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Effect of lipase treatment on the biocompatibility of microbial polyhydroxyalkanoates

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Films made from microbial polyesters polyhydroxybutyrate (PHB) and poly(hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx) were treated by lipases and NaOH solution. The change of the polyester biocompatibility was evaluated by inoculating mouse fibroblast cell line L929 on films of PHB, PHBHHx and their blends. Polylactic acid (PLA) was used as a control. It was found that untreated PHB and PLA films gave a poor support to the growth of L929 cells, viable cell density ranged from 0.1×10^4 to 0.7×10^4 per ml only. While films of pure PHBHHx and PHB blended with PHBHHx showed improved biocompatibility, viable cell density observed increased from 9.6×10^2 to 6×10^4 on blended films of PHB/PHBHHx in ratios of 0.9/0.1 to 0/1, respectively. This result showed PHBHHx has a better biocompatibility compared with PHB. Films of PHB, PLA and the blends treated with lipases and 1 N NaOH, respectively, showed an improved ability to support cell growth. Biocompatibility of PHB was approximately the same as PLA after the treatment, while PHBHHx and its dominant blends showed improved biocompatibility compared with PLA. The sensitivity of the treatments was reduced when PHBHHx content increased in the PHB/PHBHHx blends. All three lipase treatments demonstrated more biocompatibility increase on all the films compared with the results of NaOH treatment. Scanning electron microscopy showed that PHB films changed its surface from multi-porous to rough non-porous after the lipase or NaOH treatment. While PHBHHx films showed little change after these treatments. The results showed that the polyester surface morphology played an important role in affecting cell attachment and growth on these materials.
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1. Introduction

Polyhydroxyalkanoates, abbreviated as PHA, is a family of biopolyesters synthesized by many bacteria [1, 2]. Over 90 PHA consisting of various monomers have been reported and the number is still increasing [2]. Properties of some PHA are similar to polyethylene and polypropylene while others are elastomeric. All natural PHA normally possess biodegradability [2].

Many research and industrial efforts have been made to turn PHA into biodegradable plastics [3–5]. However, the high production cost of PHA has prohibited its application as ''biodegradable packaging materials''. Studies were conducted using polyhydroxybutyrate (PHB), the most common member of the PHA family as biomaterials for *in vitro* and *in vivo* studies, and results showed various degrees of biocompatibility and biodegradability [6, 7]. The application of PHA as biomaterials will add values to this novel biopolymer and thus suitable for current application exploitation [8].

Due to its biodegradability and biocompatibility, PHA may well serve as materials for applications in tissue engineering [8]. PHA, including fragile PHB, flexible copolymer consisting of 3-hydroxybutyrate and 3-hydroxyvalerate (PHBV), and elastomeric copolymer consisting of 3-hydroxyoctanoate and 3-hydroxyhexanoate (PHOH) may have promising applications as tissue scaffolds and cardiovascular tissue [8]. However, some studies showed PHB and PHBV to have induced prolonged acute inflammatory responses when implanted *in vivo* [10]. These results could be attributed to the PHA surfaces that may not be biocompatible under the *in vivo* circumstance. On the other hand, PHB and PHBV have poor mechanical properties that limit their applications in situations that require high tensile strength. Thus, PHA with better mechanical properties, such as copolymers of 3-hydroxybutyrate and 3-hydroxyhexanoate [11], may be more suitable for the tissue engineering application provided PHBHHx is biocompatible.

It is, therefore, desirable to investigate and improve the biocompatibility of PHBHHx. Effective polymer surface modifications include changes in chemical group functionality, surface charge, hydrophilicity, hydrophobicity, and wettability [8, 12, 13]. Polymer surface can be modified by means of various chemical or physical

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TABLE I Activities of three lipases used in this study

Lipase type	Activity determination			
	Substrate	Temperature °C	pH	Lipolytic activity
Lipolase 110T	Tributyrin	30	7.0	110 KLU/g
Lipolase Ultra 50T	Tributyrin	30	7.0	50 KULU/g
Lipoprime 50T	Tributyrin	30	7.0	50 KLU (P)/g

KLU and KULU are Novo Nordisk lipase activity units.

processes including plasma-ion beam treatment, electric discharge, surface grafting, chemical reaction, vapor deposition of metals and flame treatment [8, 9].

In this study, the biocompatibilities of PHB, PHBHHx, their blends and PLA were investigated before and after the surface treatment with lipases and NaOH, respectively.

2. Experimental Section

2.1. Materials

2.1.1. Preparation of PHA polymer films

PHBHHx and PHB were kindly provided by Procter & Gamble Co., Cincinnati, USA and Jiangmen Center for Biotech Development, Guangdong, China, respectively.

1 g PHBHHx (Mw 1 000 000 Da) was dissolved in 110 ml acetone and refluxed at 60 °C for 1 h. The PHBHHx acetone solution was centrifuged to remove non-soluble particles (5000 *g*, 20 min). The supernatant was added with methanol to obtain PHBHHx precipitates in high purity.

PHB (Mw 300 000 Da) in powder form was already in 99.99% purity and it was not subjected to further purification.

PLA (Mw 350 000 Da) was kindly provided by Institute of Macromolecules, Nankai University, Tianjin, China.

2.1.2. Polymer film casting

Totally 1 g of PHB and PHBHHx in a series of weight ratios (1 : 0, 0.9 : 0.1, 0.75 : 0.25, 0.5 : 0.5, 0.25 : 0.75, 0.1 : 0.9, 0 : 1), respectively, was dissolved in 110 ml chloroform. Each 110 ml chloroform PHA solution was evenly distributed into 20 petri dishes. The dishes were maintained at room temperature to allow complete evaporation of chloroform. The evaporation of solvent resulted in formation of PHA films in the petri dishes. Vacuum drying was further applied to completely remove any possible solvent remained in the films as the solvent is toxic to cells and may influence the results. Before inoculation, the films were sterilized under ultraviolet radiation overnight.

PLA was casted into film as described above.

2.1.3. Agents used to treat the polymers

Lipolase 110T, Lipolase Ultra 50T and Lipoprime 50T were kindly donated by Novo Nordisk China (Beijing, China). The optimal working conditions for the lipases were listed in Table I. Each polymer film was immersed in 11 ml of one of the above lipase solution (0.1 g/l) under 30 °C and pH 7.0 for 24 h. 1 N NaOH was also used here to treat the PHA films at 60 °C for 1 h.

2.2. Methods

2.2.1. Cell line and cell culture

The mouse fibroblast cell line L929 was purchased from the Institute of Virology Academia Sinica, Beijing. The cell line was cultured in 50 ml polystyrene flasks incubated in a CO_2 incubator (Sanyo, MCO-15 A, Japan) supplied with 5% CO_2 at 37 °C. The culture medium was Dulbecco's modified eagle medium (DMEM) supplemented with 11% fetal calf serum and 1 penicillin–streptomycin solution. After the cell line grew to completely cover the 50 ml cell culture flask, 5 ml of DMEM containing 0.01% trypsinase were added to the culture flask to digest the interconnected cells.

2.2.2. Growth of the cell line on polymer films

The mouse fibroblasts cell line L929 was treated with trypsinase to create a single cell suspension before cultivation on the films. Cell number was determined by direct counting via haemacytometer. Effects of lipases and NaOH treatment on cell growth were carried out by direct cultivation of 0.2 ml suspended mouse fibroblast cell line L929 (5×11^4 per ml) on the treated polymer films immersing in 5 ml DMEM containing 11% fetal bovine serum.

The above seeded films were then inoculated in the CO_2 incubator (5% CO_2) for 68 h. The medium was then removed and the films washed with phosphate buffer solution (PBS, pH 7.4) to completely remove serum. 1 ml 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT) and 5 ml serum-free DMEM were added to the each film and further inoculated for another 4 h. Cell morphological examinations of every dish were performed daily under an inverted phase contract microscope (JNOEC XD-111, Nanjing, China).

2.2.3. [3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide] MTT assay

Biocompatibility of untreated or treated polymers was evaluated by observing the number of mouse fibroblast cell line L929 grown on the above polymer films using the MTT assay [14–17].

MTT assay determines viable cell number and is based on the mitochondrial conversion of the tetrazolium bromide salt. MTT assay was employed in this study to quantitatively assess the viable cell numbers of L929

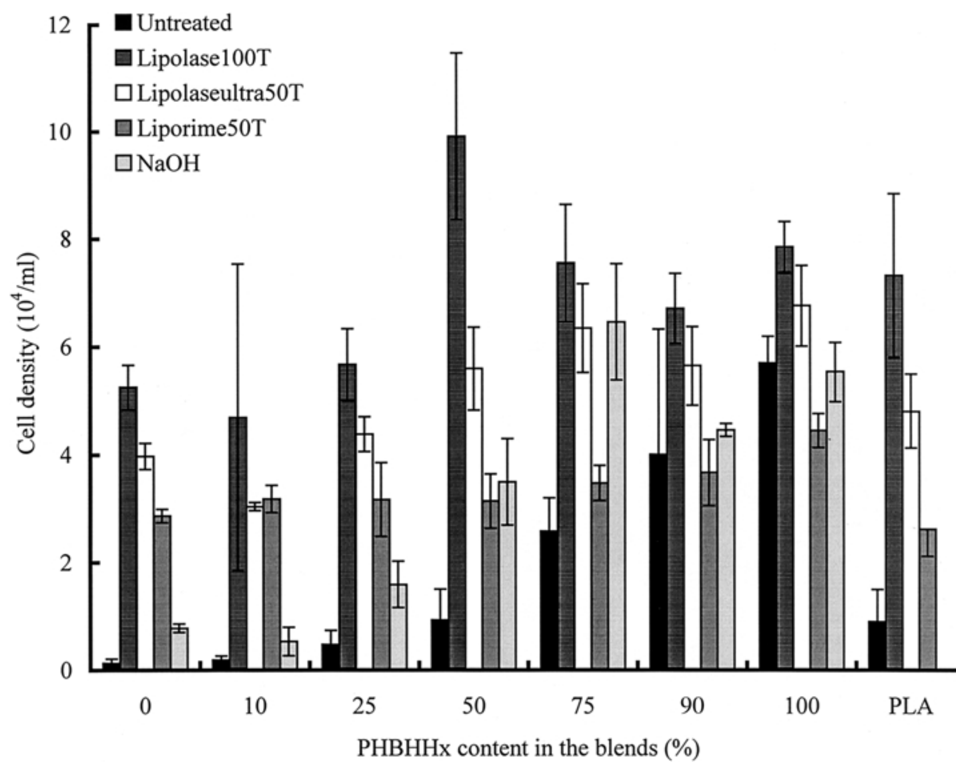


Figure 1 Effects of various lipases and NaOH treatments on the biocompatibility of PHB, PHBHHx, their blends and PLA.

attached and grown on polymer film surfaces. As stated above, the original medium was replaced by 1 ml MTT PBS solution containing 5 mg MTT in 1 ml PBS, and 5 ml serum-free DMEM medium. The films in new MTT containing medium were incubated at 37 °C for additional 4 h for MTT formazan formation. At the end of culturing period, the medium and MTT was removed and rinsed twice with PBS (pH 7.4). 11 ml dimethyl sulfoxide (DMSO), was added to each film to dissolve formazan. Formazan was completely dissolved after 30 min. 200 µl of the above formazan solution were taken from each inoculum and added to one well of the 96-well plate. Six parallel samples were prepared. The absorbancies of the samples were measured using a Microplate Reader (Bio-RAD Benchmark, USA) set at wavelength 550 nm; DMSO was used as blank. To ensure that the polymers themselves did not contribute to the absorbance, polymer films alone were assayed as background controls. Six parallel replicates were measured for each film. MTT assay was performed on a direct count of L929 cells consisting of following numbers 1×10^6, 5×10^5 2.5×10^5, 1.25×10^5, 6.25×10^4 and 3.1×10^4 and the absorbancy values were plotted against the counted cell numbers; thus a standard calibration curve was established (Fig. 1). Viable cells grown on a polymer film were determined based on their MTT.

2.2.4. Scanning electron microscopy (SEM)

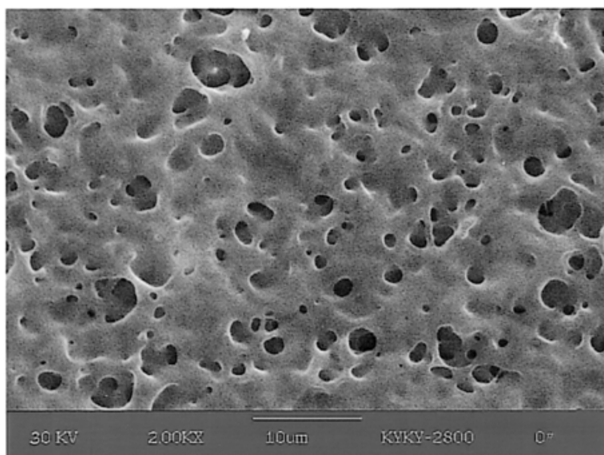
The polymer films, including untreated and treated samples, were washed twice with phosphate buffer and fixed with glutaraldehyde for 1 h. After dehydration by alcohol, the dried samples were observed under scanning electron microscope (Chinese Academy of Science, KYKY-2800, Beijing, China).

2.2.5. Statistical analysis

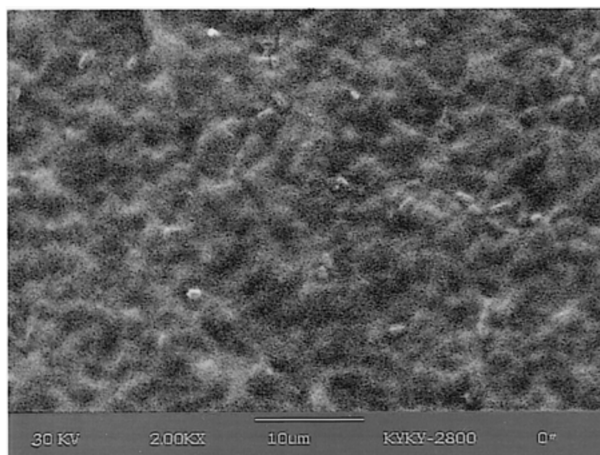
Results were expressed as mean $\pm$ standard error of the mean. Comparisons between groups were performed by one-way ANOVA test. Statistical significance was set at $p < 0.05$. Data were processed with Microcal orign 6.0 software (Microcal, USA). Linear regression analysis was utilized to evaluate the correction using Microsoft Excel software.

3. Results

Growth of mouse fibroblast cell line L929 on untreated and treated films of PHB, PHBHHx and blends of PHB/PHBHHx was significantly different (Fig. 1). Cells L929 grew poorly on untreated PHB and untreated control PLA films, viable cell density ranged only from 0.1×10^4 cells per ml on untreated PHB film to 0.7×10^4 cells per ml on untreated PLA film. Viable cells on the untreated PHBHHx film were approximately 56 times more than that on the PHB film and 8.5 times more than on PLA film. Films made from blending PHB and PHBHHx showed a dramatic improvement, viable cell density increased from 0.2×10^4 to 4×10^4 or 6×10^4 on blended film of PHB/PHBHHx in which the content of PHBHHx was 10% and 90%, respectively, depending on the lipases used. The number of viable cells increased dramatically when films of PHB, PHBHHx, their blends and PLA were treated with three different lipases and 1 N NaOH, respectively. Lipase treatment increased the viable cell number on the PHB film 30–56 times compared with the untreated PHB film. Viable cells on the PLA film also increased from 0.7×10^4 to 3×10^4 and 7.5×10^4 cells per ml after the lipase treatment, a 4.3- and 11-fold increases, depending on the type of lipase used. NaOH treatment on PHB film also indicated a nine-fold increase



(a)



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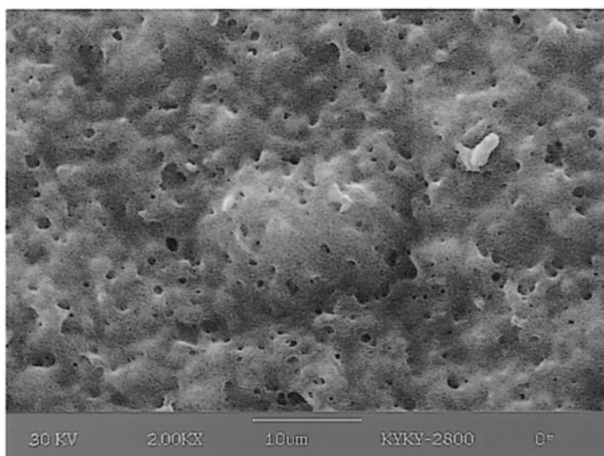

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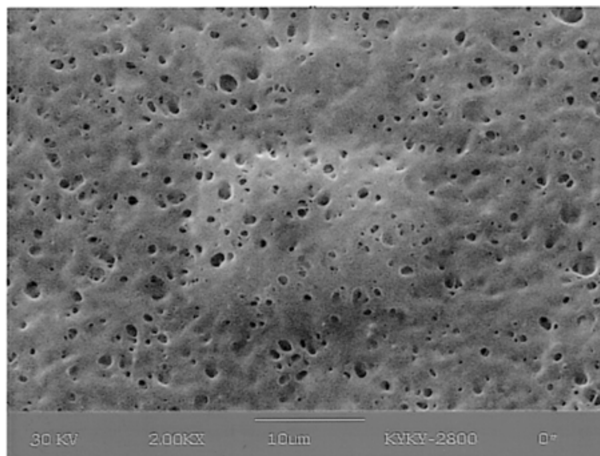

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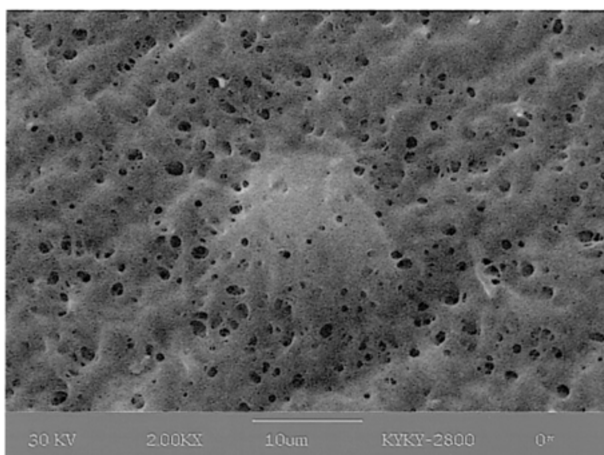

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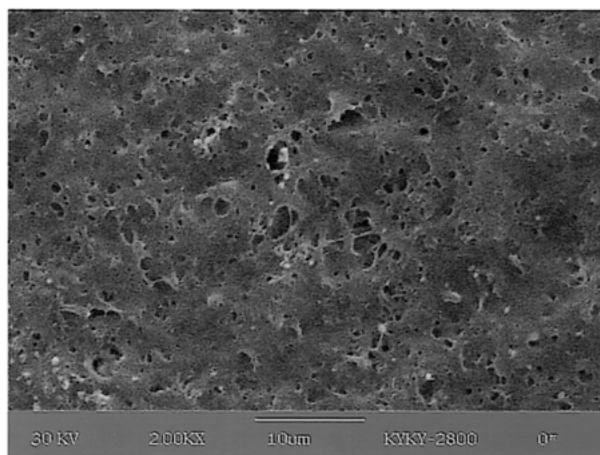
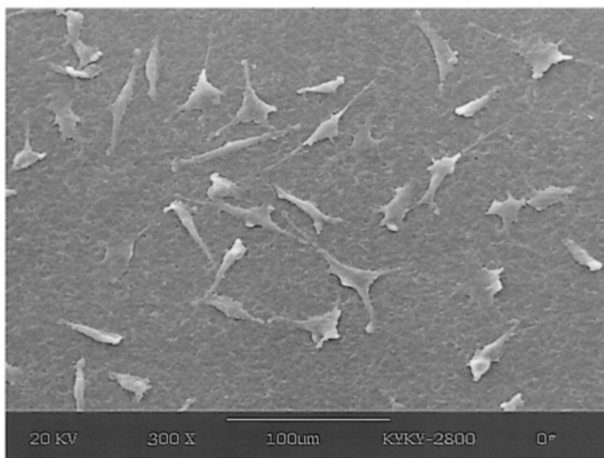


(f)

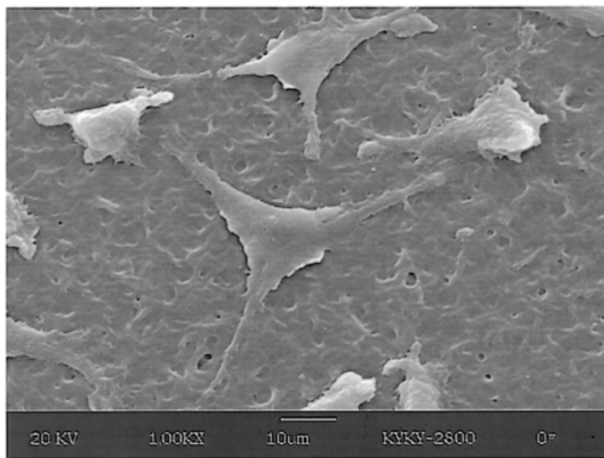
Figure 2 Scanning electron microscopy documented the surface structures of polymer films. (a) PHB film; (b) PHB film treated with lipase; (c) PHB film treated with 1 N NaOH; (d) PHBHHx film; (e) PHBHHx film treated with lipase; (f) PHBHHx film treated with 1 N NaOH. The magnification was 2000 ×.

on viable cell number compared with the untreated PHB film.

As PHBHHx content increased in the blends, the effects of lipase and NaOH treatment were significantly weakened. The lipase treatment on a blend film consisting of PHB/PHBHHx with the content of PHBHHx at 10% resulted in at least 30 fold increase in viable cells. While same treatment showed much less effect on a blend containing 90% PHBHHx, a maximum of 0.6 fold increase in viable cells was observed. The lipase treatment on PHBHHx alone showed mixed effects, lipase Lipoprime 50T even decreased viable cell number on the film, while Lipolase 100 T and Lipolase Ultra 50T improved growth of viable cells on PHBHHx very little (Fig. 1). The treated and untreated PLA films showed a similar behavior affecting viable cell growth, compared with a blend consisting of 50% PHB and 50% PHBHHx, although a little bit weaker than the blend.



(a)



(b)

Figure 3 Scanning electron microscopy (SEM) observation of L929 cells grown on untreated PHBHHx film. The magnification and the ruler bar were indicated at the bottom of the photos.

NaOH treatment showed less effective in improving growth of viable cells on all PHA film (Fig. 1). Cell number increased only 9 fold after 1 N NaOH treatment compared with 56 fold observed after lipase treatment. PLA is soluble in 1 N NaOH and no comparison was done. The effect of NaOH on blend polymers of PHB and PHBHHx behaved similar to lipase treatment, albeit weaker than lipase treatment (Fig. 1).

SEM showed that the surface of PHB film was decorated with many pores ranging from 1 to 5 μm in size (Fig. 2(a)). The lipase treatment transferred PHB film into a pore free surface (Fig. 2(b)). NaOH treatment on PHB reduced the pore size to around 1 μm, yet the surface was very rough compared with the PHB film treated with lipase (Fig. 2(c)). No apparent change of PHBHHx surface morphology was observed before and after lipase treatment, while NaOH treatment seemed to reduce the already small pore sizes further (Fig. 2(d)–(f)). SEM confirmed that cells L929 were able to attach onto the rough non-porous PHBHHx films, grew well and maintained normal healthy morphology on the films (Fig. 3).

4. Discussion

As a new member of PHA family, PHBHHx still has many properties that may be interesting for a wide range of applications. PHBHHx has much better mechanical properties compared with PHB and PHBV [11]. However, no study was done so far concerning this promising material for applications as tissue engineering material. The results of the present study have shown that PHBHHx can well support cell growth. SEM showed that the cells L929 were able to grow well and maintain normal morphology on PHBHHx films (Fig. 3). The MTT assay showed that viable cell growth on untreated PHBHHx was better than that on PHB and PLA films, indicating a promising property of PHBHHx as a biomaterial (Fig. 1). This study may provide the first hand data to compare the biocompatibility of PLA, PHB and PHBHHx.

The surface properties of a biomaterial, especially hydrophilicity, influence cell adhesion to the materials [19–22]. In general, the higher the hydrophilicity of a material surface, the stronger the cells attached to the material. In this study, lipases and NaOH were employed to improve the hydrophilicity of the polymer films. The improved hydrophilicity of the films allowed cells in its suspension to easily attach on the polymer films compared to that on the untreated ones. This is why we observed better cell growth on the treated PHB and PLA.

Lipase was an enzyme specifically acting on ester bonds of polyesters including our PHB, PHBHHx and PLA. The lipase treatment may bring two changes to the polymers surface: one is the change of chemical compositions, another is its physical properties. It was believed that the lipase treatment would produce many hydroxyl groups resulting from the hydrolysis of the ester bonds, and thus contributing to the improved hydrophilicity of the polymer films. To prove this argument, FT-IR was applied to detect the functional group changes on the treated polymer surfaces.

However, no difference was found by the FT-IR scanning of PHB and PHBHHx film surface before and after the lipase treatment (data not shown). On the other hand, SEM examination revealed significant morphological change in PHB film surface after lipase treatment (Fig. 2(a) and (b)), which suggested that the increase of hydrophilicity was caused by the physical change of surface morphology made by lipase treatment rather than the change of chemical functional group. NaOH acted as a hydrolysis catalyst, however, the effect of NaOH was much weaker compared with that of lipase (Fig. 2(c)). This may be the reason why we observed weaker growth on NaOH treated films than that of lipase treated ones.

Interestingly, the treatment with lipase gave the PHB films a much smoother surface rather than a coarser one. SEM examinations showed that PHB films treated with lipase had a pore free, relatively smooth surface compared with that of untreated films (Fig. 2(a) and (b)). The reason may be that the coarse part on the surface of PHB films (such as the pores) had a fairly large surface exposed to the lipase solution, thus it was degraded more easily. The size of the pores on the untreated PHB films range from 1 to 5 μm (Fig. 2(a)), which is a little smaller than the size of L929 cells (Fig. 3). The untreated multi-porous PHB film actually provided a smaller surface compared with the treated pore free film for the cells to attach to and grow on it. That may be the reason why cells grew better on treated

smooth films compared with the untreated, multi-porous films and NaOH treated films, which had reduced pore sizes compared with the untreated film.

PHBHHx film showed little change on its surface after the lipase and NaOH treatment (Fig. 2(d)–(f)), corresponding to an insignificant cell growth change before and after surface treatment. This may be due to a fairly lower crystallization degree of PHBHHx compared to that of PHB. The irregularity of the PHBHHx structure may interfere with the binding of lipase to the polymer molecules, and thus weaken its attack to the ester bonds of PHBHHx. In this case, an adjustment of the reaction conditions and the concentration of the lipase used may give a better performance. Further experiments are still in progress.

This study has demonstrated that PHBHHx, a microbially synthesized polyester with good biodegradability and biocompatibility, as well as strong mechanical properties, may be a very promising biomaterial for tissue engineering applications.

Acknowledgments

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References

1. A. J. ANDERSON and E. A. DAWES, *Microbiol Rev.* **54** (1990) 450.
2. A. STEINBUCHEL, in ''Biomaterials: Novel Materials from Biological Sources'', edited by D. Byrom (Stockton, New York, 1991) p. 124.
3. G. Q. GHEN, K. H. KONIG and R. M. LAFFERTY, *Antonie van Leewenhock* **60** (1991) 61.
4. D. BYROM, *FEMS Microbiol Rev.* **113** (1992) 247.
5. G. Q. CHEN and W. J. PAGE, *Biotechnol Biotechnique* **11** (1997) 347.
6. C. DOYLE, E. T. TANNER and W. BONFIELD, *Biomaterials* **12** (1991) 841.
7. J. C. KNOWLES, G. W. HASTINGS, H. OHTA, S. NIWA and N. BOEREE, *ibid.* **13** (1992) 491.
8. F. W. SIMON, D. P. MARTIN, D. M. HOROWITZ and O. P. PEOPLES, *Intl. J. Biol. Macromolecules* **25** (1999) 111.
9. D. M. ZHANG, F. Z. CUI, Z. S. LUO, Y. B. LIN, K. ZHAO and G. Q. CHEN, *Surf. Coat. Technol.* **131** (2000) 1–5.
10. S. AKNTAR, in ''Physiochemical properties of bacterial P(HB-HV) polyesters and their uses in drug delivery'', PhD Thesis, University of Bath (1990).
11. Y. DOI, S. KITAMURA and H. ABE, *Macromolecules* **28** (1995) 4822.
12. J. H. LIU, H. L. JEN and Y. C. CHUNG, *J. Appl. Polym. Sci.* **74** (1999) 2947.
13. J. SACRISTAN, H. REINECKE and C. MIJANGOS, *Polymer* **41** (2000) 5577.
14. G. ZUND, Q. YE, S. P. HOERSTRUP, A. SHOEBERLEIN, A. C. SCHMID, J. GRUNENFELDER, P. VOGT and M. TURINA, *Eur. J. Cardio-thoracic Surgery* **15** (1999) 519.
15. G. CIAPETTI, E. CENNI, L. PRATELLI and A. PIAAOFERRATO, *Biomaterials* **14** (1993) 359.
16. Y. MAROIS, R. GUIDOIN, R. ROY, T. VIDOVSKY, B. JAKUBIEC, M. SIGOT-LUZARD, J. BRAYBROOK, Y. MEHRI, G. LAROCHE and M. KING, *ibid.* **17** (1996) 1835.
17. D. SGOURAS and R. DUNCAN, *J. Mater. Sci.: Mater in Med.* **1** (1990) 61.
18. B. SAAD, P. NEUENSCHWANDER, G. K. UHLSCHMID and U. W. SUTER, *Intl. J. Biol. Macromolecules* **25** (1999) 293.
19. J. ZHAO and G. GEUSKENS, *Eur. Polym. J.* **35** (1999) 2115.
20. T. FURUKAWA, Y. MATSUSUE, T. YASUNAGA, Y. SHIKINAMI, M. OKUNO and T. NAKAMURA, *Biomaterials* **21** (2000) 889.
21. D. J. CHANVEL-LESART, P. PELLEN-MUSSI, P. AUROY and M. BONNAURE-MALLET, *ibid.* **20** (1999) 291.
22. T. W. QING, Z. M. YANG, S. X. CAI, S. R. XU and Z. Z. WU, *Chinese Reparative and Reconstructive Surgery* **13** (1999) 31.

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