Combination of Proteins on PHBV Microsphere Scaffold to Regulate Hep3B cells Activity and Functionality for an *In Vitro* Model of Liver Tissue Engineering

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*

The synergistic effects of extracellular matrix (ECM) proteins combination on Hep3B cell proliferation and functions are studied herein. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) microspheres were covalently conjugated with three types of proteins, collagen (type I), laminin and fibronectin by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide cross linkers. The densities of grafted proteins were quantified by using a Micro-BCA kit. A human hepatoma cell line, Hep3B, was then cultured *in vitro* on the ECM proteins modified microspheres for two weeks. Cell proliferation was estimated using MTT method and two hepatic functions, albumin secretion and P-450 activity, were evaluated using ELISA and EROD assays respectively. The results indicated that combination of the three ECM proteins on microsphere surfaces has a significant effect on the proliferation of Hep3B cells, thus better mimicking the *in vivo* environment for liver tissue engineering.
Key words: PHBV; protein; surface conjugation; liver tissue engineering

1. Introduction

The use of microspheres as scaffolds is a new idea in tissue engineering which is fast gaining attention because of the versatility that microspheres offer in guiding cell growth [1-4]. This versatility includes not just the ease of scaffold assembly into various shapes suitable for different tissue application, but also offers easy and controllable surface modification for enhanced cell-material interaction. We have previously demonstrated that PHBV microspheres were well suited for liver cell growth with high cell proliferation as well as improved cellular functions of the liver as compared to cells cultured on two-dimensional surfaces of thin films [4].

In this work, we aim to show the versatility of microspheres as tissue engineering scaffold by easily modifying the scaffold surfaces in a controllable manner. We have modified the whole surface of PHBV microspheres with collagen (type I), fibronectin and laminin. With the ultimate aim to mimic the *in vivo* conditions of the body, we have cultured a model liver cell line, Hep3B, either on microspheres grafted with single protein type or on microsphere mixtures grafted with three types of proteins respectively, to prove that various ECM proteins do interact with cells in a synergistic manner to regulate cell activity and functionality.

2. Materials and Methods

2.1 Microsphere preparation and proteins conjugation

A solvent evaporation technique was used to fabricate PHBV microspheres. The microspheres were hydrolyzed in 6 M NaOH solution for 15 min to introduce functional groups. The hydrolyzed microspheres were re-suspended into MES buffer (pH=6.0) containing 10mM EDC and 10mM sulfo-NHS to activate the carboxyl groups on the surfaces and subsequently

immersed in PBS buffer (pH=7.4) containing 0.05 μM proteins and shaken at 130 rpm for 24 hr at room temperature. The protein quantity on the microsphere surface was determined using a Micro BCA™ Protein Assay Kit (Pierce, Rockford, USA) according to the manufacturer's instruction.

2.2 Hep3B cells growth on PHBV microspheres

Microspheres were sterilized using 70 v/v% ethanol followed by completely washing with sterilized PBS. Five milligram of sterilized microspheres was transferred to each well to form monolayer, and cells were subsequently seeded at 3×10^4 cells/well. In order to further mimic the *in vivo* environment, individual protein conjugated microspheres were added in a mass ratio of 1:1:1 (Collagen : Fibronectin : Laminin) to form the mixed protein conjugated samples. The plate was topped up to 1mL DMEM per well after 1.5 hr of incubation.

The morphologies of cells and cell-microspheres constructs were observed under optical microscope (Leica DMIL, 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.

Hep3B cell proliferations were assessed by using the MTT assay which is based on the cleavage of a water-soluble yellow tetrazolium salt to water-insoluble purple formazan crystals by the action of dehydrogenase enzymes. The albumin secretion by liver cells was measured using ELISA (Human albumin ELISA quantification kit, Bethyl, Texas, USA). 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 Surface density of conjugated molecules

The surface densities of grafted proteins were quantified using Micro BCA™ protein assay and the results were shown in Table 1. It was observed that the surface density of grafted collagen was higher than that for laminin and fibronectin. This could be due to the larger conformational sizes of laminin (900,000 Daltons) and fibronectin (440,000 Daltons) molecules resulting in greater steric hindrance during grafting. It was also believed that self-assembly of fibronectin into fibrils may occur during the conjugation process and decrease the amount of fibronectin grafted onto the surface.

Table 1: Surface density of proteins conjugation to microspheres

Samples	Surface Density (pmol/cm^2)
Collagen	2.193 ±0.355
Laminin	0.704±0.181
Fibronectin	0.670 ±0.135

3.2 Morphologies of Hep3B cells growth on surface-modified microspheres

A human hepatoma cell line Hep3B was used as model liver cells to study the effects of the modified microspheres on liver cell proliferation and function. Cells were cultured either on single proteins grafted microspheres, or on mixture of microspheres grafted with the three types of proteins respectively in a mass ratio as Collagen: Laminin: Fibronectin at 1:1:1.

Although there were no significant differences in the morphology of the Hep3B cells cultured on the blank and proteins-conjugated microspheres, the cells spread more extensively when cultured on proteins-conjugated samples and more microspheres were bridged together to form thicker tissues (Figure 1a and 1b). Studies by others have also indicated that hepatocytes exhibited a flat and extended morphology when cultured on ECM proteins coated substrates with certain densities [5,6]. It was believed that ECM proteins coated on rigid, planar surfaces could stimulate the gene expression of major cytoskeletal proteins through binding to specific integrin receptors and therefore regulated the morphology of the cells [5].

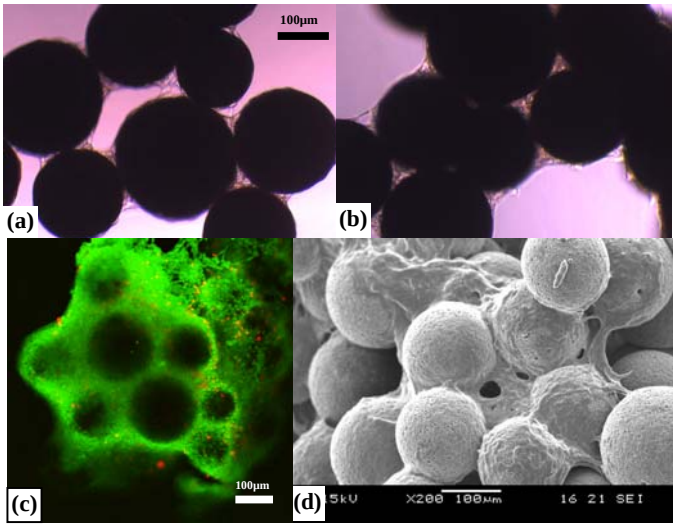

Figure 1 Optical micrographs of Hep3B cells growth on (a)Blank, (b)Collagen-conjugated microspheres. CLSM of Hep3B cells growth on (c)Fibronectin-conjugated microsphere. SEM images of Hep3B cells growth on (d) Laminin-conjugated microspheres.

CLSM images proved that most of the cells grown on blank and treated microspheres were viable. Cells were found to be better distributed on protein-grafted microspheres as compared to the blank, which indicated that the immobilization of these bioactive molecules improved the biocompatibility of PHBV microspheres.

SEM images of cells at day seven of culture on the representative laminin-grafted microspheres are shown in Figure 1d. It was observed that the cells had encapsulated the microspheres and aggregated them into three dimensional clumps. This suggested that the microspheres were good substrates for Hep3B, and thus also hepatocytes, to grow on by allowing extensive cell-cell and cell-substrate interactions to occur.

3.4 Hep3B Cell proliferation activity on microsphere scaffold

The proliferation activities of Hep3B cells grown on blank as well as on surface modified microspheres were quantitatively evaluated by MTT assay during the two weeks of culture (Figure 2). From day 4 onwards, the cells showed improved proliferation activities when cultured on proteins conjugated microspheres, especially on laminin conjugated sample over the blank, while no significance differences were found among the three proteins conjugated samples. According to Mooney, high ECM density activated hepatocytes spreading and proliferation, regardless of the type of ECM molecules used [5]. Since the surface densities of the three proteins on the microspheres were in the range of 200-600 ng/cm^2, it was believed that the cells had been stimulated to enter the S phase with higher proliferation activities. Studies by others suggested that growth and gene expression of hepatocytes were dependent on the interactions of cells with different ECM substrates, however these effects were absent at high cell density [7,8]. Taken together, the three types of proteins might be different in terms of improving cell proliferation but the differences could have been diminished by the naturally

high proliferation rate of the Hep3B cell line and strong cell-cell interactions induced by the tissue-like constructs.

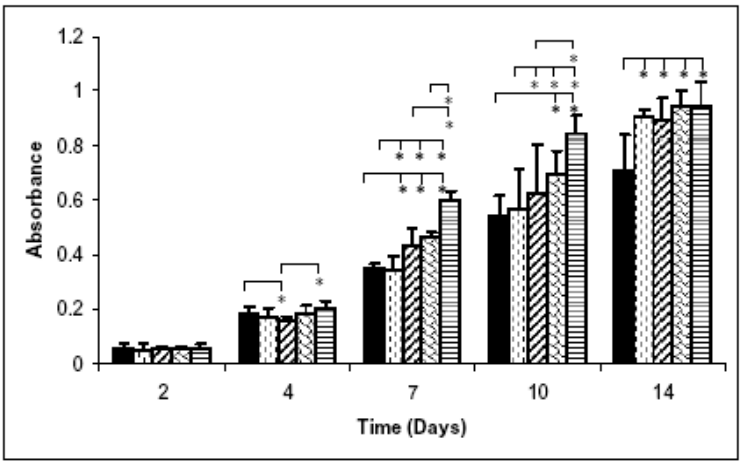

Figure 2 Proliferation of Hep3B cells cultured on blank (■), Collagen-conjugated (▥), Fibronectin-conjugated (▨), Laminin-conjugated (▩); and proteins combination (Collagen:Fibronectin:Laminin) with a ratio as 1:1:1 (▤), PHBV microspheres. Values represent means±SD, n=3 (* p<0.05 as compared by Student t-test).

In an attempt to develop an improved liver tissue engineering system, we hypothesized that culturing Hep3B cells onto microsphere scaffold with combination of different ECM proteins on the surface would improve cell proliferation activity and functionality. From the MTT results, it is interesting to note that the mixed protein-conjugated samples allowed a significant improvement in proliferation of Hep3B cells. The three types of ECM molecules used in this work contain distinct peptide sequences which can bind with different integrin receptors on the membrane of hepatocytes [5]. When microspheres conjugated with different proteins were bridged together, the Hep3B cells would have chances to interact with different proteins through multiple ECM receptors. These interactions might function to re-organize the cytoskeletons of the cells, which were associated with the regulation of gene expression and DNA synthesis. Studies by others showed that the response of hepatocytes cultured on a complex substratum (EHS gel, an ECM derived from EHS tumor) were strikingly different from on individual matrix proteins like type I collagen gel [6,8]. The results presented here were consistent with these findings in term of the synergistic effects of various proteins, but our results also demonstrated that the combination of proteins on rigid polymer surface could improve the cell attachment on the surface as well as stimulate cell proliferation activity.

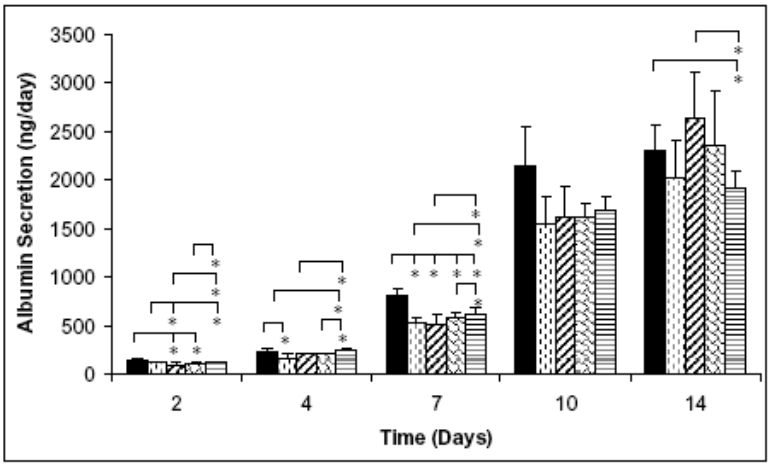

Figure 3 Albumin secretion by Hep3B cells cultured on blank (■), Collagen-conjugated (▥), Fibronectin-conjugated (▨), Laminin-conjugated (▩); and proteins combination (Collagen:Fibronectin:Laminin) with a ratio as 1:1:1 (▤), PHBV microspheres. Values represent means±SD, n=3 (* p<0.05 as compared by Student t-test).

3.5 Albumin secretion by Hep3B cells

Albumin secretion is an important indicator of hepatic function for normal liver hepatocytes, and can be similarly modeled by the Hep3B cells. As was shown in Figure 3, there was a trend of increasing albumin secretion with time elapsed for all samples. This was expected as it was known from the MTT data that cell proliferation activity

increased and thus, the greater number of living cells present would have secreted more albumins. It is interesting to note that the cells cultured on mixed proteins-conjugated microspheres did not show improved albumin secretion ability although they had a higher proliferation rates, which indicated that the synergistic effect of combination ECM proteins on rigid polymer surface functioned more on improving cell proliferation. On the contrary, the blank samples showed higher albumin secretion over most of the other samples while they showed lower proliferation rates. As discussed in section 3.5, the high surface densities of proteins may have stimulated the higher percentage of cells into S phase with a concomitant down-regulation of differentiated functions. Our results are consistent with the findings by others that hepatocytes could be switched from differentiation to proliferation cycle through binding with different ECM molecules [5, 9,10].

3.7 P-450 activity of Hep3B cells

Another important indicator of liver hepatocyte function that can be modeled by Hep3B cells is the cytochrome P-450 enzyme activity. Similar to the ELISA results, the blank samples had higher P-450 activity over most of the other samples, while the combination of proteins did not show a conclusive enhancement (Figure 4). This again suggested that high densities of proteins on rigid polymer surface could improve the cell proliferation with down-regulated differentiated functions.

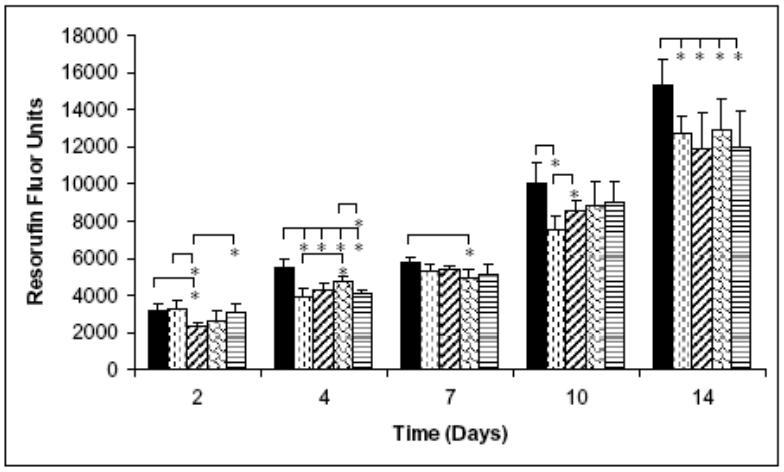

Figure 4 Cytochrome P-450 activity of Hep3B cells cultured on blank (■), Collagen-conjugated (▦), Fibronectin-conjugated (▨), Laminin-conjugated (▩); and proteins combination (Collagen:Fibronectin:Laminin) with a ratio as 1:1:1 (▤) PHBV microspheres. Values represent means±SD, n=3 (* p<0.05 as compared by Student t-test).

4. Conclusions

In this study, we have demonstrated that the viability of using PHBV microspheres as tissue engineering scaffolds can be improved through a controlled surface conjugation with ECM proteins. The improved proliferation of the cells cultured on mixed protein-conjugated samples suggested that the proliferation activity of Hep3B did not just depend on single protein, but rather, involved complex interactions with all of the ECM components. Conversely, high proliferation would tend to lower cell function. Thus, during the design of a tissue engineering system, a scaffold showing different surface properties at different cell development stages might be necessary.

Acknowledgement

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