Fabrication, Characterization and Degradation of PHB and PHBV Microspheres For Liver Cell Growth

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1. Introduction

Tissue engineering is a rapidly growing approach used to overcome the lack of available organs and donor shortages for transplantation. The task of a tissue engineer is to construct the desired scaffold for the intended tissue formation. Among various organs in our body, the liver is able to recover after injury and perform normal functions through spontaneous regeneration. The polymeric scaffolds used for tissue engineering require biodegradability, biocompatibility, non-toxicity and high porosity. Novel tissue engineering technique uses three dimensional scaffolds as structural templates for cell adhesion and subsequent tissue formation, since 3D scaffolds have more available surface area for cell attachment and adequate porosity for nutrients and oxygen transport. Three-dimensional microspheres have been widely used for biomedical applications, especially drug delivery [1-2] and tissue engineering. This research presents work on the fabrication of biodegradable porous polymer microspheres as temporary scaffolds for liver cell growth, in order to create desired engineered liver tissue for the regeneration of diseased livers.

Microbial PHB and PHBV copolymers with HV contents of 5%, 8% and 12% (PHBV(5%), PHBV(8%) and PHBV(12%)) have been shown to be a suitable for tissue engineering [3-4]. The PHB and PHBV microspheres were fabricated by an oil-in-water (o/w) emulsion solvent evaporation technique. To ensure that PHB and PHBV are biocompatible for medical purposes, a direct contact cytotoxicity test was conducted by following the ISO 10993-5 method using L-929 mouse fibroblasts. To test for liver tissue engineering, human hepatoma cells, from the Hep3B cell line, were cultured on the microspheres. Polymer thin films were also prepared as 2D scaffolds in comparison with the 3D microspheres. Liver specific functions such as albumin production, cytochrome P450 activity and cell proliferation were evaluated for both the microspheres and thin films.

2. Experimental

Polymer microspheres were prepared by using an oil-in-water (o/w) emulsion solvent evaporation technique. The four different polymers are dissolved in chloroform before PVA was added as emulsifier. The solution was subsequently homogenized and poured into a PVA buffer solution for solvent evaporation. The resultant microspheres were filtered, washed, lyophilized in liquid nitrogen and freeze dried for 72 h and stored at 4ºC for further use. Polymer thin films were prepared as 2D scaffolds by dissolving the polymers in chloroform and poured into a 50 mm glass Petri dish. The solutions were dried under a fume hood overnight to obtain polymer thin films of 200 μm thickness. The resulting polymer films were freeze dried for 72 h to remove any remaining solvent.

To prepare a positive control, a sterilized glass coverslip was coated with 1 mL of 1 mg/mL poly(L-lysine) aqueous solution for 3 days at room temperature. Then, 20 μL of a 1 mg/mL aqueous solution of laminin was added and incubated for 12 h at 37ºC. Polyurethane film containing 0.1% zinc diethyldithio-carbamate (ZDBC) was used as a negative control.

Human hepatoma cell line (Hep3B) was obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% of the FBS, 2 mM L-glutamine and 1% antibiotic antimycotic solution (100 units/mL penicillin G, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B). For the microspheres, the cell density of 1×10^5 cells/2mL was put into a 15 mL conical tube with the sterile microspheres and shaken gently for 10 min, allowing the cells to adhere onto the microspheres. The microspheres with attached cells were transferred into a 30 mm diameter polystyrene Petri dish and incubated in a 5% CO_2 incubator at 37ºC. For thin films and controls, the cells were seeded at a density of 1×10^5 cells/2mL and incubated. The culture medium was renewed every 3 days. Cell adhesion, growth and proliferation on the scaffolds were studied under an optical microscope, SEM and LSCM.

3. Results

Three-dimensional PHB and PHBV microspheres were made in the size range between 100-200 µm (± 30 µm) which was observed to be the most suitable size for Hep3B (20-30 µm ± 5 µm) growth. External morphologies of the microspheres were examined by SEM. All the spherical microspheres were in the size range between 150-230 µm (± 30 µm), having multi-vesicles on the external surface due to the removal of internal water droplets after lyophilization.

Cytotoxicity test was performed to determine the biocompatibility of the polymers. The ISO 10993-5 test is based on a neutral red dye which penetrates the cell membranes and stains the lysosomes of the viable cells red. The quantitative cytotoxicity test results were calculated by averaging five replicates and the inhibition zone of the specimen was recorded as diameter (mm)/area (mm^2). Figure 1 shows the quantitative results of the cytotoxicity test for the positive control (PC), negative control (NC) and the polymer thin films. While the cytotoxicity was 100% for the positive control, PHB, PHBV(5%), PHBV(8%) and PHBV(12%) films showed cytotoxicities of 18.4%, 12.7%, 10.6% and 12.7% respectively, which clearly illustrates very low cytotoxic effects of PHB and PHBV polymers. Therefore, the polymers used here for tissue engineering are deemed non-toxic.

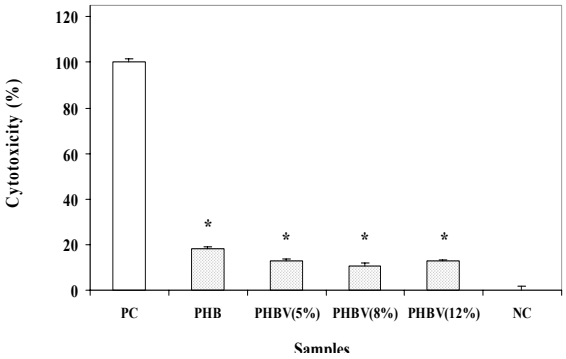

Figure 1. Cytotoxicity results for positive control (white bar), negative control (black bar) and polymer thin films (dotted bars). Values represent means ± SD, $n = 5$. Statistical analysis was performed by Student *t*-test. $*p < 0.01$.

Hep3B cells spread and flatten to form polygonal shape, 20-40 µm in dimension after 4 days of cultures on tissue culture polystyrene dishes. The cells firmly adhered to the substrate and forming tight contact with each other in a confluent monolayer of cells. SEM scans of Hep3B cells grown on the PHBV(5%) thin film showed that they were spread and flattened to form a monolayer. The same phenomena were observed for the PHB, PHBV(8%) and PHBV(12%) films.

The growth of the Hep3B cells on the PHBV(12%) microspheres at various days of culture were shown as the optical micrograph images in Figure 2. Few cells were found attached to the microspheres after 2 days of culture. Cell-cell contacts between two microspheres occurred after 4 days of culture. From day 6 onwards, cells were observed to bridge one microsphere to other microspheres as well as stretching to fill the gaps between the microspheres, forming multilayers of cells. After 2 weeks of culture, the cells were seem to become confluent on the microspherical scaffolds and developed cell-polymer aggregates that led to a tissue-like structure. SEM images confirmed the results, showing strong cell-cell interaction as well as cell-substratum interaction of the liver cells and the polymer microspheres. Multilayers of cells were observed to bridge microspheres and covered the surfaces.

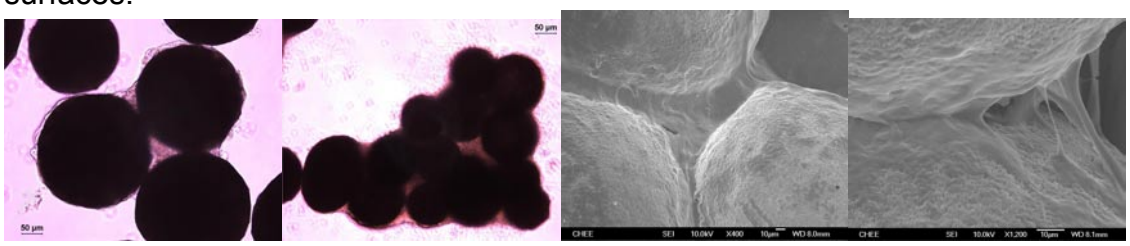

Figure 2. Optical light microscopy images and SEM images of Hep3B cells grown on PHBV (12%) microspheres

4. Discussion

Microbial PHB and PHBV polyesters have attracted much attention for biomedical applications due to their biodegradability, biocompatibility, low toxicity and thermoplasticity. PHB, PHBV(5%), PHBV(8%) and PHBV(12%) were specifically chosen to be fabricated into two-dimensional thin films and three-dimensional microspheres to be used as artificial scaffolds. The porous microspherical polymer scaffolds are hypothesized to assist in enabling a significant increase in liver cell growth, proliferation and liver specific functions. Human hepatoma cell lines, Hep3B, were cultured in-vitro and seeded on both polymer microspheres and thin films to create artificial liver tissue *in vitro*. MTT results showed that the cell proliferation on the microspheres were more than 2-5 times higher than on thin films at 6 days of culture. At the same culture period, EROD and ELISA assays showed that cytochrome P-450 activity and albumin secretion respectively were 1 to 3 times higher on the microspheres than on thin films. In conclusion, the PHB, PHBV(5%) and PHBV(8%) microspheres can be considered as promising polymer scaffolds for liver tissue engineering

5. References

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