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Biocompatibility and Biodegradation of novel PHB porous substrates with controlled multi-pore size by emulsion templates method

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Abstract PHB porous substrates were prepared based on the mono-membrane fabricated by emulsion templates method. The key factors of the method affecting the pore size and porosity of the PHB porous substrates were studied. The surface of PHB porous substrates were investigated by scanning electron microscope (SEM), which showed the even pore size and regular arranged pore. The transect of the PHB porous substrates prepared using the templates method was good. Moreover, the effects of variation of surfactant content (P%) and water content (R) on the pore size and porosity of PHB films were discussed. Preliminary studies showed that when P% is less than 20%, the pore size made by emulsion templates ranged from 5 μm to 30 μm with the value of P increasing. As P% is up to 20%. It was interesting to see that the porous substrates had muti-pore size distribution, i.e., median pore sizes were about 5 μm and inside the wall of pore, there existed numerous micro-pores size can be controlled from 100 nm to 500 nm only by adjusting the parameter R of the microemulsion. The cell-compatibility was evaluated via Chinese Hamster Lung (CHL) fibroblast cultivation *in vitro*. The Cells were cultured on both the mono-pore size membrane prepared by emulsion templates and the multi-pore size membrane prepared by microemulsion templates. It can be seen that the cells cultured on multi-pore size membrane stretched their morphology and proliferated better than that of mono-pore size membrane. These results indicated that the multi-pore size membrane had better cell-compatibility and was more suitable for tissue engineering. The degradation experiment indicated that the degradation of PHB porous substrates were accelerated by enzyme *in vitro* and the porous configuration was favorable to its degradation.

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1 Introduction

Fundamental knowledge of cell-substrate interactions is important for tissue engineering. Topographical cues, independent of biochemistry, generated by an extracellular matrix (ECM) may have significant effects on cellular behavior [1, 2]. In general, the tissue development is controlled in three matrix size scales. The gross shape and size of tissue is decided by the macroscope shape (cm to mm scale) of matrix; cell invasion and growth is controlled by the size and structure of the matrix pore (μm); The adhesion and gene expression of cells are adjusted by the surface chemistry of the matrices (nm scale). Thus, the pore size of porous substrates used in tissue engineering has important effects on cell behavior. To fabricate synthetic micro- and nano-structured porous substrates will be a key field for tissue engineering research.

Numerous technologies have been applied to process macroporous biodegradable polymer including solvent casting/salt leaching [3], phase separation [4], membrane lamination [5], melt molding [6] and fiber bonding [7]. Solvent casting/salt leaching involves mixing solid impurities, such as sieved sodium chloride particles, into a polymer solvent solution, and casting the dispersion to produce a membrane of polymer and salt particles. The salt particles are then leached out with water to yield a porous membrane. Porosity and pore size (100–500 μm in diameter) have been shown to be dependent on salt weight fraction and particle size [3]. Phase separation involves dissolving a polymer in a suitable solvent, placing it in a mold, and then cooling the mold rapidly until the solvent is frozen. The solvent is removed

by freeze-drying, leaving behind the polymer as a foam with pore size of 1–20 μm in diameter [4].

The nature poly(3-hydroxybutyrate)(PHB) is a biodegradable polyester produced by a number of bacteria as a reserve of carbon and energy [8]. Due to its excellent properties, for example, biodegradability [9], biocompatibility [10, 11], optical activity, piezoelectricity and non toxicity, PHB has been evaluated for a variety of medical applications, which include controlled release system [12–14], surgical sutures [15], wound dressing, orthopedic uses [16] and as a pericardial substitute [17]. In recent years, PHB has been studied as biopolymer porous substrates in tissue engineering applications [18–22]. The PHB biopolymer provides a means to target a wide range of tissues with potential product applications for the cardiovascular system, cornea, pancreas, gastrointestinal system, kidney and genitourinary system, musculoskeletal system, nervous system, teeth and oral cavity, skin and so forth.

An initial goal of this research was to develop a novel processing technique to fabricate highly porous implantable PHB porous substrates. More specifically, it involves fabricating porous substrates with porosity greater than 90% and the ability to control pore size ranging between 5 μm and 30 μm. This paper addresses a novel method of fabricating biodegradable porous substrates with variable porosity and pore size. Qualitative evaluation of fabrication parameters that control porosity, pore size and characterization of the physical properties of this material is also a subject of this report.

2 Experiment

2.1 Materials

The poly(3-hydroxybutyrate)(PHB), a white powder sample was kindly provided by Tianjin TianLu Co. Ltd (Tianjin, China), $M\mathrm{w} = 4.3 \times 10^5$, $M\mathrm{n} = 2.9 \times 10^5$, $M\mathrm{w}/M\mathrm{n} = 1.49$ (obtained by G.P.C. in chloroform at 30°C). It was purified by precipitation in *n*-hexane from chloroform solution, subsequently precipitated in methanol from chloroform solution. Chloroform, Span85, Tween60, n-hexane was AR grade and used without further purification.

2.2 Preparation of PHB porous membranes by emulsion templates method

The H_2O/Span85-Tween60/chloroform reverse emulsion templates were prepared, using Span85-Tween60 as composite surfactant. The ultraviolet (UV)/visible light spectromometer was applied to investigate the effects of surfactant content P% and water content R on the properties of emulsion templates.

The PHB porous membranes were prepared by solution-cast technique using chloroform as the solvent. Since PHB is insoluble in chloroform at room temperature. An autoclave was used to stir it for 150 min at 70°C. The polymer concentration of each solution was 30 g/L. After the PHB solutions were well homogenized, the emulsion templates wad added. The mixed solution was dispersed sufficiently and then dipped on a poly (tetrafluoroethylene) dish, left at room temperature for 48 h and subsequently the dried membranes was immersed into *n*-hexane to extract the templates. Finally, the membranes was dried at 60°C for further 120 h under vacuum to eliminate the solvent completely.

2.3 Scanning electron micrographs (SEM)

The microscopic morphology and pore size of t PHB porous substrates were investigated using scanning electron microscope (SEM) (Philips XL-30) at a magnification of 2000~50000 at 20 kV. The samples were fractured in liquid nitrogen so that its surfaces were not affected by external stress. For the morphological measurement, the samples were then coated with gold to make an electric flow on its surfaces.

In order to measuring the pore size, the eight different partial SEM photographs were scanned into computer in BMP format. The boundary of each pore was detected by gray gradient method using the software designed by us. The area of each pore, defined as Si, was cumulated by pixel. The diameter of each pore di, was calculated by the equitation $di = (4Si/\pi)^{1/2}$. Then, the average pore size can be calculated by equitation $d = (\sum_{i=1}^{n} di)/n$. The pore size distribution of each SEM photographs was also plotted in statistical charts form.

2.4 Porosity

The porous substrates to be measured for porosity were soaked in ethanol for 48 h, then removed and placed on a dry paper towel. Another towel was immediately placed on the porous substrates and a constant gentle pressure was applied. After placing the porous substrates on a second dry paper towel and repeating the procedure, the porous substrates were then weighed immediately. The porosity of the PHB porous substrates was determined by the following formula.

$$\text{Porosity}(\%) = \frac{(W_2 - W_1)\rho_1}{\rho_1 W_2 + (\rho_2 - \rho_1)W_1} \times 100\% \quad (1)$$

Where W_1, W_2 is the weight of PHB porous substrates before and after immersed in alcohol for 24 hrs, ρ_1, ρ_2 is the density of PHB and alcohol respectively.

2.5 Evaluation of cell compatibility

2.5.1 Preparation of samples

The PHB membranes prepared previously were cut into 1 cm × 1 cm squares. These were sterilized by gamma irradiation.

Chinese Hamster Lung cells (CHL), passages 4, were used in the study and were cultured in Dulbecco's Modified Eagles Medium (DEME/F-12), supplemented with gentamycin (50 μg/ml), 10% fetal calf serum (FCS) and 5.6% sodium bicarbonate adjusted to pH 7.0∼7.4.

2.5.2 Cell culture

The square films of 1 cm × 1 cm and 300 μm in thickness were placed in the bottom of each well of a 24-well tissue-culture plate. Single-cell suspensions were added to the polymers at a density of 1.5×10^4 cells/ml. And the polymer membranes were incubated in 1 ml medium for 48 h at 37°C/5% CO_2.

2.5.3 Determination of cell adhesion and growth

For the measurement of cell adhesion, cells were washed twice with PBS to remove nonadherent cells and the attached cells were fixed with 2.5% glutaraldehyde buffer solution (pH 7.4) at 4°C for 12 h. Furthermore, the membranes were rinsed in distilled water and dehydrated by immersing them in increasing concentrations of ethanol (10%, 30%, 50%, 60%, 70%, 80%, 90%, 95% and 100%) for about 20 min at each concentration. For each experimental value, three independent experiments were conducted.

The cells' morphology on polymer surface was observed by scanning electron microscope SEM (Philips XL-30), and the cells number, at eight random fields, were counted visually.

2.6 Biodegradation studies

The dried membranes and porous substrates were cut into squares (1 cm × 1 cm) and cubes (1 cm × 1 cm × 1 cm) and incubated in the following solutions at 37°C: Sterilized non-ion water; PBS (PBS, Oxoid PH: 7.2∼7.4); lysozyme (Sigma, 0.2% solution in PBS). The samples were exposed to constant roller mixing at 37°C and at various time points the samples were removed, washed in distilled water and allowed to dry in air. The degradation rate was determined by the ratio of the weight loss to the initial weight of samples as shown below.

$$S = \frac{W_0 - W_t}{W_0} \times 100\% \quad (2)$$

Where S is the degradation rate, W_t and W_0 are the weight of samples after dried and the initial weight respectively.

These degraded samples were coated with gold and viewed under a scanning electron microscope (SEM).

3 Results and discussion

3.1 Emulsion templates

In this study, the H_2O/Span85-Tween60/chloroform reverse emulsions were prepared, using Span85-Tween60 as composite surfactant. The properties of emulsion were decided by the parameters given below.

$$\text{Surfactant content (P\%)} = [\text{surfactant}]/[\text{chloroform}] \times (\text{weight ratio}) \times 100$$

$$\text{Water content (R)} = [H_2O]/[\text{surfactant}](\text{molar ratio})$$

$$\text{Composite Hydrophilic-Lipophilic Balance (HLB)} = \mu_1 \text{HLB}_1 + \mu_2 \text{HLB}_2$$

where the μ_1, μ_2 are the weight fraction of Span85 and Tween60, HLB_1, HLB_2 are the HLB of Span85 and Tween60 respectively.

The properties of emulsion templates were affected significantly by the surfactant content (P%) and water content (R). Using ultraviolet (UV)/visible light spectromometer, we measured the variation of transmittance with surfactant content P (Fig. 1) and water content R (Fig. 1). As seen in Figs. 1 and 2, the transmittance of the emulsion increases sharply when the surfactant content is up to 16%. According the transmittance curve, when the value of P% is in the range of 18%∼30% and $R < 1.5$, we can get stable H_2O/Span85-Tween60/chloroform reverse microemulsions.

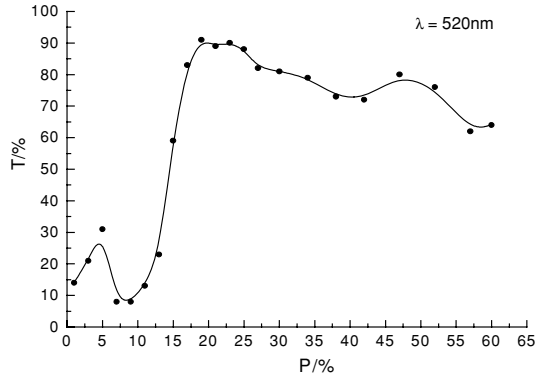


Fig. 1 Variation of transmittance with surfactant content P%.

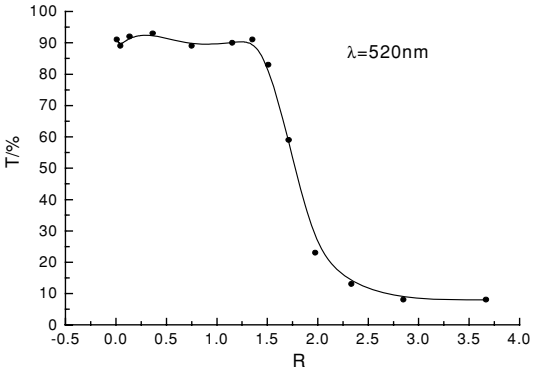


Fig. 2 Variation of transmittance with water content R.

3.2 The morphology of the porous substrates prepared by emulsion templates

Figure 3 shows the porous architecture of PHB porous substrates prepared by emulsion template, which the P% is 5%, 7%, and 10% respectively. It can be seen that the average pore size was less than 30 μm in these three porous structures. With the P% increased from 5% to 10%, the average pore size decreased from 20 μm to 5 μm, and the distribution of pore size became smaller. The pore size was well controlled by utilization of parameter of the emulsion. However, at the low concentration of surfactant content the pore interconnectivity was not desired and the porosity was less than 90%. As seen in Fig. 3, with an increase in the concentration of surfactant content, the pore interconnectivity became better together with a decrease in the thickness of pore walls. Hence, the effect of emulsion stability on porous substrates pore structure has to be considered since emulsions are usually not thermodynamically stable systems. When the content of surfactant was at low level, the flocculation and coalescence resulted in the increase of thickness of pore walls as well as pore size, at the same time, the pore interconnectivity and pore dispersion decreased. Figure 4 shows the scanning electron micrograph of PHB porous substrates, in which the surfactant content was up to 20%. It was interesting to see that the porous substrates had multi-pore size distribution, i.e., median pore sizes were about 5 μm and inside the wall of pore, and there existed numerous micro-pores. More interests we found that the micro-pores size can be controlled from 100 nm to 500 nm only by adjusting the parameter R of the emulsion (Fig. 5). It is clear that emulsions present a large interfacial area, and any reduction in interfacial free energy will reduce the driving force towards coalescence, promoting stability. The presence of an emulsifier should reduce the interfacial free energy and thereby stabilize the emulsions. Likewise, the more emulsifier is used, the more stable emulsion is. With the emulsifier content increasing to a high level, the milk like emulsion becomes transparent. In our study, when the P% is up to 20%, the reserve emulsion

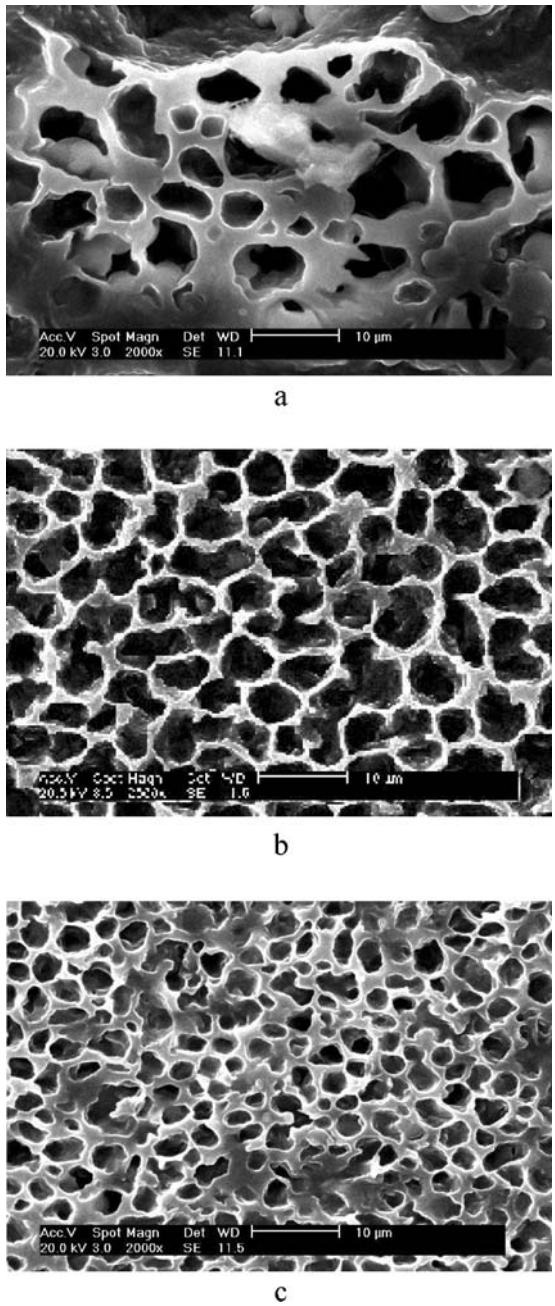


Fig. 3 SEM micrographs of porous PHB porous substrates made using the emulsion template method. (a) The average pore size is 20 μm with the emulsion parameter P% = 5%, R = 10. (b) The average pore size is 10 μm with the emulsion parameter P% = 7%, R = 7. (c) The average pore size is 5 μm with the emulsion parameter P% = 10%, R = 5.

become reverse microemulsions, the size of aqueous microdroplets that form in apolar solvents containing a surfactant depends on the R.

The difficulty countered in the emulsion templates is that the congregation of micelles during the dying step results in the pore size and geometry distorted, which is also happened in the extracting process. We also use the freeze-drying

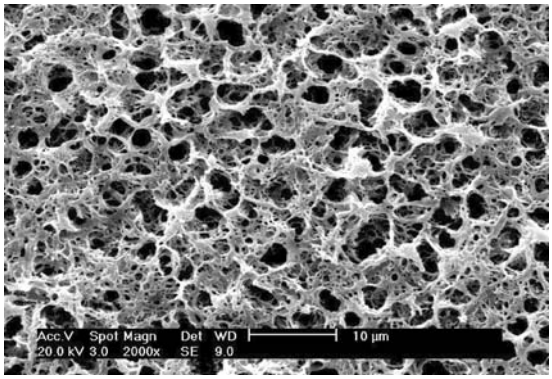


Fig. 4 SEM micrographs of porous PHB porous substrates prepared with the emulsion parameter P% = 20%, R = 4.8.

technique to lock the micelles locations. That result will be reported later.

In addition, some pores were caused by air bubbles because of the dispersion of the air during blending the emulsion template with the viscous polymer solution. These pores were beneficial for extracting templates more effectively.

3.3 Cell compatibility

Cell cytotoxicity testing is one of important factor that affected the use of polymers in tissue engineering. In this study, Chinese Hamster Lung (CHL) fibroblast was used to evaluate PHB porous membranes' cell-compatibility via cultivation in vitro.

In generally, cells do not grow on a solid polymer surface. In fact, cells grow on a layer of protein that interacts with cellular receptors. As nanometer-pore appeared in the PHB porous substrate, the PHB substrate became more hydrophilic and easy to desorption proteins.

Figure 6 shows the morphology of CHL cultured on mono-pore PHB substrate (substrate A) and multi-pore PHB porous substrate (substrate B) at 37°C for 48 h. As shown in Fig. 6, in the case of substrate A, some of the cells were still in round-shaped forming cluster which indicate that the mono-pore PHB substrate has poor cell adhesion. However, in the case of substrate B, no round-shaped cells and clusters can be seen. The cells stretched their morphology and were proliferating. At the same time, live cell adhesion on the surface of multi-pore size substrate was much more than that of on mono-pore size substrate. Thus, the multi-pore size substrate exhibited favorable cell-compatible properties. Cells cultured on the matrix stretched their morphology maintained their phenotype and the cellular interaction between the cells and the polymer was inseparable. This indicates that multi-pore size porous substrate has better cell compatibility than mono-pore size substrate. The reason is the appearance of nm scale pores in the multi-pore size substrate which can adjust the

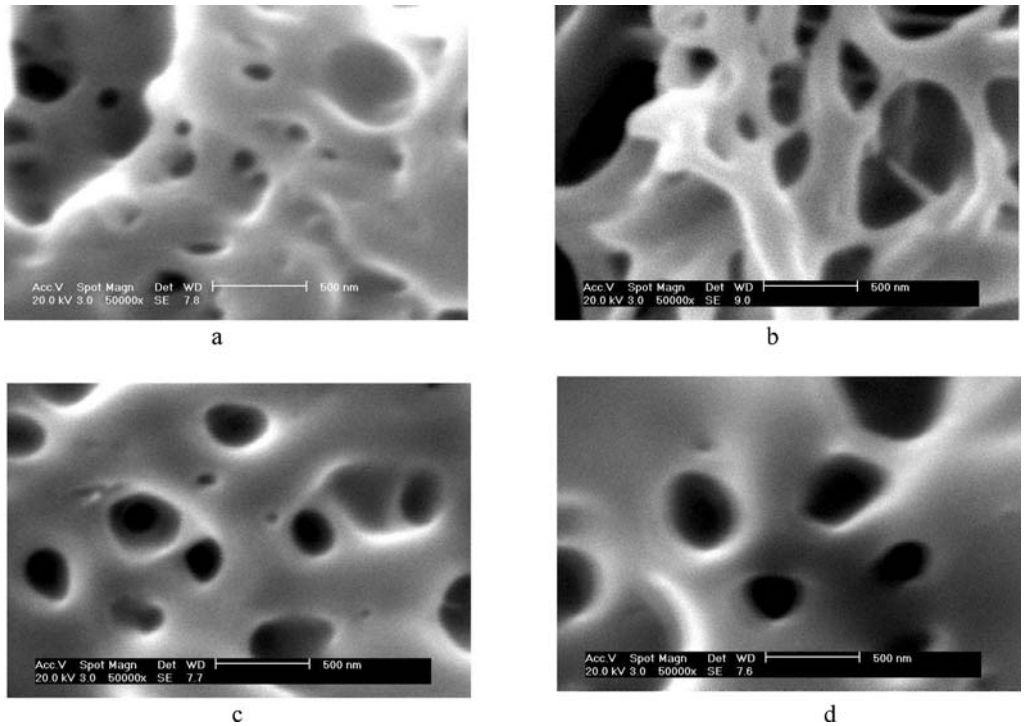
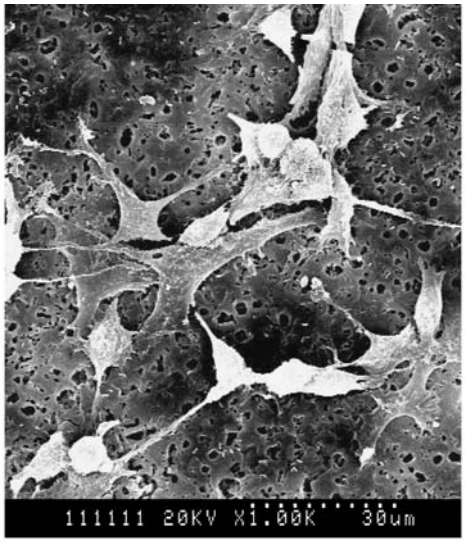
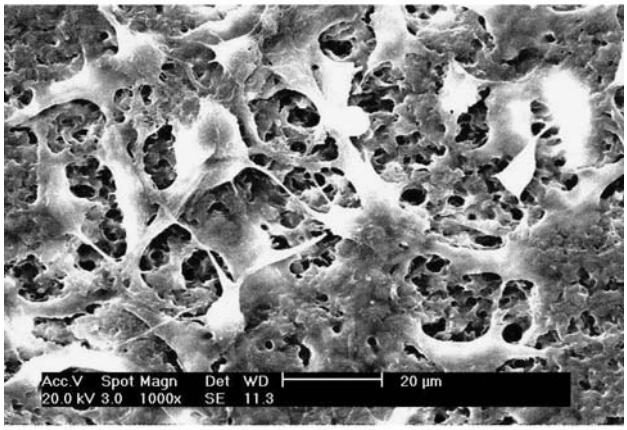


Fig. 5 Pore walls of porous porous substrates made using the emulsion template method. (a) The average pore size is 100 nm with the emulsion parameter P% = 20%, R = 1.2. (b) The average pore size is 200 nm with the emulsion parameter P% = 20%, R = 1.5. (c) The average pore size is 350 nm with the emulsion parameter P% = 20%, R = 2.4. (d) The average pore size is 420 nm with the emulsion parameter P% = 20%, R = 4.8.



A



B

Fig. 6 SEM of Chinese Hamster Lung (CHL) fibroblasts cultured on PHB based films in vitro. (A, CHL cell cultured on PHB; B, CHL cell cultured on PHB50).

surface chemistry to improve the adhesion of cells. Moreover, the multi-pore size substrate can offer the diffusion of molecules important to cell survival if the porosity if truly interconnected. We also use the freeze-drying technique which commonly was used to prepare three-dimensional scaffold to prepare PHB scaffold with multi-pore size distribution. We believe that this multi-distribution of pore size may be in favor of cell adhesion and transferring nutrient fluid and waste effectively so as to benefit the cell growth in three-dimensional porous substrates. These works are still in investigation and the results will be reported later.

3.4 Biodegradability of porous films PHB

Biodegradability is a remarkable characteristic of PHB. It has been reported that PHB can be biodegraded by many

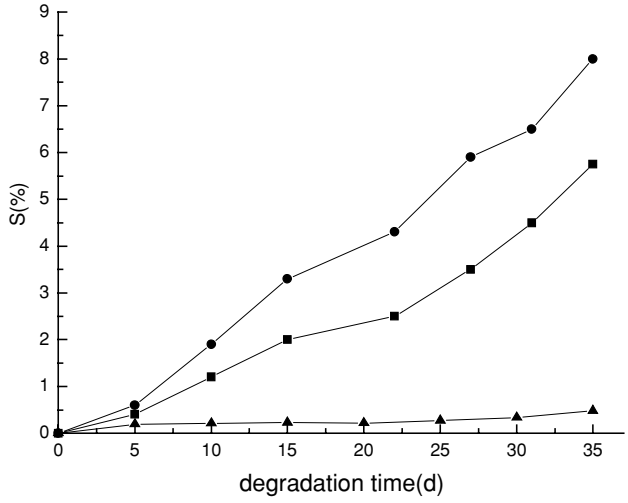


Fig. 7 Biodegradation test of PHB at 37°C (▲ PHB membrane in sterilized non-ion water; ■ PHB membranes and ● porous substrates tested in PBS/lysozyme buffer solution).

enzymes, for example, depolymerase from *Pesudomonas lemoignei* [23] etc. In vivo, enzyme such as lysozyme may also accelerate the degradation rate of PHB. In this study, biodegradability of PHB membrane and porous substrates were tested by measuring the weight loss as they were degraded by the sterilized non-ion water and PBS/lysozyme buffer solution (pH:7.2∼7.4) separately at 37°C. As seen in Fig. 7, after degradation for 35 days, the weight loss ratio is less than 0.5% of original weight in sterilized non-ion water, which indicates that PHB exhibits a long-term degradation prolife in hydrolytic degradation as reported previously. While in PBS/lysozyme buffer solution, the weight loss ratio is about 6% that is much larger than that of in non-ion water after 35 days degradation. These results revealed that enzyme accelerate the degradation of PHB.

Figure 7 also shows the PHB membranes and porous substrates degraded by PBS/lysozyme buffer solution. Compared with the two curves, it can be seen that the degradation rate of porous substrates is faster than that of membranes. This is perhaps because of more specific surface area that can be attacked by enzyme. Meanwhile, the morphology of porous substrates before and after degradation is shown in Fig. 8. Before degradation, inner pores in porous substrates were arrayed tightly and regularly, but after dipped into lysozyme buffer solution, the pore size become larger. After degradation for 35 days, the porous substrates' configuration varied greatly. The pores become larger and irregular. Some part of the porous substrates even was dented.

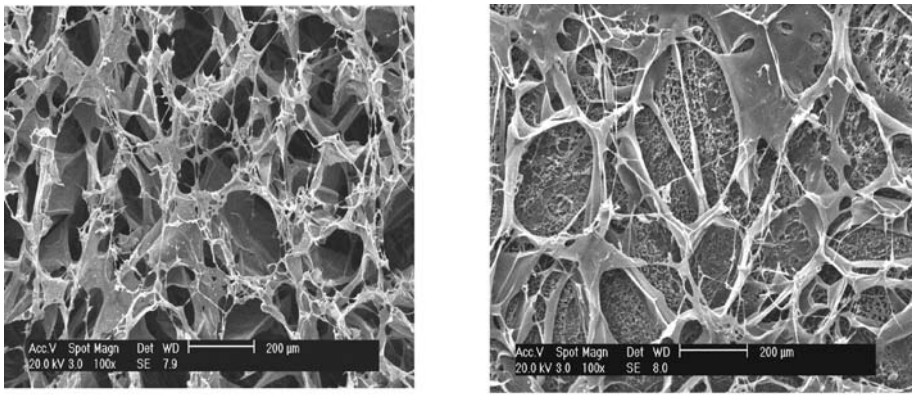


Fig. 8 SEM of PHB porous substrates in the degradation process (A, before degradation; B, degradation for 40 days).

4 Conclusion

A novel porous substrates fabricating method was developed. This technique was shown to be capable of producing porous substrates of PHB with porosity greater than 90%, median pore sizes ranging from 5 μm to 30 μm in diameter with micro-pore size ranging from 100 nm to 500 nm inside the pore wall, and good interconnections between the pores. It can be seen that the cells cultured on multi-pore size membrane stretched their morphology and proliferated better than that of mono-pore size membrane. These results indicated that the multi-pore size membrane had better cell-compatibility and was more suitable for tissue engineering. The reason is the appearance of nm scale pores in the multi-pore size substrate which can adjust the surface chemistry to improve the adhesion of cells. The biodegradation experiment indicated that the degradation of PHB porous substrates were accelerated by enzyme in vitro and the porous configuration was favorable to its degradation.

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