J Mater Sci: Mater Med (2008) 19:67–73
DOI 10.1007/s10856-007-3170-y

In vitro biocompatibility assessment of PHBV/Wollastonite composites

Haiyan Li · Wanying Zhai · Jiang Chang

Received: 25 April 2006 / Accepted: 11 September 2006 / Published online: 19 June 2007

Abstract Biodegradable and biocompatible materials are the basis for tissue engineering. As an initial step for developing bone tissue engineering scaffolds, the in vitro biocompatibility of degradable and bioactive composites consisting of polyhydroxybutyrate-co-hydroxyvalerate (PHBV) and wollastonite (W) was studied by culturing osteoblasts on the PHBV/W substrates, and the cell adhesion, morphology, proliferation, and alkaline phosphatase (ALP) activity were evaluated. The results showed that the incorporation of wollastonite benefited osteoblasts adhesion and the osteoblasts cultured on the PHBV/W composite substrates spread better as compared to those on the pure PHBV after culturing for 3 h. In the prolonged incubation time, the osteoblasts cultured on the PHBV/W composite substrates revealed a higher proliferation and differentiation rate than those on the pure PHBV substrates. In addition, an increase of proliferation and differentiation rate was observed when the wollastonite content in the PHBV/W composites increased from 10 to 20 wt%. All of the results showed that the addition of wollastonite into PHBV could stimulate osteoblasts to proliferate and differentiate and the PHBV/W composites with wollastonite up to 20 wt% were more compatible than the pure PHBV materials for bone repair and bone tissue engineering.

Introduction

As bone is an apatite-collagen composite material, the polymer matrix composite containing a particulate bioactive component appears a natural choice for substituting cortical bone. The bioactivity of the composite, which is rendered by the bioactive component in the composite, will promote the tissue growth adjacent to the implant and the formation of a strong bond between the tissue and the implant. The polymer will provide ductility and other associated properties that are required of hard tissue replacement materials [1].

There are a number of studies about cell responses to polyhydroxybutyrate-co-hydroxyvalerate (PHBV) and it was found that porous PHBV scaffolds were adequate as substrates for cell cultures [2, 3]. In addition, being thermoplastic polyesters, PHBV can be easily processed by conventional techniques such as compression moulding, extrusion and injection. In some previous studies, hydroxyapatite (HAp) and tricalcium phosphate (TCP), as bioactive ceramics, have been incorporated into PHBV to produce bioactive, degradable, and biocompatible composite biomaterials for potential medical application [4]. Recently, we have reported the incorporation of wollastonite into PHBV to prepare bioactive and degradable composite scaffolds [5, 6]. The results showed that the incorporation of wollastonite with PHBV could not only result in scaffolds with enhanced bioactivity and tailored mechanical properties but also alter the degradation behavior of PHBV and formed scaffolds with controlled degradability for specific application by adjusting the mount of wollastonite.

However, other conditions should be met for a material to be considered suitable for biomedical use. For successful functioning biomaterials, the biocompatibility of the materials is an essential requirement. The performance of a biomaterial is controlled by two sets of characteristics, which are biofunctionality and biocompatibility. The former is to determine the ability of a biomaterial to perform

H. Li · W. Zhai · J. Chang (✉)
Biomaterials and Tissue Engineering Research Center, Shanghai Institute of Ceramics, Chinese Academy of Sciences, 1295 Dingxi Road, Shanghai 200050, P.R. China
e-mail: jchang@mail.sic.ac.cn

the appropriate and specific function and the latter is to determine the compatibility of the materials within the body [7]. Non-biocompatible materials have been shown to recruit T cells, macrophages and other immune cells to generate inflammatory reactions [8]. Being considered as ''the ability of a material to perform with an appropriate host response in a specific application'', the biocompatibility of biomaterial is closely related to cell behaviors in contact with them, such as cell adhesion, morphology, proliferation and differentiation [9].

In this paper, the biocompatibility of the PHBV/W composites were evaluated by culturing osteoblasts on PHBV/W substrates for up to 14 days. The adhesion, proliferation and differentiation of osteoblasts on the surfaces of the pure PHBV and PHBV/W composites were investigated.

Materials and methods

Raw materials

Poly-3-hydroxybutyrate-co-3-hydroxyvalerate containing up to 3% mol of β-hydroxyvalerate unites was obtained from TIANAN BIOLOGIC MATERIAL co. LTD, (China, Ningbo). Wollastonite powders were prepared by chemical coprecipitation method as described previously [5]. Briefly, continuous mixing of an aqueous solution of Na_2SiO_3 (1 mol/L) with an aqueous solution of $Ca(NO_3)_2$ (1 mol/L) at ambiance temperature was carried out overnight (mol ratio: Na_2SiO_3:$Ca(NO_3)_2$ = 1:1). Then the stirring was stopped and the resulting calcium silicate suspension was filtered and washed with deionized water and ethanol. After being dried at 80 (°C overnight followed by sintering at 800 °C for 2 h, the obtained wollastonite powders were sieved to obtain particles between 98 and 154 um and characterized by X-ray diffraction (XRD, Geigerflex, Rigaku Co., Japan).

Preparation and characterization of PHBV/W composites

The process for preparation of composites by polymer coagulation and hot compression moulding is schematically shown in Fig. 1. With this method, wollastonite particles are homogeneously incorporated in a polymer matrix through precipitation of a polymer solution containing dispersed wollastonite particles into a non-solvent. Briefly, PHBV was dissolved in $CHCl_3$ at a concentration of 10% (w/v) at room temperature. A certain amount of wollastonite particles were added to rapidly stirred polymer solution and continued to stir for at least 24 h. The rapid stirring ensured a homogeneous dispersion of the wollastonite particles in the polymer solution, which was then precipitated into a 10-fold excess of rapidly stirred methanol. The composite powders containing PHBV and wollastonite were isolated from the non-solvent by filtering and drying the precipitates in fume hood at room temperature to allow the solvent to evaporate for 1 h. Then, the precipitates were sieved to size range 250–400 μm before using. After vacuum-drying at 60 °C for 48 h to remove any remaining solvent, the composite powders were hot-compression moulded into discs with diameter of 6 mm and height of 2 mm at a pressure of 10 MPa at 200 °C for 30 min and cooled to room temperature. Then, the formed polymer surface on the discs were ground off by sand paper (800 grit). The resultant composites are denoted by PHBV/wollastonite composites (PHBV/W).

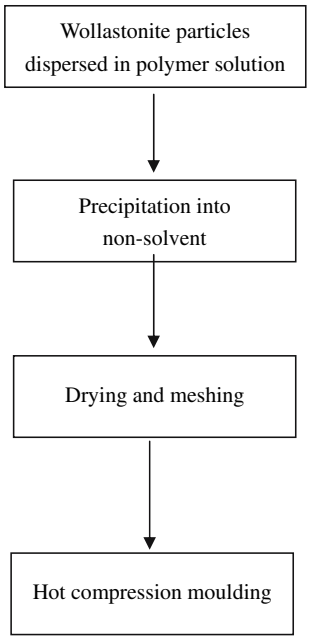

Fig. 1 Preparation of composite substrates by polymer coagulation and hot compression moulding

The surface of the as obtained substrates was coated with gold and SEM examination was carried out in a scanning electron microscope (Model JSM-6700F, Japan) at an accelerating voltage of 10 kV to observe the microstructures.

In vitro experiments

Cell culture

Osteoblasts were isolated from calvaria of neonatal (less than 2 days old) Sprague-Dawley rats. The calvaria were dissected into 1 cm^3 chips and washed with Hank's buffered salt solution for three times. The chips were digested at 37 °C with 0.25%(m/v) trypsin for 20 min in order to

diminish fibroblastic contamination, and then were treated with 1 mg/mL TypeII collagenase at 37 °C for 90 min. After that, the supernatants were centrifuged at 1,000 rpm for 10 min. The cells were suspended in the RPMI Medium 1640 (GIBCO) containing 15% heat-inactivated calf serum and incubated at 37 °C in a humidified atmosphere consisting of 5% CO_2 in air. The medium was changed every other day.

Cell adhesion

The PHBV and PHBV/W composite substrates were soaked in 75% ethanol for 48 h and sterilized under ultraviolet radiation overnight followed by washing with sterile phosphate-buffered saline (PBS, pH 7.4). Then, the osteoblasts were seeded onto the substrates in a 48-well plate at a density of 70 cells/mm^2. The cells were maintained in the CO_2 incubator for 3 h before 1 ml fresh medium was added to each well.

The number of living cells was measured using MTT-based colorimetric assay [10]. This assay relies upon the ability of living cells to reduce a tetrazolium salt into soluble colored formazan product. After the 1 ml fresh medium was added to each well, the supernatant containing non-adhering cells was removed, and 100 µl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added in each well. Crystal formazan was formed after incubation for 4 h at 37 °C and the samples were transferred from the original tissue culture plate and placed into the wells of a new one. This way only the crystals formed on the samples were dissolved. To dissolve the formazan product, 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The optical density (OD) was measured at the wavelength of 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (EL× 800, BIO-TEK, USA). The number of viable cells was calculated by converting the optical density (OD) values of the MTT assay into cell numbers according to a calibration curve. Osteoblasts with different known cell numbers were used to obtain the straight-line calibration curve (cell number versus absorbance at 490 nm) in order to know the cell number corresponding to the absorbance data.

Cell morphology

At the end of incubation period, culture medium was aspirated and cultured osteoblasts were washed twice with PBS. Cells on substrates were fixed with 250 µl of 2.5% glutaraldehyde in PBS at 4 °C overnight. After fixation, dehydration was performed by slow water replacement using series of ethanol solutions (30, 50, 70, 90, 95, 100%) for 10 min, allowing samples to dry at room temperature and under vacuum. The samples were mounted on stubs and coated in vacuum with gold before being observed by SEM.

Cell proliferation

Cells were incubated on the different substrates for 1, 3 and 7 days before analysis. The cell proliferation was assessed at different time points using above-mentioned MTT method. Results are reported as cell numbers, which were converted from the OD values measured by MTT according to the calibration curve.

Alkaline phosphatase activity assay

To determine the effect of the substrates on early events of osseous tissue formation in vitro, alkaline phosphatase (ALP) activity, a marker of osteoblasts differentiation, was analyzed. After culturing osteobpasts on composite substrates for 7 and 14 days, the substrates were transferred to a new tissue culture plate and the cells on the substrates were lysated with a solution containing 0.1% Triton ×-100. Then, ALP activity was assayed by the method of Lowry (1954) [11]. Aliquots (200 ul) of the cell lysates were incubated with 900 ul of reaction solution (containing 450 ul of 3 mmol/l *p*-nitrophenyl phosphate and 450 ul of 0.1 $MgCl_2$-HCl-diethanolamine) at 37 °C for 30 min. The conversion of *p*-nitrophenylphosphate to *p*-nitrophenol was stopped by adding 900 ul of 0.1 M NaOH [12], and the absorbance at 405 nm was measured with the UV-visible spectrophotometer. The visible light absorbance at 405 nm was utilized for calibration and quantification, the correlation between *p*-nitrophenol concentration (x, in umol/L) and UV/VIS absorbance (y) could be expressed as: $y = 0.0024x–0.0185$. Since the cells attained confluent after being cultured for 7 days as shown Fig. 4, and the cell number after 7 days was almost the same as that of 14 days for all of the samples (data not shown), the ALP activities were expressed in nanomoles of *p*-nitrophenol produced per minute per well.

Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Ionic concentrations of released silicon (Si) and calcium (Ca) in culture medium (2.5 cm^2/ml) were determined using ICP-OES (Varian Co., USA) after immersing substrates for 24 h at 37 °C in static conditions to mimic cell culture conditions [13]. The samples were diluted 1:10 in a 2 mol/L HNO_3 matrix before analysis. The instrument detection limits for the elements of interest were 0.05 (Si) and 0.01 (Ca).

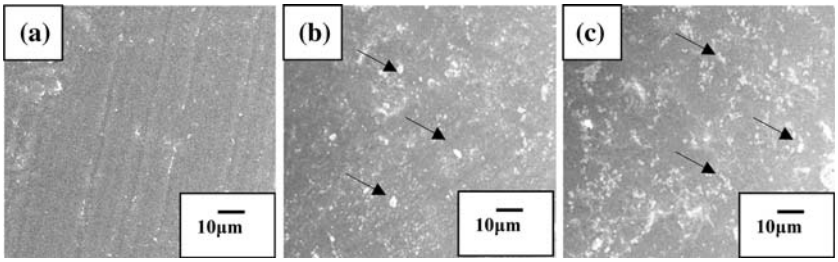

Fig. 2 SEM micrographs of PHBV and PHBV/W composite substrate surfaces: (**a**) PHBV, (**b**) PHBV/10%W and (**c**) PHBV/20%W. Arrows in the (**b**) and (**c**) indicated some of the wollastonite particles

Statistic analysis

About five biological replicates were used for each sample. All results are expressed as means ± standard deviation (SD) deviation. The results were analyzed using standard analysis of Student's *t*-test. Differences were considered significant when $p < 0.05$ (*).

Results

The SEM micrographs of the PHBV and PHBV/W composite substrates were shown in Fig. 2. It can be seen that white wollastonite particles uniformly dispersed on the surface of the PHBV/W composite substrates (arrows in Fig 2B and C) while there was no particles in the pure PHBV substrate. The surfaces of the all substrates were rather rough because of the hot compression moulding process. The method used here for preparation of PHBV/W composite substrate combined polymer coagulation and hot compression moulding, which resulted in homogenous distribution of the wollastonite particles in the polymer matrix.

Figure 3 shows the percentage of cell adhesion on PHBV substrates with different content of wollastonite. Results were determined by measuring the optical density and reported as percentage of cells adhered relative to the cells seeded. After 3 h of culture, an increase of the cell adhesion percentage was observed with the increase of the wollastonite content in the PHBV substrates. Only 60% of cell adhesion was obtained on the pure PHBV substrates, while 70 and 82% were obtained on the composites with 10 and 20 wt% wollastonite content, respectively.

Representative SEM images of cells cultured on the PHBV and PHBV/W composite substrates were shown in Fig. 4. After culturing on substrates for 3 h, it can be seen that the cells attached well on the PHBV/W composite substrates and began to spread while those on the pure PHBV substrates were round and weakly attached. On day 7, the spreading cells maintained physical contact with each other through filopodia or lamellipodia.

Cell proliferation studies were performed at different time points over a period of 7 days. Results presented in Fig. 5 show the total cell number changes after culturing on the pure PHBV and PHBV/W composite substrates for 1, 3 and 7 days. After culturing for 1 day, it can be seen that the cell number on the pure PHBV substrates was similar to that on the PHBV/10 wt%W composite substrates, which were significantly lower than that on the PHBV/20 wt%W composite substrates. After culturing for 3 days, the cell number on all of the substrates increased quickly and the osteoblasts cultured on the PHBV/20 wt%W composite substrates showed the highest proliferation rate. In addition, fifteen-fold increase of the osteoblasts on the PHBV/10 wt%W composite substrates was observed, which were significantly higher than that on the pure PHBV substrates. After culturing for 7 days, the osteoblasts continued to proliferate and the cell numbers on the PHBV/10 wt%W and PHBV/20 wt%W composite substrates were still significantly higher than that on the pure PHBV substrates. It is also clear that the cell number increased with the increase of the wollastonite content in the composites.

Figure 6 shows the changes of ALP activity of the osteoblasts cultured on the pure PHBV and PHBV/W

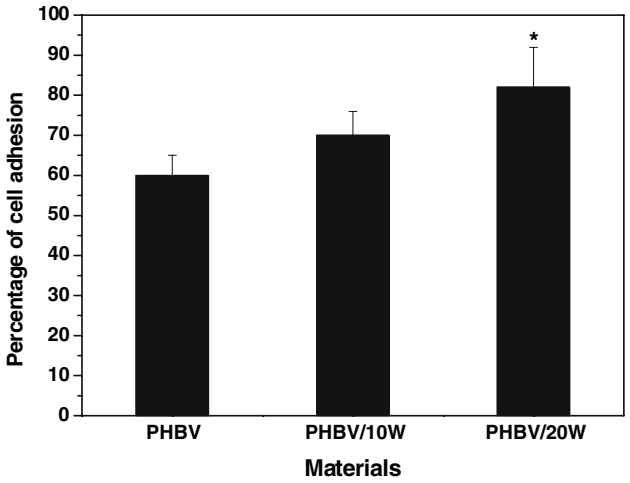

Fig. 3 Cell adhesion on PHBV substrates with different content of wollastonite. The results presented in this figure are expressed in percentage of cells adhered on the materials relative to the cells seeded. *$p < 0.05$ vs persentage of cell adhesion on the pure PHBV

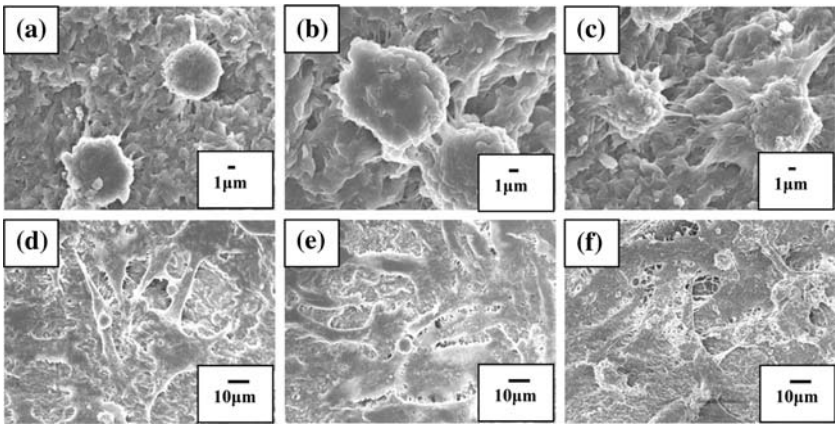

Fig. 4 SEM images of osteoblasts after culturing on the substrates for 3 h: (**a**) PHBV, (**b**) PHBV/10% W and (**c**) PHBV/20% W and for 7 days: (**d**) PHBV, (**e**) PHBV/10% W and (**f**) PHBV/20% W

composite substrates for 7 and 14 days. It can be seen that the ALP activities of osteoblasts on all substrates increased over time. On day 14, osteoblasts cultured on all substrates presented significantly higher levels of ALP than those of the osteoblasts cultured for 7 days. The results indicated that the ALP activities of the osteoblasts on composite substrates were significantly higher than that of the osteoblasts on the pure PHBV substrates, and an increase of osteoblasts ALP activity was also observed with the increased percentage of wollastonite content in the PHBV substrates.

Table 1 shows concentrations of ionic products released from PHBV/Wollastonite composites containing different wollastonite content incubated in DMEM for 24 h. Si concentration was found to be 31.42 μg/ml for PHBV/10 wt%W composites and 49.39 μg/ml for PHBV/20 wt%W composites, respectively. The Ca concentration was 46.08 μg/ml for PHBV/10 wt%W composites and 60.8 μg/ml for PHBV/10 wt%W composites, respectively. In contrast, the Ca concentration in the cell culture medium containing the pure PHBV was only 4.87 ug/ml and there was no Si ions detected, which were the similar to those in the pure cell culture medium.

Discussion

After seeding on materials, the cell/materials interactions, including adhesion, proliferation and differentiation, indicate the biocompatibility of the materials. Cell adhesion is known as an important cellular process because it directly influences cell growth, differentiation and migration that lead to colonization [14–16]. The cell adhesion and spreading belong to the first phase of cell/material interactions and

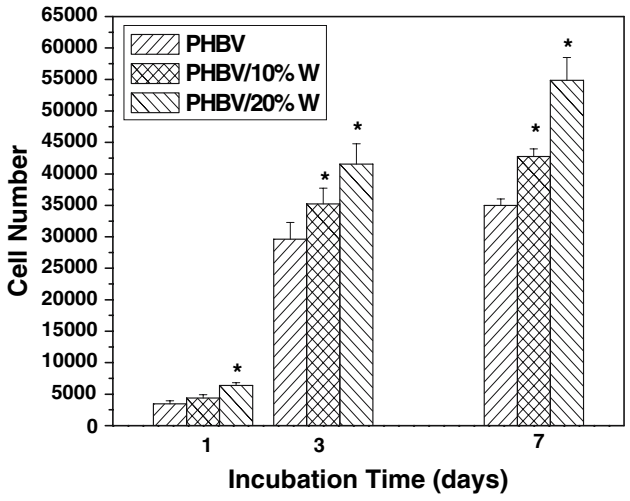

Fig. 5 Total cell number changes of the osteoblasts after culturing on the pure PHBV and PHBV/W composite substrates for different time. * indicated that the cell number on the PHBV/W composite substrates was significantly higher than that on the pure PHBV substrates ($p < 0.05$)

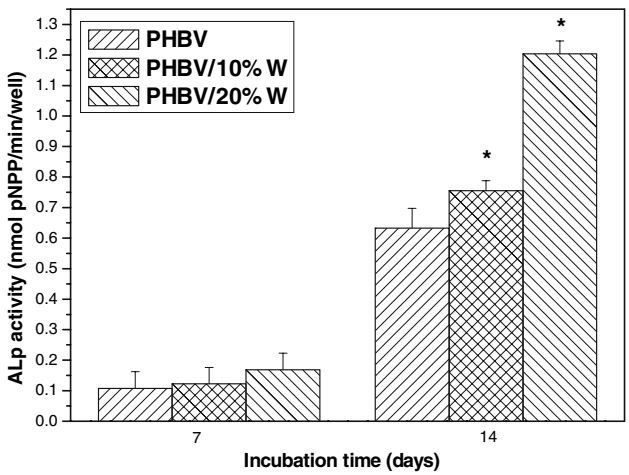

Fig. 6 Alkaline phosphate activity of osteoblasts cultured on pure PHBV and PHBV/W composite substrates for different time. * indicated that the ALP activity on the composite substrates was significantly higher than that on the pure PHBV substrates ($p < 0.05$)

Table 1 Ion concentrations of culture medium containing the different substrates (24 h)

Sample	Si (μg/ml)	Ca (μg/ml)
PHBV	0	4.87
PHBV/10 wt% W	31.42	46.08
PHBV/20 wt% W	49.39	60.8
Medium	0	4.93

surface characteristics of materials play an essential part in this first phase [17–20]. There are two phases in the process of cell adhesion. The first is non-specific adsorption of cells on the materials, which was mainly mediated by the physicochemical interaction between cells and materials. One of the physical properties of the materials is hydrophilicity, which influences the physicochemical interaction strongly. Many studies have reported that hydrophilic material surfaces are better for cell attachment, spreading, and cytoskeletal organization than hydrophobic ones [21], since the hydrophilicity of the materials was helpful for the absorption of fibronectin [22], which is essential for osteoblasts adhesion in vitro [23]. In addition, recent studies showed that the composition of material surface could also affect the interactions between cell and materials. Feng et al. reported that the osteoblasts amount and activity on the surfaces containing calcium are higher than those on the surface solely containing phosphate ions. They demonstrated that Ca ion sites on the material surfaces favor protein adsorption, such as fibronectin and vitronectin as ligands of osteoblasts, onto the surface due to positive electricity, chemical and biological function [24]. In the present study, the results showed that more osteoblasts adhered on the PHBV/W composite substrates as compared to that on the pure PHBV after culturing for 3 h. All of these results showed that the PHBV/W composite substrates were more suitable for the osteoblasts adhesion. The improved hydrophilicity and different composition of the composite substrates might contribute to these results. In our previous study, we have shown that the addition of wollastonite into PHBV could result in a more hydrophilic composite as compared to the pure PHBV [5]. In addition, it is undoubted that incorporation of wollastonite into PHBV introduced calcium ions in the surfaces of the PHBV/W composite substrates. Therefore, the addition of wollastonite into PHBV improved the interactions between cells and materials.

In our study, we demonstrated that the osteoblasts proliferated and differentiated more quickly after being cultured on PHBV/W composite substrates than on the pure PHBV substrates. In addition, the cells on PHBV substrates containing more wollastonite showed higher proliferation and differentiation rate. As the SEM results showed that the substrate surfaces were covered by the osteoblasts after being cultured for 7 days, the effects of the surface properties on the proliferation and differentiation of the cells were weakened. Then, the higher proliferation and differentiation levels of the composite substrates are mostly due to the addition of wollastonite. Previous studies have shown that the ionic products released from bioactive glasses containing Si and Ca can stimulate the proliferation and differentiation of osteoblasts. Xynos, Verries and Silver et al have reported that the certain dissolution product (Si and Ca in particular) of bioactive glass (45S5) dissolution could stimulate osteogenesis in vitro by inducing the proliferation and osteogenic differentiation of human osteoblasts [25, 26, 10]. Gough et al. has even shown that the certain concentration of Si and Ca ions could stimulate the mineralization and nodule formation during the incubation of osteoblasts [27]. In the present study, the ICP results showed that Si and Ca ions could be released from the PHBV/W composite substrates. These ionic products will react with $PO4^{3-}$ in medium easily and an apatite layer will deposit on the composite substrate surface. Many studies have confirmed that the formed apatite layer could give a positive effect for cellular responses, such as adhesion, proliferation and differentiation [28–30]. In addition, ions with higher concentrations could be released from the PHBV/20 wt% W composite substrates as compared to that of the PHBV/10 wt%W composites and no ionic product was released from the pure PHBV substrates. Therefore, it can be concluded that the higher proliferation and differentiation levels of osteoblasts on the PHBV/W composites are due to the ionic products of the wollastonite dissolution from the composites and the proliferation and differentiation behavior of osteoblasts was dependent on the concentration of ionic products. Gough et al. [27] also demonstrated that the ionic products of 58 bioactive glass had strong effects on the osteoblast behavior and reported that nodule formation was only demonstrated in medium with a 1:4 ratio of 58S dissolution products to DMEM, which contained approximately 47 ug/ml Si ions, and cell apoptosis was observed in conditioned medium with higher Si concentration. In our work, the Si concentrations of the PHBV/W composites were very close to the data reported by Gough et al. [27], which confirmed that Si ions played a key role in the stimulatory effect on osteoblast proliferation and differentiation.

Furthermore, the addition of wollastonite into PHBV might strongly affect the mineralization of extracelluar matrix as ALP activity has a crucial role in the initiation of matrix mineralization [31–33] and Gough et al. has demonstrated that certain ionic concentrations of dissolution product of bioactive glasses have a highly beneficial effect to osteoblasts but higher concentrations appear to cause programmed cell death. Therefore, the future work is to carry out experiments with a dilution of wollastonite extract in contact with osteoblasts to obtain the optimal Si

and Ca concentration and to evaluate the effects of wollastonite on the mineralization of extracelluar matrix.

Conclusion

In this study, the biocompatibility of the PHBV/W composites was evaluated by culturing osteoblasts on them. The results showed that the PHBV/W composites were more suitable for the cells adhesion than the pure PHBV due to their improved hydrophilicity and inorganic component. In addition, the osteoblasts on the PHBV/W substrates proliferated and differentiated more quickly than those on the pure PHBV substrates and the Si and Ca containing ionic products of wollastonite in the composites might be the reason for this stimulatory effect. Therefore, it can be concluded that the addition of wollastonite into PHBV stimulates the proliferation and differentiation of osteoblasts and the PHBV/W composites are biocompatible and might be suitable for preparation of bone implant and tissue engineering scaffolds.

Acknowledgements This work was financially supported by the National Basic Science Research Program of China (973 Program) (Grant No: 2005CB522700) and the Science and Technology Commission of Shanghai Municipality (Grant No: 02JC14009 and 05DJ14005).

References

1. M. WANG, *Biomaterials* **24** (2003) 2133–2151
2. S. GOGOLEWSKI, M. JOVANOVIC, S. M. PERREN, J. G. DILLON and K. HUGHES, *J. Biomed. Mater. Res.* **27** (1993) 1135–1148
3. G. T. KOSE, H. KENAR, N. HASIRCI and V. HASIRCI, *Biomaterials* **24** (2003) 1949–1958
4. L. J. CHEN and M. WANG, *Biomaterials* **23** (2002) 2631–2639
5. H. Li and J. CHANG, *Biomaterials* **25** (2004) 5473–5480
6. H. Li and J. CHANG, *Polym. Degrad. Stabil.* **87** (2005) 301–307
7. D. F. WILLIAMS, Medical, Dental Materials, edited by R. W. CAHN, P. HAASEN and E. KRAMER *J. Mater. Sci. Technol*.—a comprehensive treatment. (Weinheim, New York, Basel, Cambridge: VCH, 1992) p.1–27
8. S. L. ABBONDANZO, V. L. YOUNG, M. Q. WEI and F. W. MILLER, *Mod. Pathol.* **12** (1999) 706–713
9. D. F. WILLIAMS, The Williams Dictionary of Biomaterials. (Liverpool, UK: University Press, 1999) p. 40
10. S. VERRIER, J. J. BLAKER, V. MAQUET, L. L. HENCH and A. R. Boccaccini, *Biomaterials* **25** (2004) 3013–3021
11. O. H. LOWRY, N. R. ROBERTS, M. WU, W. S. HIXTON and E. J. CRAWFORD, *J. Biol. Chem.* **207** (1954) 19–37
12. H.F. WANG An ATLAS of Bone of Cells and Cell Culture Techniques. (Shanghai Science and Technique press, 2001) p. 63
13. ISO/EN 10993–5: Biological evaluation of medical devices-part 5: Tests for cytotoxicity: in vitro methods, 1992
14. K. BURRIDGE and K. FATH, *Bioessays* **4** (1989) 104–108
15. K. ANSELME, *Biomaterials* **7** (2000) 667–681
16. S. VERRIER, R. Bareille, A. Rovira, M. DARD and J. AMEDEE, *J Mater. Sci. Mater. Med.* **7** (1996) 46–51
17. D. A. ULEO and R. BIZIOS, *J. Biomed. Mater. Res.* **26** (1992) 291–301
18. A. HUNTER, C. W. ASCHER, P. S. WALKER and G. W. BLUNN, *Biomaterials* **6** (1995) 287–295
19. K DERHAMI, J. F. WOLFAARDT, A. WENNERBERG and P. G. SCOTT, *J. Biomed. Mater. Res.* **52** (2000) 315–322
20. C. H. THOMAS, C. D. Mcfarland, M. L. JENKINS, A. REZANIA, J. C. STEELE, K. E. HEALY, *J. Biomed. Mater. Res.* **37** (1997) 81–93
21. K. WEBB, V. HLASY and P. A. TRESCO, *J. Biomed. Mater. Res.* **41** (1984) 422–430
22. M. YANG, S. ZHU, Y. CHEN, Z. CHANG, G. CHEN, Y. GONG, N. ZHAO and X. ZHANG, *Biomaterials*. **25** (2004) 1365–1373
23. J. G. STEELE, C. MCFARLAND, B. A. DALTON, G. JOHNSON, M. D. EVANS and C. R. HOWLETT, *J. Biomater. Sci. Polym. Ed.* **5** (1993) 245–257
24. B. FENG, J. WENG, B. C. YANG, S. X. QU and X. D. ZHANG, *Biomaterials* **17** (2004) 3421–3428
25. I. D. XYNOS, M. V. J. HUKKANEN, J. J. BATTEN, L. D. K. BUTTERY, L. L. Hench and J. M. Polak, *Calcif. Tissue. Inter.* 67 (2000) 321–329
26. I. A. SILVER, J. DEAS and M. ERECINSKA, *Biomaterials* **22** (2001) 175–185
27. J. E. GOUGH, J. R. JONES, L. L. HENCH, *Biomaterials* **25** (2004) 2039–2046
28. A. EL-GHANNAM, P. DUCHEYNE and I.M. SHAPIRO, *J. Biomed. Mater. Res.* **36** (1997) 167–180
29. C. LOTY, J. M. SAUTIER, H. BOULEKACHE, T. KOKUBO and H. M. KIM, *J. Biomed. Mater. Res.* **49** (2000) 423–434
30. N. OLMO, A. I. MARTÍN, A. J. SALINAS, J. TURNAY, V. R. MARÍA and M. A. LIZARBE, *Biomaterials* **24** (2003) 3383–3393
31. G. R. BECK Jr, E. C. Sullivan, E. MORAN and B. ZERLER, *J. Cell. Biochem.* **68** (1998) 269–280
32. J. E. AUBIN, F. Liu, L. MALAVAL and A. K. GUPTA, *Bone* **17** (2 Suppl) (1995) 77S–83S
33. L. MALAVAL, F. LIU, P. ROCHE and J. E. AUBIN, *J. Cell. Biochem.* **74** (1999) 616–627