Mater. Res. Soc. Symp. Proc. Vol. 845 © 2005 Materials Research Society AA5.33

Enhanced Osteoblast Functions on Nanophase Titania in Poly-lactic-co-glycolic Acid (PLGA) Composites

Huinan Liu[1], Elliott B. Slamovich[1] and Thomas J. Webster[1,2]
[1]School of Materials Engineering, 501 Northwestern Avenue
[2]Weldon School of Biomedical Engineering, 500 Central Drive
Purdue University, West Lafayette, IN 47907, U.S.A.

ABSTRACT

Much work is needed in the design of more effective bone tissue engineering materials to induce the growth of normal bone tissue. Nanotechnology offers exciting alternatives to traditional bone implants since bone itself is a nanostructured material composed of nanofibered hydroxyapatite well-dispersed in a mostly collagen matrix. For this purpose, poly-lactic-co-glycolic acid (PLGA) was dissolved in chloroform and nanometer grain size titania was dispersed by various sonication powers from 0 W to 166 W. Previous results demonstrated that the dispersion of titania in PLGA was enhanced by increasing the intensity of sonication and that greater osteoblast (bone-forming cells) adhesion correlated with improved nanophase titania dispersion in PLGA. However, adhesion of osteoblasts to material surfaces, alone, is not adequate to determine long-term functions of implant materials. For this reason, and as a next step to determine the efficacy of nanocomposites in bone applications, subsequent functions of osteoblasts on nanophase titania/PLGA composites were investigated *in vitro* in this study. For the first time, results correlated better osteoblast long-term functions, specifically the deposition of calcium-containing mineral, with improved nanophase titania dispersions in PLGA. In this manner, the present study demonstrated that PLGA composites with well-dispersed nanophase titania can improve osteoblast functions necessary for the further investigation of these materials in orthopedic applications.

INTRODUCTION

The scientific challenge of bone regeneration encompasses not only understanding cell functions but also the development of suitable scaffold materials that can improve cell adhesion, growth and proliferation. Several physicochemical and biological requirements have to be fulfilled by the scaffold, depending on the particular application under consideration. Specifically, for orthopedic applications, scaffolds should have the following characteristics: (i) biocompatible and bioresorbable with a controllable degradation and resorption rate to match cell/tissue growth *in vitro* and/or *in vivo*; (ii) suitable surface chemistry and roughness for cell attachment, proliferation and differentiation; and (iii) bioactivity and osteoconductivity to facilitate the migration of osteoblasts from surrounding bone into the implant site and hence assist in the healing process [1-3]. To satisfy these demanding criteria, investigators have been studying a wide variety of natural and synthetic biomaterials, like polymers and ceramics, for the design and construction of scaffolds for orthopedic tissue engineering. These include naturally occurring polymers (e.g., hydrogels like gelatin, fibrin or collagen [4-6]), synthetic bioresorbable polymers (e.g., polylactic acid, polyglycolic acid and poly-lactic-co-glycolic acid [7-9]), bioactive ceramics (e.g., bioglasses and hydroxyapatite derivatives [10-12]) and naturally occurring ceramics (such as coral [13]). As a means to repair defects in bone, the design of

polymer/ceramic composites offers an exceptional opportunity to combine biodegradability and bioactivity to optimize physical and biological properties of scaffolds for bone regeneration. The addition of a ceramic phase to a biodegradable polymer may be exploited to alter the polymer degradation behavior towards favorable directions [14]. Moreover, functions of bone-forming cells can be increased in polymer/ceramic composites due to better cell seeding and growth environments as a result of improved osteoconductivity provided by the bioactive ceramic phase [15-20].

In attempts to further emulate the characteristics of natural bone, a number of groups have been investigating the use of nanophase ceramics (grain sizes less than 100 nm) in orthopedic applications. Specifically, reports in the literature demonstrated enhanced osteoblast functions (adhesion, proliferation, synthesis of alkaline phosphatase, deposition of calcium-containing mineral, etc.) on nanophase alumina and titania that mimic the size scale of constituent components in bone, i.e. less than 100 nm in diameter [21-23]. Bone itself is a nanostructured material composed of materials like proteins (collagen Type I) and hydroxyapatite crystals that have nanometer dimensions. For example, the dimensions of crystalline hydroxyapatite in natural bone are from 50-100 nm in length and 1-10 nm in diameter. Thus, it stands to reason that osteoblasts are naturally accustomed to interacting with nanostructured surface roughness in the body. Therefore, one promising consideration for the next generation of orthopedic implants with improved efficacy is degradable polymer and nanophase ceramic composites.

Poly-lactic-co-glycolic acid (PLGA) was chosen as the model polymer in the current study since it is biodegradable and widely utilized in tissue engineering applications. Nanophase titania was utilized as the model ceramic due to its excellent biocompatibility [21, 22]. Since nanophase ceramics tend to significantly agglomerate when added to polymers, different sonication output powers were investigated in this study to enhance titania dispersion. The results from a previous study by this research group demonstrated that osteoblast adhesion on nanophase titania/PLGA composites was enhanced at higher sonication powers [25]. Since osteoblast adhesion results alone are not enough for evaluating cell responses to the composites, osteoblast long-term functions (specifically calcium deposition) on the nanophase titania/PLGA composites were studied here.

MATERIALS AND METHODS

Substrate preparation

PLGA pellets (50/50 wt.% polylactic/glycolic acid; Polysciences, Warrington, PA) were dissolved in chloroform at 50 °C in a water bath for 40 minutes. Nanophase titania powder (Nanophase Technologies, Romeoville, IL) was then added to the PLGA solution to give a 70/30 polymer/ceramic weight ratio. The purity of the titania powder was above 99.5%, the particle size was 32 nm according to Brunauer, Emmett, and Teller (BET) measurements and the crystal phase was 80% anatase/20% rutile. The composite suspensions were sonicated using a W-380 sonicator (Heat System – Ultrasonics, Inc.) with output power settings of 119 W to 166 W. After sonication, the suspension was cast into a Teflon petri dish, evaporated in air at room temperature for 24 hours and dried in air in a vacuum chamber at room temperature for 48 hours.

Finally, the composite films (0.3 mm in thickness) were cut into 1×1 cm squares for cell experiments.

PLGA films, green and sintered titania compacts were used as control materials. PLGA pellets were dissolved in chloroform, cast, evaporated and vacuum dried for 48 hours at room temperature. Then, the films (0.2 mm in thickness) were cut into 1×1 cm squares for use. Green titania (TiO_2) disks were prepared by dry pressing nanophase titania powders in a tool-steel die via a uniaxial pressing cycle from 0.6 to 3 GPa over a 10 min period into 0.8 mm in thickness. Then sintered disks were heated in air at the rate of 10 °C/min from room temperature to a final temperature of 600 °C, holding at 600 °C for 2 hours and cooling down at the same rate as the heating rate. Glass coverslips were etched in 1 N NaOH and used as reference materials according to standard protocols [24]. All the substrates used in the experiments are shown in Table I.

Composites and PLGA substrates were sterilized by soaking in 70% ethanol for 30 minutes before performing experiments with cells. Titania substrates were sterilized by exposing them to UV light for 1 hour for each side. Glass substrates were sterilized in a steam autoclave at 120°C for 30 minutes.

Table I. PLGA, TiO_2 and PLGA/TiO_2 composites substrates

Substrate	Parameters
PLGA	Pure PLGA, control
TCG	Green pure titania compacts, control
TCS	Sintered pure titania compacts, control
PTC25	PLGA/titania composites sonicated at 119 W for 10 min
PTC35	PLGA/titania composites sonicated at 166 W for 10 min

Cell culture

Human osteoblasts (bone-forming cells; CRL-11372 American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin (P/S; Hyclone) under standard cell culture conditions, that is, a sterile, 37 °C, humidified, 5% CO_2/95% air environment. Cells at population numbers 8-9 were used in the experiments.

Quantification of extracellular calcium

Osteoblasts were seeded at a density of 100,000 cells/cm^2 onto the substrates of interest and were cultured in complete DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 50 μg/ml L-ascorbic acid (Sigma) and 10 mM β–glycerophosphate (Sigma) under standard cell culture conditions for 7, 14, and 21 days. At the end of the prescribed time periods, cells were lysed through three freeze-thaw cycles while the substrates of interest to the present study were treated with 0.6 N HCl at 37 °C overnight. The amount of calcium present in the acidic supernatant was quantified using a commercially available kit (Sigma) and following the manufacturer's instructions. Light absorbance of the samples was

measured at 575 nm using a spectrophotometer. Total calcium was calculated from standard curves of absorbance versus known concentrations of calcium standards (Sigma) run in parallel with the experimental samples. Calcium concentration values were normalized by total protein synthesis. All experiments were run in triplicate and repeated at least three separate times.

Statistical analysis

Numerical data were analyzed using standard analysis of variance (ANOVA) techniques; statistical significance were considered at $p<0.05$.

RESULTS

Calcium deposition

After 7 days of culture, as show in Figure 1, there were no detectable amounts of calcium deposited on all the composites, PLGA substrates and the glass reference; while there were significantly higher amounts of calcium on the TCG and TCS substrates than all the other substrates. After 14 days of culture, there were significantly higher amounts of calcium deposited on the TCG and TCS substrates than all the other substrates. Most importantly, after 21 days of culture, there were significantly higher amounts of calcium deposited on the PTC35 composites than the PTC25 composites. Higher amounts of calcium were also measured on the PTC25 compared to the PLGA after 21 days of osteoblast culture; significantly higher amounts of calcium on TCG and TCS substrates than all the other substrates was also observed. Calcium deposition was significantly greater after 14 days than after 7 days of culture on the PTC25, PTC35 composites, PLGA, TCG and TCS controls as well as glass substrates.

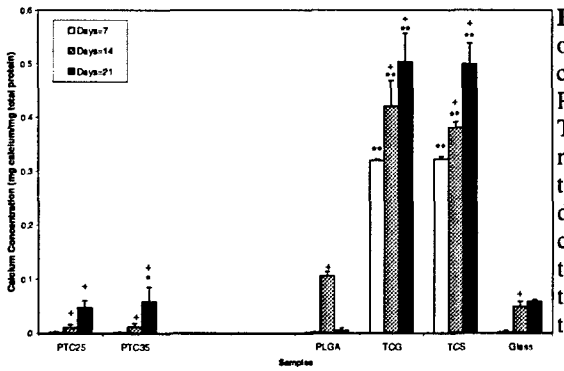

Figure 1. Calcium deposited by osteoblasts cultured on PLGA/titania composite films: 1) PTC25 and 2) PTC35; controls: 3) PLGA, 4) TCG, 5) TCS; and reference: 6) Glass. Values are mean ± SEM; n = 3; $^{*}p < 0.05$ compared to PTC25 according to corresponding days; $^{**}p < 0.05$ compared to all the composites, PLGA and glass, according to respective days; $^{+}p < 0.05$ compared to the same substrates at the previous time point.

DISCUSSION

Compared to metals and metal alloys (such as titanium and titanium alloys) conventional ceramics (grain sizes greater than 100 nm), nanophase ceramics (such as alumina and titania) have improved compatibility with bone cells [21-23]. In these studies, the biocompatibility of nanophase titania was documented by greater osteoblast adhesion over conventional titania

compacts [21, 22]. However, practically, ceramics themselves are very brittle and difficult to form stable scaffolds with acceptable mechanical properties for orthopedic applications. Therefore, nanophase titania/PLGA composites offer a chance to take advantage of great biocompatibility properties of nanophase titania with improved mechanical properties due to the addition of a polymer. In addition, through the use of PLGA, the polymer may degrade as new bone grows thus allowing for increased interlocking and potential higher implant success.

Results of a previous study conducted by this research group demonstrated that the dispersion of nanophase titania in PLGA was significantly enhanced by increasing the intensity of sonication [25]. Specifically, in that study, 10% compared to 5.7% of the surface area occupied was titania on the PTC35 and PTC25 composites, respectively. In addition, higher ultrasonic energy broke larger titania agglomerations into smaller titania particles, which were more easily dispersed in PLGA suspensions. For example, there were approximately 500 nm compared to 125 nm particle agglomerations on the PTC25 compared to PTC35. Moreover, previous results correlated greater osteoblast adhesion with increased nanophase titania dispersion in PLGA. That is, when sonication power increased, ceramic agglomeration decreased which promoted titania dispersion and subsequently enhanced osteoblast adhesion [25]. We also did not measure any appreciable increase in temperature with increases in these sonication powers (thus suggesting that we did not change the polymer chemistry between the two sonication powers).

Initial events during cell-biomaterials interactions, such as cell adhesion affect long-term functions, such as proliferation, synthesis of proteins and calcium mineral deposition. In the present study, enhanced deposition of calcium-containing mineral was observed on the titania/PLGA composites with increased nanophase titania dispersions in PLGA.

It is intriguing to consider why osteoblast functions were promoted on PLGA composites with well-dispersed titania. Increased surface coverage of nanophase titania particles and subsequently increased nanometer roughness may be plausible explanations [25]. Another explanation for increased osteoblast functions may be greater surface area. Previous studies have shown that, compared with larger grain size titania compacts, nanophase titania had about 35% more surface area for cell adhesion [24]. However, when normalized to this increased surface area, osteoblast adhesion, proliferation, and deposition of calcium-containing mineral were still enhanced on nanometer compared to conventional titania [24]. This indicates that increased surface area was not the contributing factor to greater osteoblast functions on nanophase titania compacts. Future studies will have to determine why osteoblast functions are promoted on PLGA composites with well-dispersed titania. Whatever the reason, this study suggests that nanophase titania in PLGA composites should be further considered in bone tissue engineering applications.

ACKNOWLEDGMENTS

The authors would like to thank the NSF for a Nanoscale Exploratory Research (NER) grant.

REFERENCES

1. A. R. Boccaccini and V. Maquet, *Composite Science and Technology*, 63, 2417-2429 (2003).
2. D. W. Hutmacher, *Biomaterials*, 21, 2529-2543 (2000).

3. R.C. Thomson, M.J. Yaszemski, J.M. Powers, and A.G. Mikos, *Biomaterials*, 19, 1935-1943 (1998).
4. R. Landers, U. Huebner, R. Schmelzeisen, and R. Muelhaupt, *Biomaterials*, 23, 4437-4447 (2002).
5. J. Weng and M. Wang, *Journal of Materials Science Letters*, 20, 1401-1403 (2001).
6. Y. Zhang and M. Zhang, *Journal of Biomedical Materials Research*, 55, 304-312 (2001).
7. M.A. Slivka, N.C. Leatherbury, K. Kieswetter, and G. Niederauer, *Tissue Engineering*, 7(6), 767-780 (2001).
8. R.J. Vance, D.C. Miller, A. Thapa, K.M. Haberstroh and T.J. Webster, *Biomaterials*, 25, 2095-2103 (2004).
9. H. Kim, H.W. Kim, and H. Suh, *Biomaterials*, 24, 4671-4679 (2003).
10. V. Maquet, A.R. Boccaccini, L. Pravata, I. Notingher and R. Jerome, *Biomaterials*, 25, 4185-4194 (2004).
11. W.W. Lu, F. Zhao, K.D.K. Luk, and Y.J. Yin, et al., *Journal of Materials Science: Materials in Medicine*, 14, 1039-1046 (2003).
12. P.X. Ma, R. Zhang, G. Xiao and R. Franceschi, *Journal of Biomedical Materials Research*, 54(2), 284-293 (2001).
13. H. Petite, V. Viateau, W. Bensaid, and A. Meunier, et al., *Natural Biotechnology*, 18(9), 959-963 (2000)
14. A.G. Stamboulis, L.L. Hench, and A.R. Boccaccini, *Journal of Materials Science: Materials in Medicine*, 13(9), 843-848 (2002).
15. J. J. Blaker, J. E. Gough, V. Maquet, I. Notingher, and A. R. Boccaccini, *Journal of Biomedical Materials Research - Part A*, 67(4), 1401-1411 (2003).
16. K.G. Marra, J.W. Szem, P.N. Kumta, P.A. DiMilla, and L.E. Weiss, *Journal of Biomedical Materials Research*, 47(3), 324-335 (1999).
17. S.J. Kalita, S. Bose, H.L. Hosick, and A. Bandyopadhyay, *Materials Science and Engineering C*, 23(5), 611-620 (2003).
18. A.R. Boccaccini, J.A. Roether, L.L. Hench, V. Maquet, and R. Jerome, *Ceramic Engineering and Science Proceedings*, 23(4), 805-816 (2002).
19. A.J. Dulgar, R. Bizios, and R.W. Siegel, *Materials Research Society Symposium Proceedings*, 740, 161-166 (2003).
20. S. Kalita, J. Finley, S. Bose, H. Hosick and A. Bandyopadhyay, *Materials Research Society Symposium Proceedings*, 726, 91-96 (2002).
21. T.J. Webster, R.W. Siegel, and R. Bizios, *Biomaterials*, 20, 1221-1277 (1999).
22. T.J. Webster, C. Ergun, R.H. Doremus, and R.W. Siegel, *Biomaterials*, 21, 1803-1810 (2000).
23. T.J. Webster, R.W. Siegel, and R. Bizios, *Nanostructured Materials*, 12(5), 983-986 (1999).
24. T.J. Webster and T.A. Smith, *Journal of Biomedical Materials Research*, (2004) (in press).
25. H. Liu, E.B. Slamovich and T.J. Webster, *American Ceramic Society Annual Meeting Proceedings*, (2004) (in press).