Growing tissue-like constructs with Hep3B/HepG2 liver cells on PHBV microsphere scaffold

Xin Hao Zhu[a], *Chi-Hwa Wang*[a], *Yen Wah Tong*[a,b,*]

[a]*Department of Chemical & Biomolecular Engineering, National University of Singapore*
[b]*Division of Bioengineering, National University of Singapore*
21 Lower Kent Ridge Road, Singapore 119077

[*] *Corresponding author: Yen Wah Tong. E-mail: chetyw@nus.edu.sg*

In this study, a solvent evaporation technique was used to fabricate poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV, 8% PHV) microspheres as scaffold to guide liver cell growth. Human hepatoma cell lines, HepG2 and Hep3B, were cultured in vitro on both the microspheres and polymer films. SEM and optical microscope images showed that multilayer cells were formed among the microspheres to bridge them together and developed into cell-construct aggregates after one week of culture. MTT results showed that the cell proliferation on the microspheres was more than two times higher than that on the films after 12 days of culture. The cells seeded on microspheres secreted albumin 2-4 times more than that on the positive control after one weeks of culture, which indicated that this hepatic function was greatly improved by the aggregation of cells on microspheres. Although HepG2 failed to express P-450 activity, this hepatic function was preserved when Hep3B cultured on microspheres. All the results indicated that PHBV microspheres are appropriate scaffolds for liver tissue engineering.
Key words: PHBV; microspheres; liver cells; liver tissue engineering.

1. Introduction

As temporary replacement of extracellular matrix (ECM), polymer scaffolds play an essential role in tissue engineering. The use of microspheres as scaffolds is a new idea in tissue engineering with many advantages due to its versatility [1-3]. The microspheres can be easily modified in a controllable manner to introduce various ECM proteins for enhanced cell-scaffold interaction [4]. Growth factors and other molecules can be delivered controllably from the microsphere scaffold to regulate cell growth as well as to promote vascularization. Additionally, the microspheres can be easily assembled into various shapes with controlled cell structures by growing different cell types on different sets of microspheres.

Our previous works have demonstrated that water-in-oil-in-water double emulsion solvent evaporation is a suitable technique to produce porous PHBV microspheres [3]. In this work, we continued the study by preparing three size distributions of microspheres using different homogenizing speeds to determine the optimum dimension of microspheres for the growth of liver cells. Methylthiazol tetrazolium (MTT) and total DNA quantification assays were used to measure the liver cell proliferation rate, while enzyme-linked immunosorbent assay (ELISA) and ethoxyresorufin-O-deethylase assays (EROD) were used to determine the functionality of the liver cells. The objectives of this paper are to examine the effects of microspheres sizes on cells growth and also to compare the functions of two human hepatoma cell lines, HepG2 and Hep3B cultured on PHBV microspheres.

2. Materials and Methods

A solvent evaporation technique was used to fabricate PHBV microspheres. The size distributions of microspheres produced by different homogenizing speeds were measured by a particle size analyzer (Coulter LS-230, Florida, USA). The external surface morphologies of the microspheres were characterized by a scanning electron microscope (SEM) (JSM-5600VL, JEOL, Tokyo, Japan). To observe the internal structure of the microspheres, samples were sectioned with a cryostat microtome (Leica CM3050S, Germany) with blade step setting at 30 μm and air dried.

Human hepatoma cell lines, HepG2 and Hep3B (American Type Culture Collection, Virginia, USA), were selected as models for the culture of primary hepatocytes. Cells were seeded at 2.5 × 10^4 cells/well, or approximately 1×10^4 cells/cm^2 of microsphere surface. The cells attached on the microspheres were counted by a trypan blue exclusion method to estimate the seeding efficiency. The morphology of cells and cell-microspheres construct was observed under optical microscope (Leica DMIL, GmbH, Germany) and a scanning electron microscope (SEM) (JSM-5600VL, JEOL, Tokyo, Japan). Hep3B cells viability was assessed using a live/dead assay (Molecular Probes, USA). The fluorescent green-colored cells were live cells while the fluorescent red-colored cells were dead ones.

Cell proliferation were assessed by using the MTT assay and total DNA quantification. The albumin secretion by liver cells was measured using ELISA (Human albumin ELISA quantification kit, Bethyl, USA), which is based on the antibody-sandwich mechanism. The P-450 activity (CYP1A1/2) represents the detoxification ability of liver cells, which was measured by using an ethoxyresorufin-o-deethylase (EROD) assay.

3. Results and Discussion

3.1 Preparation and characterization of PHBV microspheres

Three size distributions of microspheres were prepared using different homogenizing speeds to study the effects of size and shape of microspheres on liver cell proliferation and functions. Figure 1 shows an example the internal and external morphology of the PHBV microspheres produced. It can be seen that the microspheres have spherical shapes with uniform sizes, and the surfaces have rough topography with nano-pores. The porous structure of the microspheres was induced by the removal of internal water droplets through lyophilization, which is a crucial feature of tissue engineering scaffold because the pores will benefit the exchange of nutrient, oxygen, cellular signals as well as removal of metabolic wastes.

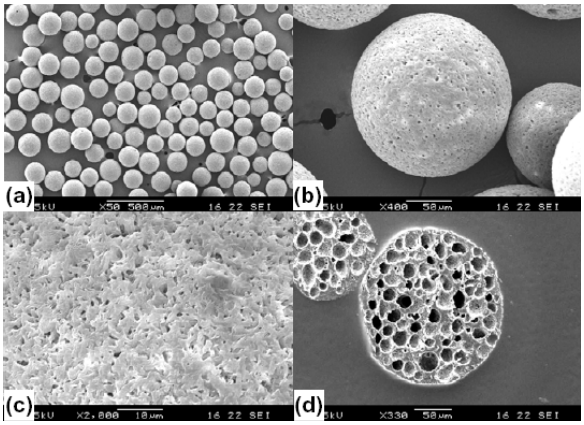

Figure 1. (a) and (b) SEM images of PHBV microspheres illustrating their spherical shape and uniform size; (c) magnified image of (b) showing a rough surface with nano-pores; (d) cross-section of the microsphere;

3.2 Culturing HepG2 and Hep3B cell lines on PHBV microspheres

Approximately 20-30% of cells attached onto the microsphere scaffold and the highest seeding efficiency was found on the smallest microsphere (Figure 2). As HepG2 cells are anchorage-dependent, the initial cell adhesion on microspheres is critical because it precedes other events, such as cell spreading, proliferation and differentiation. Compared to two dimensional scaffolds such as polymer films, seeding efficiency on most three dimensional scaffolds is generally lower and it is more difficult to distribute the cells uniformly onto the scaffolds.

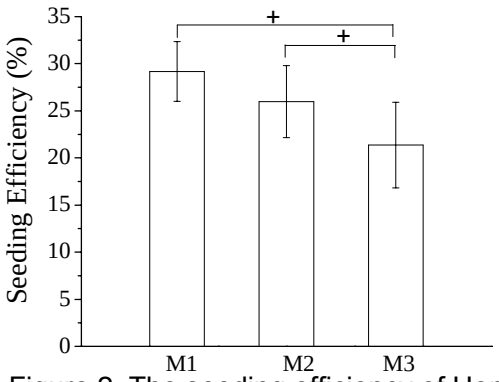

Figure 2. The seeding efficiency of HepG2 cells on microsphere scaffolds ($^{+}p<0.05$).

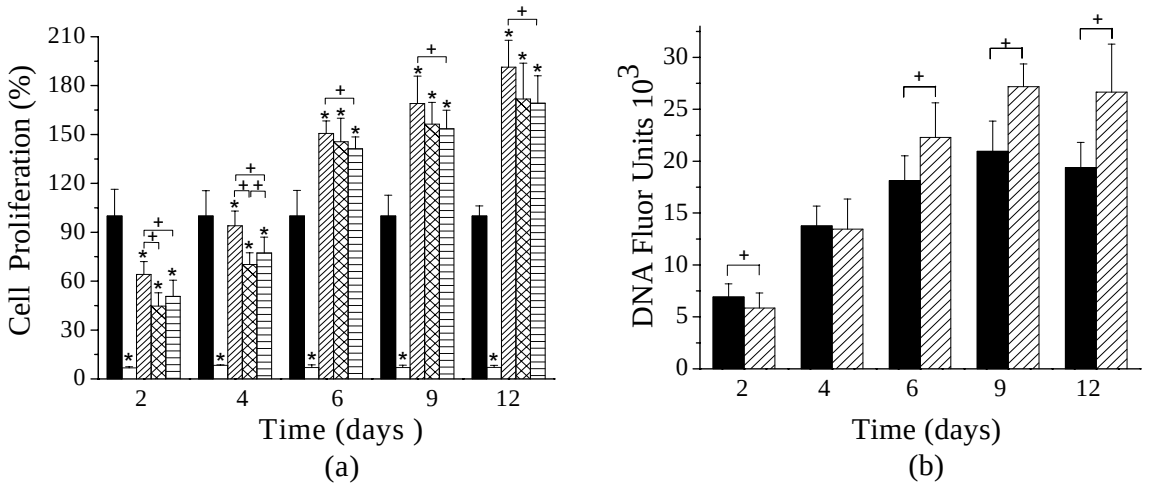

Figure 3. Proliferation of HepG2 cells cultured on positive control (■), negative control (□), M1 (▨), M2 (▩) and M3 (▤) as assessed by (a) MTT assay; (b) total DNA quantification. Values represent means±SD, n=3 (* p<0.05 as compared to the positive control by ANOVA, +p<0.05 by t-test comparison between the two samples).

The proliferation of HepG2 cells on microspheres as well as the positive control (laminin coated PLL films) and negative control (ZDBC films) were quantitatively evaluated by MTT and total DNA quantification during the 12 days of culture (Figure 3). It can be seen that the cell proliferation on the three types of microspheres were lower than that of the positive control at day 2 and day 4 of culture, while after one week, the proliferation on the three sizes of microspheres were significantly increased to 150.7 %, 145.5 % and 141.3 % respectively. From day 6 onwards, cell proliferation increased steadily on the microsphere scaffolds. By the last day of culture, the proliferations on M1, M2 and M3 reached 191.3%, 171.9% and 169.3%. Figure 5(a) also indicated that HepG2 showed much lower proliferation rate on the negative control, where all values was lower than 10%. The proliferation of Hep3B cells showed a similar profile (data not shown). To more accurately quantify the cell proliferation, a total DNA

assay was conducted. Figure 3(b) shows a similar profile of an increase in cellular DNA which is indicated by the fluorescence intensity as the MTT results for the 2-D positive control and the M1 samples. T-tests between these two samples show a significantly greater amount of cell growth on M1 which confirmed that cell proliferation was improved when cultured on 3-D microsphere scaffolds. HepG2 cells showed much higher proliferation rates on microspheres than on positive controls (Figure 3). One reason for this is that compared to two-dimensional positive control, microspheres have a larger surface area, which allow larger number of cell growth. More importantly, it is believed that the shape of the scaffold affects cell adhesion, proliferation and function greatly. Smaller microspheres improved cell-cell interaction and cell-substrate interaction, which may stimulate cell proliferation. The cells would also be more active in aggregate forms.

Albumin production by HepG2 and Hep3B cells cultured on controls and microspheres were assessed by ELISA and the results are shown in Figure 4 (a) and (b) respectively. HepG2 cells cultured on microspheres maintained a steady increase of albumin secretion throughout the study although there was a slight drop at 12 days of culture. No significant increases in albumin secretion were found on the positive controls. It can be seen that Hep3B cells seeded on microspheres secreted albumin 2-4 times more than that on the positive control after 7 days of culture, which indicated that this hepatic function was greatly improved by the aggregation of cells on microspheres.

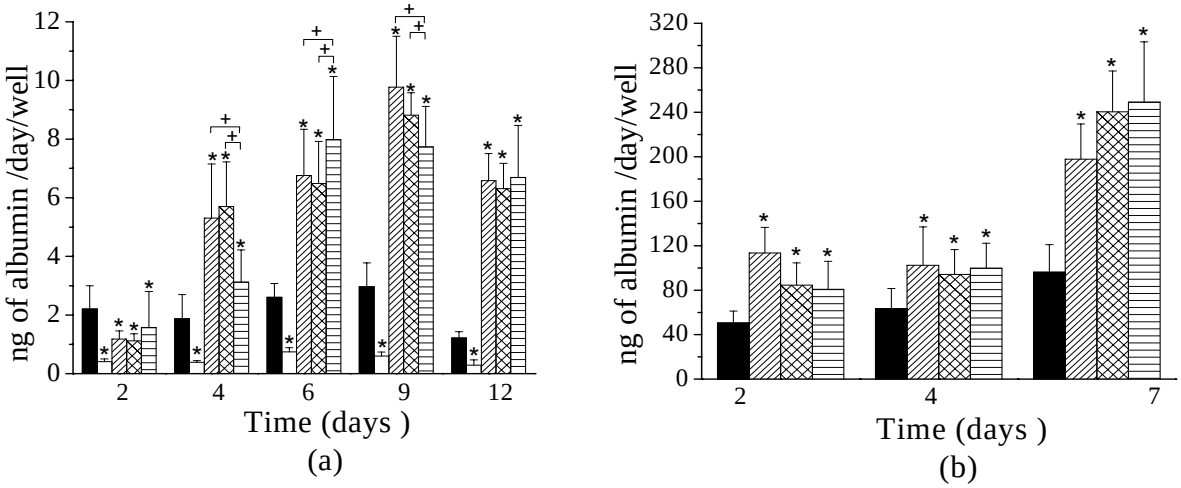

Figure 4. Albumin secretion by (a) HepG2 and (b) Hep3B cells cultured on positive control (■), negative control (□), M1 (▨), M2 (▩) and M3 (▤). Values represent means±SD, n=3 (* $p<0.05$ as compared to the positive control by ANOVA, +$p<0.05$ by t-test comparison between the two samples).

HepG2 and Hep3B cells secreted significantly more albumin when cultured on microspheres than on positive control. The results from cell proliferation assays indicated that HepG2 cells proliferated faster on microspheres than on the positive control (Figure 3), and the larger number of cells might secrete a higher amount of albumin (Figure 4a). More importantly, it is believed that the cell-scaffold aggregate can improve the hepatic functions of liver cells [5]. From 4 days onwards, HepG2 attached onto microspheres secreted albumin 2-4 times more than that on the positive control. However, the proliferation was only 0.8-1.9 times of the

positive control (Figure 3), so the higher level albumin secretion could be attributed mainly to the improvement of hepatic function by the cell-polymer aggregation. Compared to HepG2, Hep3B cells expressed much higher levels of albumin secretion. After 7 days of culture, Hep3B cell grown on microspheres secreted albumin up to 30 times more than that by HepG2 cells. According to the results obtained by ELISA, it can be seen that albumin secretion by HepG2 and Hep3B cells were well preserved on microsphere scaffolds and the levels were much higher than that on the positive control

Our experiments indicated that HepG2 cells could not express P-450 functions. Therefore only Hep3B cells were used to test this hepatic function. The cytochrome P-450 enzyme activities of Hep3B grown on the positive control and microsphere scaffolds were measured over one week in culture and the results are shown in Figure 5. Although there are no statistically significant differences among the three different sizes microsphere scaffold ($p>0.05$), the P-450 activities of Hep3B cultured on microspheres were significantly higher than two-dimensional positive control. The higher P-450 activity which Hep3B cells performed on 3D microsphere scaffold than on positive control again proved that the aggregation of cells on microspheres can improve hepatic cellular functions. Therefore, 3D microsphere scaffolds are proved to be suitable substrates to guide liver cell proliferation and function, with a potential role in regenerating liver tissue in vitro.

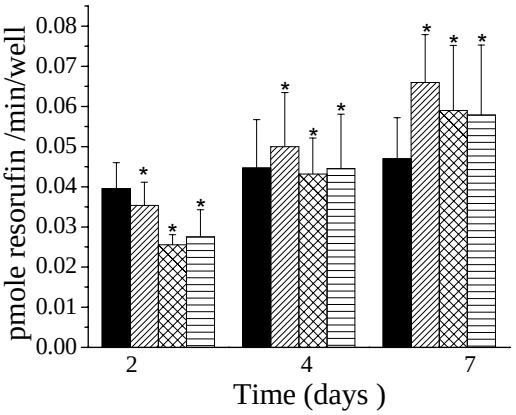

Figure 5. Cytochrome P-450 activity of Hep3B cells cultured on positive control ( ), M1 ( ), M2 ( ) and M3 ( ). Values represent means±SD, n=3 (* $p<0.05$ as compared to the positive control by ANOVA).

5. Conclusions

Our study have demonstrated that PHBV microspheres with the size range between 100-300μm are suitable scaffolds to support liver cells growth. Optical and SEM images showed that cell-scaffold constructs were formed after 2 weeks of culture, while CLSM images confirmed that most of the cells were alive. Compared with cells cultured on 2-D positive control, cells growth on microsphere scaffold showed higher proliferation as well as improved hepatic functions.

Acknowledgement

This work was funded by the National University of Singapore under the grant number R279000096112.

References

[1] Barrias CC, Ribeiro CC, Lamghari M, Miranda CS, Barbosa MA. Proliferation activity and osteogenic differentiation of bone marrow stromal cells cultured on calcium titanium phosphate microspheres. J Biomed Mater Res 2005; 72A: 57-66.

[2] Mercier NR, Costantino HR, Tracy MA, Bonassar LJ. Poly(lactide-co-glycolide) microspheres as a moldable scaffold for cartilage tissue engineering. Biomaterials 2005; 26:

1945-1952.
[3] Tong YW, Thwin CS. Fabrication, characterization and degradation of PHB and PHBV microspheres for liver cell growth. Transactions of the American Institute of Chemical Engineers Annual Meeting (2004).
[4] Zhu XH, Gan SK, Wang CH, Tong YW. Proteins combination on PHBV microsphere scaffold to regulate Hep3B cells activity and functionality: a model of liver tissue engineering system. Submitted to J Biomed Mater Res 2006 (under review).
[5] Bhandari RNB, Riccalton LA, Lewis AL, Fry JR, Hammond AH, Tendler SJB, Shakesheff KM. Liver tissue engineering: A role for co-culture systems in modifying hepatocyte function and viability. Tissue engineering 2001; 7(3): 345-357.