrication of PHA-whole-cell matrix was also carried out using similar technique by mixing two phases which consists of an organic phase (50 mg PHA crude polymer in 10 mL of dichloromethane) and an aqueous phase containing 100 mL distilled water with 0.5% (w/v) polyvinyl alcohol and vaccine *P. multocida* B:2. The mixture was homogenized (IKA Ultra-Turrax, Germany) at 24,000 rpm for 5 min. Then, the emulsion was continuously mixed mechanically overnight until the solvent was completely evaporated. Microparticles-vaccine matrix were collected by centrifugation at 10,000 rpm for 5 min, rinsed 5 times in distilled water and lyophilized.

Field Emission Scanning Electron Microscope (FESEM)

The microparticles sizes and surface morphology were analysed using FESEM (Zeiss Leo Supra 50VP, Germany). Samples were mounted on aluminium stumps and coated with gold.

Zetasizer Analyzer (PHA Microparticle Size Analysis)

The size and distribution of microparticles was determined using Zetasizer Nano ZS (Malvern, UK). Sample was prepared by mixing liquid sample with distilled water at 1:9 ratio and loaded into folded capillary tube, before being analyzed.

In Vitro Cytotoxicity

The L6 cells was cultured in tissue culture flasks, incubated at 37 °C with 5% CO_2 in an incubator (ESCO, Germany). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and the medium was changed every 2-3 days. The L6 cells were cultured until confluence, followed by detachment using trypsin-EDTA. Cells were then seeded in 96-well plate at $1x10^5$ cells/ml concentration and incubated at 37°C with 5% CO_2. Viability of cells was assayed with 3-[4,5-dimethylthioazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT). Twenty µL of MTT was added into each well and was incubated for another 4 hours. Formed blue formazan crystal was dissolved with DMSO and the absorbance was measured using a microplate reader (Thermo Labsystems Multiskan Ascent, Finland) at 570 nm. Following the 72 hours treatment, a standard curve of L6 cells viability against sample concentrations was plotted and the IC_{50} values were determined.

Results and Discussion

Characterization of PHA Micro Particles

The shake-flask cultures of *Cupriavidus* sp. USMAA1020 had resulted in the production of 54±0.6 wt% P(3HB-*co*-27% 4HB) copolymer while the *B. megaterium* UMTKB-1 cultivation produced 3.6±0.1 wt% of P(3HB) homopolymer. Solvent evaporation is known as one of the first method established to produce polymeric micro and nanoparticle (Yadav *et al*., 2012). The different PHA polymer in our study was used to formulate the PHA microparticle. Table 1 shows the particle size, polydispersity index and zeta potential of four different types of PHA matrix. The microparticle sizes varied from 300–600 nm according to the different composition of PHA. *Cupriavidus* sp. USMAA1020 yielded the largest particle size of 519±17 nm for P(3HB) microparticles with the smallest size of 314±10 nm for P(3HB-*co*-85% 4HB) copolymer. The highest zeta potential was recorded for P(3HB-*co*-85% 4HB) from *Cupriavidus* sp. USMAA1020 at 0.03 mV while P(3HB) from *B. megaterium* KB-1 strain recorded the lowest zeta potential at -0.07 mV. The zeta potential value was used to evaluate the microparticle surface charge (Mainardes *et al*., 2010). The polydispersity index was within the range of 0.28 to 0.41, indicating a broader microparticle size distribution (Budhian *et al*., 2008).

Table 1: The properties of PHA microparticles produced using solvent evaporation technique

Strain	Type of PHA	Particle size (nm)	Polydispersity index (PdI)	Zeta potential (mV)
Cupriavidus sp. USMAA1020	P(3HB)	519±17	0.41	-0.01
	P(3HB-*co*-27%4HB)	323±3	0.33	-0.01
	P(3HB-*co*-85%4HB)	314±10	0.28	0.03
Bacillus megaterium UMTKB-1	P(3HB)	432±16	0.40	-0.07

The particle size is influenced by the type and concentrations of stabilizer, homogenizer speed and polymer concentration (Yadav *et al*., 2012). Utilizing similar method, an average diameter ranging from 700 nm up to 2.6 µm of P(3HB) was obtained (Murueva *et al.*, 2013), P(3HB-*co*-6.1% 4HB) and P(3HB-*co*-16% 4HB) can be used as matrices for cytostatic drug where the polymeric nano or microparticle could aid in promoting effective permeation through the cell membrane with higher stability in blood stream (Yadav *et al*., 2012).

Observation of PHA-Whole-Cell Matrices Using SEM

The SEM images of all the four types of PHA-whole-cell matrices are shown in Figure 1. The formations of microparticle in all the four matrices were heterogenous in shape. The surface structures of P(3HB) from both strains appeared smoother and spherical in shape. There was also less surface deformation on the particle as compared to P(3HB-*co*-4HB). On the other hand, the structures of both P(3HB-*co*-4HB) from *Cupriavidus* sp. USMAA1020

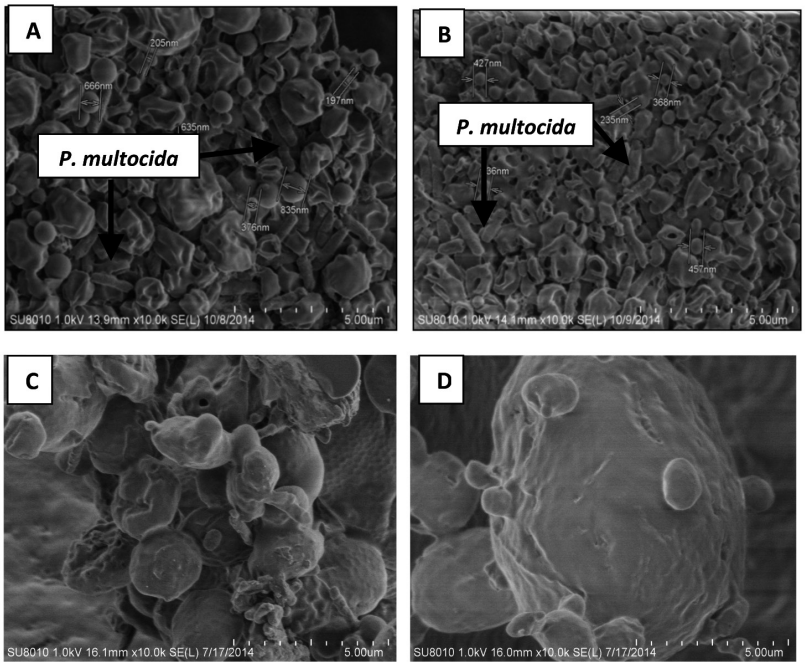

Figure 1: SEM images of PHA microparticles with vaccine *P. multocida* B:2: **A** P(3HB) produced by *Cupriavidus* sp. USMAA1020, **B** P(3HB) produced by *B. megaterium* UMTKB-1, **C** P(3HB-*co*-27%4HB) produced by *Cupriavidus* sp. USMAA1020, **D** P(3HB-*co*-85%4HB) produced by *Cupriavidus* sp. USMAA1020

strain showed rough surface and less uniform with undefined irregular shape. Similar observation with spherical shape and smoother surface has been reported in the formulation of P(3HB) microparticle for drug delivery purpose. However, P(3HB-*co*-4HB) at 6.1 mol% and 16 mol% show almost the same physical properties as P(3HB) obtained (Murueva *et al*., 2013). Even though both P(3HB-*co*-4HB) in our study exhibited smaller particle sizes, the P(3HB) microparticles form better matrix layout for vaccine to embed onto as compared to P(3HB-*co*-4HB).

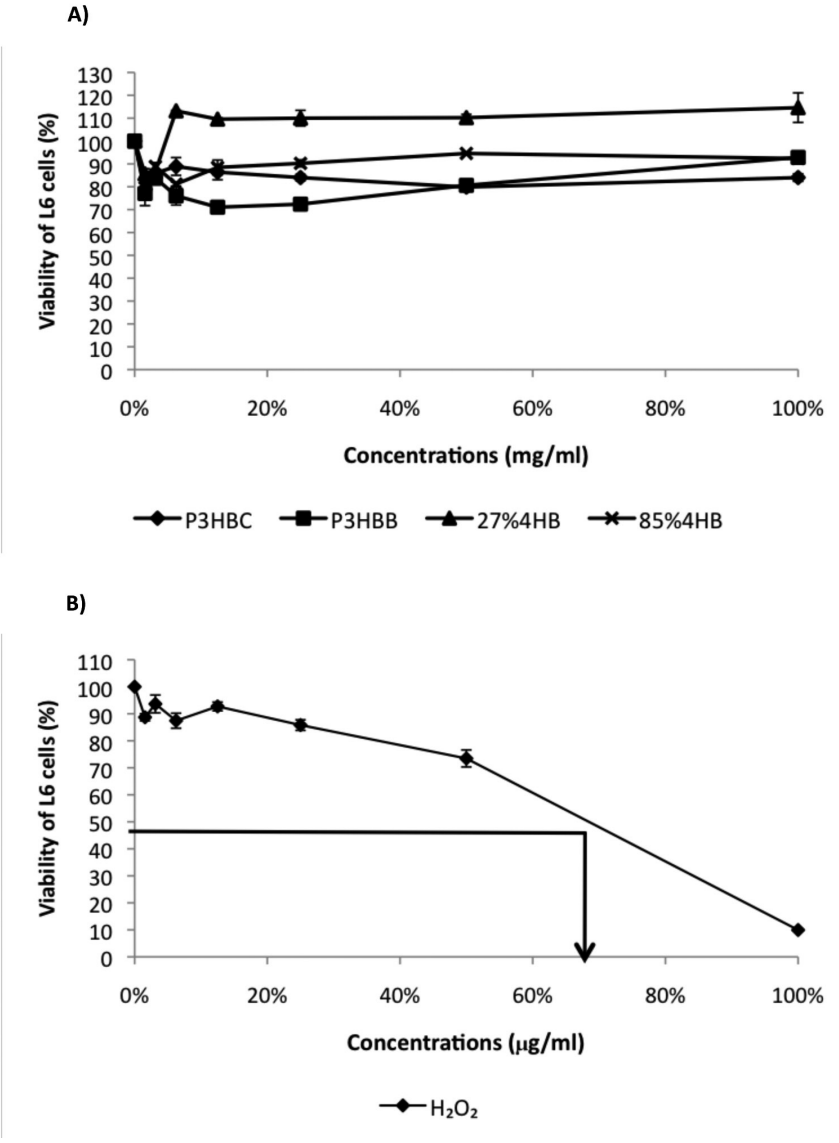

Figure 2: Cytotoxicity of (A) the four types of PHA-whole-cell matrix and (B) H_2O_2 against L6 cells. P3HBC indicates P(3HB) produced by *Cupriavidus* sp. USMAA1020; P3HBB indicates P(3HB) produced by *B. megaterium* UMTKB-1; 27% 4HB indicates P(3HB-*co*-27% 4HB) produced by *Cupriavidus* sp. USMAA1020; 85% 4HB indicates P(3HB-*co*-85% 4HB) produced by *Cupriavidus* sp. USMAA1020. Data shown is the mean of triplicates

Cytotoxicity Study on L6 Rat Skeletal Muscle Cells

The cytotoxicity effects of four different types of PHA-whole-cell matrices and hydrogen peroxide (H_2O_2) against the rat skeletal muscle cells (L6) after 72 hours treatment was assessed using MTT assay (Figure 2). The L6 cells were used due to the potential use of rat as animal model to understand the effects of PHA micro particle as adjuvant in the future *in vivo* study. There was no reduction in the cell viability in all the four types of PHA polymeric emulsion as compared to the H_2O_2 positive control, which showed reduced cell viability in a concentration-dependent manner. At the highest concentration, P(3HB-*co*-27%4HB) recorded the highest cell viability (114.5±6.4%). P(3HB) polymer from *Cupriavidus* sp. USMAA1020 recorded the lowest cell viability among all the emulsion (84±1.6%), On the other hand, the H_2O_2 positive control showed only 9.94±0.3% cell viability. While H_2O_2 recorded an IC_{50} value of 68 µg/mL, there were no IC_{50} values observed for all the four polymeric emulsions.

In this study, MTT assay did not suggest any cytotoxic effects from the emulsions formulated based on the four different types of PHA. Instead, cell proliferation was observed from P(3HB-*co*-27%4HB) microparticle emulsion, following the 72 hours incubation. These results are in good agreement with the data reported in the cytotoxicity studies of P(3HB-*co*-4HB) consisting of 50mol% of 4HB monomer against V79 and L929 fibroblast cells with no IC_{50} value recorded (Siew *et al*., 2006). Similarly, the microparticles formulated from P(3HB), P(3HB-*co*-6.1% 4HB) and P(3HB-*co*-16% 4HB) show no cytotoxic effects against mouse fibroblast NIH 3T3 cells when analysed with MTT assay (Murueva *et al*., 2013).

Conclusion

In this study, P(3HB) was found to exhibit better properties as matrices in terms of smoother particle surface and stable layout as compared to P(3HB-*co*-4HB) in embedding the whole-killed vaccine. No significant cytotoxic effects were observed for all the four polymeric emulsions when treated on the L6 cells as compared to the H_2O_2 positive control. The PHA-whole-cell matrix has high potential to be used as adjuvant in vaccination against pneumonia pasteurellosis.

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