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Mesenchymal stem cellular adhesion and cytotoxicity study of random biopolyester scaffolds for tissue engineering

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Abstract Mesenchymal stem cells (MSCs) were isolated from the bone marrow of rabbits and inoculated respectively on 3D scaffolds of poly(3-Hydroxybutyrate-co-3-Hydroxyvalerate) (PHBV), poly(butylenes succinate) (PBS) and different blends (100/0, 80/20, 50/50, 20/80, 0/100) (Wt%) in vitro. It was found that the (50/50) blends possessed the best performance on adhesion and cytotoxicity of MSCs. The scanning electronic microscopy (SEM) results showed that the (50/50) blends had the appropriate roughness for MSCs to attach and grow, which may be used as a suitable biomaterial to create small caliber vascular grafts.

1. Introduction

Atherosclerotic vascular disease such as coronary artery disease and peripheral vascular disease, remains the largest cause of mortality in the world. This led to the widespread clinical use of Dacron (polyethylene terephthalate) (PET) and Teflon (expanded polytetra-fluoroethylene) (ePTFE) grafts in cardiovascular surgery [1]. However, small diameter grafts (< 6 mm) of both Dacron and Teflon failed rapidly due to thrombotic occlusion [2]. Various synthetic grafts have been seeded with vascular cells in attempts to reduce the thrombotic response. To select appropriate polymers, it is necessary to understand the influence of the polymer on cell viability, growth, and function [3].

Bioabsorbable polymers degrade following their implantation. This feature may be important for many tissue regeneration applications, because the polymer will disappear as functional tissue restoration occurs [4]. According to the difference in the preparation methods, biodegradable polymers can be classified into two types. One is the biosynthetic polymers, such as bacterial poly (hydroxybutyrate) (PHB). PHB is a truly biodegradable and biocompatible polymer with relatively high melting point and crystallinity. However, practical application of PHB has often been limited by its brittleness and narrow processing window. Therefore, blending of PHB with other polymers has been often reported in the literature. Among such copolymers, PHBV is characterized by better impact resistance and offer the possibility of processing at lower temperatures than PHB [5]. As a biodegradable polyester produced by microorganisms, which has recently been investigated to improve blood vessel restoration [6, 7].

The other type of biodegradable polymers is the chemosynthetic polymer, such as the linear aliphatic polyesters. PBS, as an aliphatic polyester, was selected to blend with PHBV [8]. B. Q. ZHAO *et al.* studies the miscibility and crystallization behavior of PHBV/PBS blends [9]. In blended polymers, it can improve the brittleness and hydrophility of PHBV. According to these studies, we think the PHBV/PBS blends maybe a suitable scaffold for tissue engineering vessels. To the best of our knowledge, no attention has been paid to the study of cells cultured on the blending of PHBV and PBS till now in the literature.

MSCs, which are readily isolated from bone marrow, have attracted much attention for tissue engineering because they are able to proliferate and have the inherent potential to differentiate into the cell lineage of various types, for example,

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bone, cartilage, muscle, tendon, and other connective tissue. Many research trials have been reported to induce tissue regeneration by use of MSCs or their combination with scaffolds and culturing [10–14].

The objective of the present study aimed to evaluate the adhesion of MSCs to the novel blended polymers and the cytotoxicity of these materials to MSCs.

2. Materials and methods

2.1. Polymers scaffolds preparation

The PHBV/PBS blends were produced in the Institute of Polymer Science and Engineering, Tsinghua University. Briefly, 1 g (100/0, 80/20, 50/50, 20/80, 0/100) materials, for example, were dissolved in 20 ml chloroform and refluxed at 60 °C for 0.5 h. After the blended process, the microporous polymer scaffolds were obtained and dried to vacuum. The blended polymers were punched out into 6-mm-diameter disks. The 6-mm disks were prewetted with aquous ethanol solution (70 vol%) for 30 min to sterilize and enhance their water uptake, and completely rinsed twice with 0.1 M phosphate balanced solution and twice with serum free medium for 1 h each to remove ethanol.

2.2. Cell seeding

MSCs were isolated from the femurs of young (30–50 days old) New Zealand white rabbits, as described by Maniatopoulos *et al.* [15]. The released cells were cultured for 2 weeks in DMEM (Dubecco's Modified Eagle Medium, GIBICO, USA) supplemented with 10% fetal calf serum (FCS, ChuanYe Biotechnology, Tianjing, China), 100 U/ml penicillin (Jingke Biotechnology, Beijing, China), and 100 μg/ml streptomycin (Jingke Biotechnology, Beijing, China). Cells were incubated at 37 °C in a 5% CO_2 incubator and the medium was changed every 2 days. After 7 days of primary culture, cells were detached using trypsin/EDTA (0.25% w/v trypsin/0.02% EDTA) and plated on top of the experimental substrates that were positioned at the bottom of a 96 wells-plate. Blends were placed in the center of the wells added with 200 μl cell suspension and pretreated with culture medium for 3 h prior to cell implant.

2.3. Cellular adhesion assay

Cell adhesion was assessed using a methylthiazol tetrazolium (MTT) assay. Briefly, the different sterilized PHBV/PBS blends, $n = 12$ (Costar Corporation, USA) were inoculated with culture medium for 24 h at 37 °C in a 96 well culture plate. After this incubation time, 200 μl of cell suspension (1.0×10^5 cell/ml) in DMEM with 10% FCS was inoculated in the wells with different PHBV/PBS blends for 72 h. Cell-free wells with the same culture conditions were used as control reactions. The cells were washed twice with 0.1 M phosphate buffered saline and then received 200 μl serum free medium and 20 μl MTT solution (5 mg/ml in phosphate balanced solution) (Sigma, USA) were added to each sample, followed by incubation at 37 °C for MTT formazan formation. The medium and MTT were replaced by 200 μl 10% dimethylsulfoxide (DMSO) (Sigma, USA) to dissolve the formazan crystals. After 30 min, the optical density (OD) at 490 nm was determined against dimethylsulfoxide solution blank. As a positive control the culture plate itself (polypropylene) was used while silicone membranes served as a negative control. Five parallel replicates were read for each sample after cultured for 72 h.

2.4. Measurement of LDH

The extent of cell damage and death during the seeding process was assessed by measuring lactate dehydrogenase (LDH) levels used a commercial kit (AU1000, OLYMPUS, Japan). Briefly, the different sterilized PHBV/PBS blends, $n = 12$ (Costar Corporation, USA) were inoculated with culture medium for 24 h at 37 °C in a 96 well culture plate. After this incubation time, 200 μl of cell suspension (1.0×10^5 cell/ml) in DMEM with 10% FCS was inoculated in the wells with different PHBV/PBS blends for 72 h. Cell-free wells with the same culture conditions were used as control reactions. The cell suspension of each sample was sent to measure LDH. Five parallel replicates were read for each sample after cultured for 72 h.

2.5. Scanning electron microscopy observation (SEM)

The specimens were washed twice by phosphate buffered saline and immersed in phosphate buffered saline containing 3% glutaraldehyde (pH 7.4) for 4 h. They were then dehydrated in increasing concentration of ethanol (from 30%, 50%, 70%, 90%, and 95% to 100%) and followed by lyophilization. They were then mounted on aluminum stumps, coated with gold in a sputtering device for 1.5 min at 15 mA and examined under a scanning electron microscope (KYKY-2800, Apparatus Factory, Chinese Academy of Sciences, Beijng, China).

2.6. Statistical analysis

Data were presented as means ± standard error of mean. Statistical analysis was performed with SPSS 10.0 package. Statistical comparisons were performed using ANOVA. When a significant difference was found ($P < 0.05$), the groups were compared using Tukey's test.

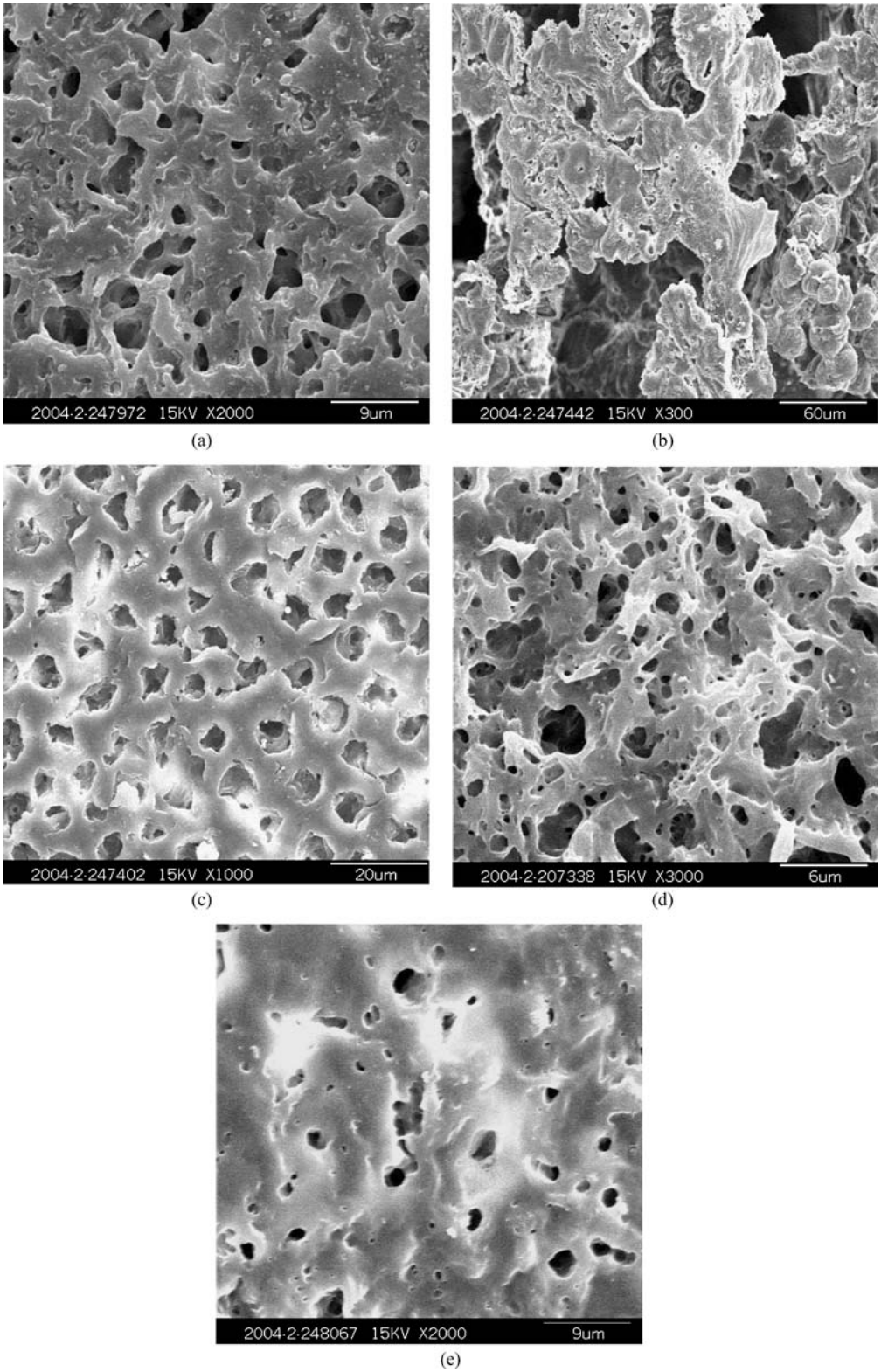
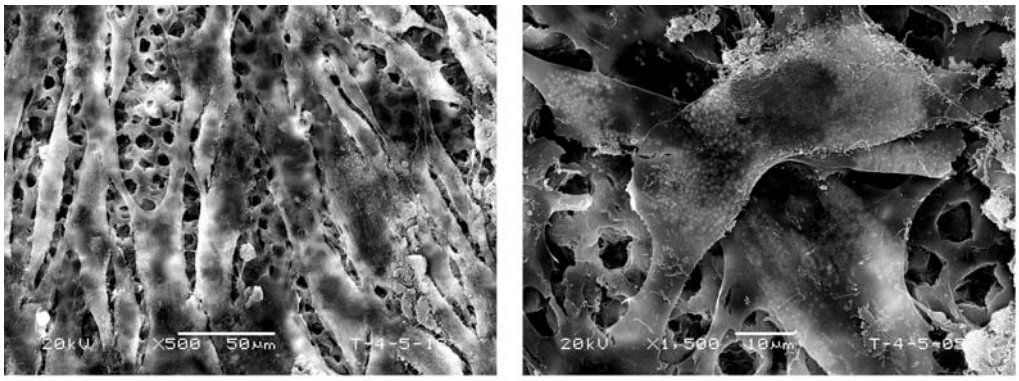


Fig. 1 Scanning electron microscopy of different scaffolds without implanted MSCs, All the experiment was done three times: (A) PHBV/PBS (100/0), pore sizes approximately 2–3 μm in diameter; (B) PHBV/ PBS (0/100), the crudest surface, nearly without visible pores; (C) PHBV/PBS (50/50), pore sizes approximately 2–3 μm in diameter; (D) PHBV/PBS (80/20), the different pores sizes from 1 μm to 6 μm; (E) PHBV/PBS = 20/80, the least pores in all the blends.



(a) (b)

Fig. 2 Scanning electron microscopy of different scaffolds with implanted MSCs for 10 d, the experiment was done three times: (A) Polygonal mesenchymal stem cells and cells clumps were observed on the (50/50) blend polymers scaffold; (B) MSCs have large nucleoluses, and bridging each other with fibrillar collagen they synthesized and organized.

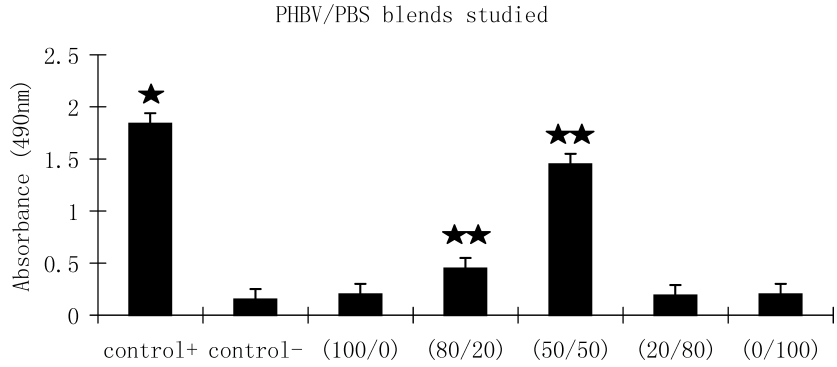


Fig. 3 Ahesion of MSCs cultured on different PHBV/PBS blends. The experiment was done three times. Five parallel replicates were read for each sample. All samples studied displayed the capacity to stimulate less adhesion than to the positive control used (1.8400 ± 0.0045, $P < 0.001^{*}$). The (50/50) (1.4500 ± 0.0035) and (80/20) (0.4500 ± 0.0035) blends were significantly different to the negative control used (0.1520 ± 0.0045, $P < 0.001^{**}$), while the (50/50) (1.4500 ± 0.0035) were significantly different to the (80/20) (0.4500 ± 0.0035, $P < 0.001$) blends.

3. Results

3.1. Surface topology

In this study, surface topologies of different blends were also examined. As shown in Fig. 1, the (50/50) blends had different roughness compared with the other blends. The (100/0) blends contained the most pores with pore sizes approximately 2–3 μm in diameter on the surface (Fig. 1a), while the (0/100) blends had the crudest surface, nearly without visible pores (Fig. 1b). The (80/20) blends had the different pores sizes from 1 μm to 6 μm(Fig. 1d), while the (20/80) blends had the least pores in all the blends (Fig. 1e). In contrast, the (50/50) blends contained pores with approximately 5–10 μm in diameter (Fig. 1c).

3.2. Cell morphology

In order to reveal the morphology of cells cultured on three-dimensional different blends scaffolds, cells after cultured for 10 days on the scaffolds were examined by SEM.

Polygonal MSCs and cells clumps were observed on the (50/50) blend polymers scaffold (Fig. 2a). Cells presented typical MCSs phenotype: MSCs have large nucleoluses, and bridging each other with fibrillar collagen they synthesized and organized (Fig. 2b). Cell morphology on the (50/50) blends polymers appeared to be much better than the other blends whose photographs did not be supplied.

3.3. Cell adhesion

MTT assay was used to compare cell adhesion on different blends. All samples studied displayed the capacity to stimulate less adhesion than to the positive control used (1.8400 ± 0.0045, $P < 0.001^{*}$). The (50/50) (1.4500 ± 0.0035) and (80/20) (0.4500 ± 0.0035) blends were significantly different to the negative control used (0.1520 ± 0.0045,$P < 0.001^{**}$), while the (50/50) (1.4500 ± 0.0035) were significantly different to the (80/20) (0.4500 ± 0.0035, $P < 0.001$) blends. These results can be seen in Fig. 3.

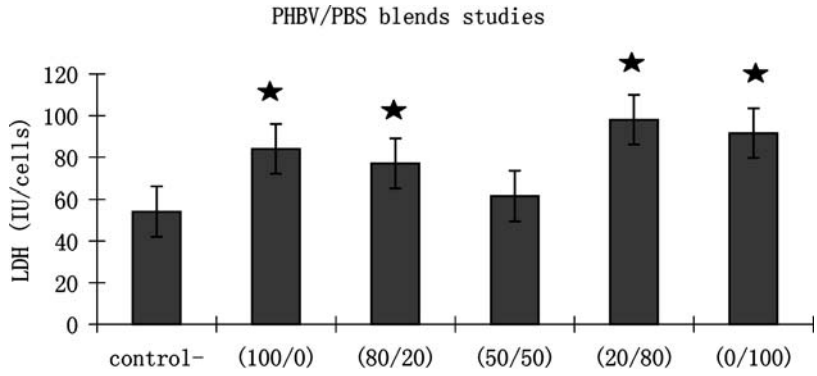


Fig. 4 LDH levels of MSCs cultured on different PHBV/PBS blends. The experiment was done three times. Five parallel replicates were read for each sample. The (100/0) (84.00 ± 0.55), (0/100) (75.00 ± 0.35), (80/20) (77.00 ± 0.51)and (20/80) (88.00 ± 0.25)blends showed were significantly different to the negative control (20.00 ± 1.90, $P < 0.05^{*}$). The (50/50) (23.00 ± 0.45) blends showed similar values to the negative control.

3.4. Metabolism of cells

Measuring lactate dehydrogenase (LDH) can assess the extent of cell damage and death during the seeding process. Figure 4 shows LDH leakage of cells after seeding of the five scaffolds. The (100/0) (84.00 ± 0.55), (0/100) (75.00 ± 0.35), (80/20) (77.00 ± 0.51)and (20/80) (88.00 ± 0.25)blends showed were significantly different to the negative control (20.00 ± 1.90, $P < 0.05^{*}$). The (50/50) (23.00 ± 0.45) blends showed similar values to the negative control. These results can be seen in Fig. 4.

4. Discussion

The development of biomaterials capable of directing cell behavior is a growing research area. Applications include device for tissue replacement and regeneration as well as substrates for cell culture in tissue engineering. In both situation cell interactions with biomaterials need to analyzed [16]. In our study, we didn't use the common sodium chloride particles to produce the pores, for the sodium chloride may affect the cells growing up. The SEM studies showed the structure of the different polymers pores in the PHBV/PBS blends. It was surprising that the membranes made by blended polymers have a structure of uniform pore. It may be leaded by the different shrinking and crystalling rate between PHBV and PBS.

Because the different PHBV/PBS blends show structural variations, they could induce variations in cell adhesion, cytotoxicity. We showed that all blends studied have a low capacity to stimulate cell adhesion. The PHBV/PBS (50/50) blends were the exception, being more receptive to cell interaction. Our data indicate that addition of PBS increase the cell adhesion for the PHBV substrate up to a limit of around 66 times of blend composition.

Cell adhesion on biomaterials is extremely important in biomaterial science. Only upon adhesion to the substrate can cells migrate and/or proliferate on it or even exert special physiological activities, such as the production of an extracelluar matrix (ECM)[17]. The adhesion is the consequence of protein adsorption to the substrate. These interactions involve electrical charge interactions, H-bonds, and electrostatic forces[18]. Other factors which can modulate cell adhesion, are the types of chemical groups present on the polymer surface and its relative hydrophilicity/hydrophobicity [19]. So cell adhesion to biomaterials is quite a complex process. Once the cells were able to proliferate on PHBV/PBS blends, they were able to attach to the polymers.

The utilization of a bioabsorbable polymer for tissue restoration depends mainly on three parameters: first, cell adhesion on the substrate; second, ECM production; and third, scaffold degradation [3]. Ideally, the scaffold must degrade at the same time as the occurrence of damaged tissue regeneration. In an in vitro situation, the importance of ECM should be noted once cell growth and differentiation in two- or three-dimensional culture conditions requires the presence of a structural environment with which the cell can interact. The ECM is therefore a functional structure that can modulate cell morphology as well as cell proliferation, migration, and differentiation [20]. In our study, the SEM showed that (50/50) PHBV/PBS blends contained pores with approximately 5–10 μm in diameter and produced much fibrillar collagen after planted with MSCs. The results obtained indicated that the (50/50) blends polymers was a good cytocompatible material that allows the adhesion and growth of MSCs. Surface roughness is an important parameter for MSCs attachment on biomaterials. Comparing the roughness on the blank blends scaffolds and the cell adhesion results, it was found that the appropriate roughness of the blended polymers maybe one of the reasons for better performances of MSCs adhesion and growth on the blended polymers. The blended polymer scaffold contained pores with approximately 5–10 μm in

diameter which were suitable to the attachment of MSCs. This appropriate roughness affects cell attachment as it provides the right space for MSCs growth.

LDH leakage as an index of cell damage and death was lower on the (50/50) blends polymers than on the blends, it is possible that the wastes generated by cells on the (100/0), (80/20), (20/80) and (0/100) blends are more accumulated than on the (50/50) blends.

In conclusion, the (50/50) blends scaffolds had shown better performance for MSCs attachment and proliferation than the (100/0), (80/20), (20/80) and (0/100) blends prepared using the same preparation procedure. Combined with the better elasticity and processibility, the blended polymer (PHBV/PBS = 50/50) could be employed as a potential biomaterial for vascular tissue engineering.

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