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Studies on Poly(*D*, *L*-lactic acid)/Wollastonite Composites as a Biomaterial

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ABSTRACT

The aim of the study was to prepare bioactive and bioabsorbable composites of polylactide/wollastonite (PDLLA/W) and evaluate their mechanical properties and biocompatibility in vitro. PDLLA and wollastonite were physically mixed and the mechanical properties of the composition showed the influence of wollastonite, and that a partial composite, e.g., a PDLLA/wollastonite composition with 15 wt% wollastonite (PW-15) has similar mechanical properties compared to pure PDLLA. However, an in vitro biocompatibility testing showed that the composite has no deleterious effects on the osteoblast cells and facilitates both adhesion and proliferation of the osteoblast cells on the PDLLA/wollastonite film surface. The introduction of wollastonite could also induce the deposition of hydroxyapatite (HA) on the surface of the composite in simulated body fluids (SBF) which has important implications on bone regeneration. These mechanical and biocompatibility properties represent PDLLA/wollastonite composites as potential biomaterials.

Key Words:

polylactic acid;
wollastonite;
biomaterial;
mechanical properties;
biocompatibility.

INTRODUCTION

Recent years, absorbable bone substitute composite materials have been the subjects of intense study in surgical reconstruction and bone tissue engineering [1]. A suitable temporary substitute material is required to have adequate mechanical and biological properties which enable tissue regeneration by exploiting the body's inherent repair mechanisms [2].

Many synthetic bioabsorbable materials have been investigated for tissue engineering and tissue repair because adequate control of material properties is relatively easy [3-5]. Polylactic acid (PLA), polyglycolic acid (PGA) and their

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copolymers [4,5] have attracted increasing attention. These materials have already demonstrated promising results in clinical use, for example as absorbable surgical sutures and meshes or in drug delivery systems [4]. However, a number of problems have been encountered due to the release of acidic degradation products leading to inflammatory responses [3-5]. Another limitation of these biodegradable polymers is that they lack a bioactive function, i.e., they do not allow for bone apposition or bonding on their surface [6].

Certain ceramic materials, such as hydroxyapatite (HA), tricalcium phosphate (TCP) and selected compositions of silicate and phosphate glasses and glass-ceramics, for example the commercially available bioglass react with physiological fluids and form tenacious bonds to hard and soft tissues through cellular activities. These materials are therefore known as "bioactive" [7]. The surfaces of these bioactive ceramics are covered with a layer of carbonate hydroxyapatite (HCA) when soaked in SBF which can connect with the tissue's collagen to form a tight combination layer. But the interaction between the bone and these materials takes place only on their surfaces. The ingrowth of new bone into the implants requires an appropriate porous structure of materials [8]. However, pure porous inorganic materials are very fragile. A new approach to overcome these problems is to design a two-phase scaffold, combining bioactive ceramic materials with absorbable polymer and developing a porous structure [9]. Moreover, composite materials can be engineered in such a way that their absorption rate in the body matches the formation rate of new tissue.

Various approaches have been introduced to develop bioabsorbable and bioactive composites, including combinations of polylactide (PLA), polyglycolide (PGA) and other absorbable polymers with HA, TCP, bioactive glasses, and glass-ceramics [9-17].

Wollastonite is a naturally occurring calcium silicate which has been widely used as filler in polymers and cement to fabricate composites with improved mechanical properties [18-20]. Recent studies have shown that wollastonite is bioactive, and it might be used as bioactive material in tissue repair or tissue engineering research [21-26].

In this article, novel bioactive and bioabsorbable composite materials were fabricated with poly(*D*,*L*-lactide) (PDLLA) and bioactive particle: wollastonite. The materials are intended for bone repair and bone tissue engineering applications. Here we report preparation, mechanical properties, and degradation behaviour of this composite, and the in vitro biocompatibility was evaluated via the cell growth assay.

EXPERIMENTAL

Materials

Poly(*D*,*L*-lactide)(PDLLA, η=3.94 dL/g) was provided by Sichuan Dikang Sci & Tech Pharmaceutical Co. Ltd. (Chengdu, China). Wollastonite powders (2500 meshes, L/D: 6-10) with particle size of 2-5 μm was purchased from LongFa super slim power Co. Ltd. (Youxi, Fujian, China). The components of the wollastonite include SiO_2 (48-52%) and CaO (42-46%).

Composite Preparation

The wollastonite powder was pre-dried at 80ºC for 24 h to remove any traces of moisture and mixed with an acetone solution of PDLLA (5 g/100 mL) in different compositions namely 95/5, 90/10, 85/15, 80/20, 75/25, 70/30, 60/40 and 50/50 in a tumbler mixture for 3.5 h. Then the composites were deposited by a large amount of ethanol. The resulting composites powder was dried in vacuum at 25ºC for 48 h to remove the remaining solvent.

FTIR Studies of Composite Microstructure

FTIR Spectra analysis for pure PDLLA, wollastonite, and the PDLLA/W composites were recorded using NICOLET 200SXV FT-IR (USA) spectrometer with a wavenumber resolution of 2 cm^{-1}. The samples were mixed with KBr and made into slices. The wavenumber was between 4000 and 450 cm^{-1}.

Testing of Mechanical Properties

Dumbbell-shaped test specimens with effective dimensions of 25×5×2 mm were prepared by compression-moulding under 10 MPa pressure. The processing temperatures for PDLLA and the composites were 155ºC and 190ºC, respectively. These specimens were placed at room temperature for 3 days before characterization. The tensile tests were conducted on

the testing machine (Testometric AX 350-10KN) at a crosshead speed of 2 mm/min. The 3-point bending strength and bending modulus were measured at a crosshead speed of 2 mm/min with a span of 20 mm. The values presented are the mean values of three replicate samples.

Deposition of HCA

Preparation of Specimens

The wollastonite powder and PDLLA solution in acetone were mixed at different wollastonite/PDLLA combinations (10/90, 30/70 and 50/50 w/w) in a mixing tumbler for 3.5 h. Then the mixture solutions were cast on flat disk-shaped glass plates (10 cm in diameter). Uniform film thickness was obtained by casting the same volume (10 mL) of the solution into each plate. Plates were dried at 25ºC for 24 h, and then the dried films were peeled intactly. The pure PDLLA films were obtained with the same method.

Immersion in SBF

The specimens were immersed in 20 mL SBF [27] of pH 7.40 and ion concentrations (Na^+: 142.0, K^+: 5.0, Mg^{2+}: 1.5, Ca^{2+}: 2.5, Cl^-: 147.8, HCO_3^-: 4.2, HPO_4^{2-}: 1.0, and SO_4^{2-}: 0.5 mM) approximating the human blood plasma, at 37.5ºC for 3 and 6 days, respectively. The SBF was prepared by dissolving NaCl, $NaHCO_3$, KCl, K_2HPO_4 .$3H_2O$, $MgCl_2$.$6H_2O$, $CaCl_2$, and Na_2SO_4 in ultra-pure water and buffered to pH 7.40 at 37.5ºC with tris(hydroxymethyl) aminomethane (final concentration of 50 mM) and 1M HCl aqueous solution. The specimens, after being removed from the fluid, were repetitiously washed with ultra-pure water and then dried in air at room temperature for 24 h. The surface deposition was scraped off the film with a knife and studied by FTIR.

Scanning Electron Microscopy

The surface of pre-deposited and deposited PDLLA films and the PDLLA/wollastonite composites films were examined using scanning microscopy (SEM, KYKY-AMRAY 1000B).

X-ray Photoelectron Spectroscopy

The component of the deposition is also certified by the X-ray photoelectron spectroscopy diffractometer (XPS, X SAM-800) using an X-ray source (Al K_α 12 kV×12 mA) with a 45.0 source analyzer angle.

The Thin-film X-ray Diffraction

The thin-film X-ray diffraction (TF-XRD) experiments were carried out using a Philips X'Pert Pro X-ray diffractometer which was equipped with a Ni-filtered CuK_α (λ = 0.1542 nm) radiation source operated at a voltage of 40 kV and a current of 30 mA. Samples were scanned from 15 to 80° (2θ). The step size and time for each step were 0.02° and 1s per step, respectively.

Cell Culture

Rat osteoblast cells line ROS 17/28 was employed in this experiment. Culture media and supplements were obtained from Invitrogen (Paisley, UK). Osteoblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, 100 U/mL penicillin [28]. The PDLLA/wollastonite composites and pure PDLLA were in the form of round discs, and after disinfection with γ-ray, these samples were placed into 24-well plate, then 1×10^4 cells in 1 mL medium were added to the well and osteoblasts allowed to settle onto composite and pure PDLLA discs. All samples were cultured at 37ºC in a humidified atmosphere with 5% CO_2. The culture medium was changed every 3 days. Time points of 1, 3, and 6 days were studied from the day of cell seeding.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay measures mitochondrial succinate dehydrogenase activity to assess cell number [29]. Osteoblast cells were seeded onto the pure PDLLA and composite (PW-15) films (1×10^4 cells/well) in a 24-well plate in triplicate and incubated at 37ºC. The culture medium was replaced every 3 days. After days 1st, 3rd, and 6th, 200 µL of MTT (5 mg/mL, Sigma) was added to each well. Cells were then cultured at 37ºC in a humidified atmosphere with 5% CO_2 for 4 h. The culture medium was then aspirated off, the polymer was washed with PBS and 1 mL of DMSO (dimethylsulphoxide) and put into each well. Then, the plate was left on a shaking platform for 10 min to solubilize the tetrazolium. Finally a portion of 200 µL of the solution was collected and

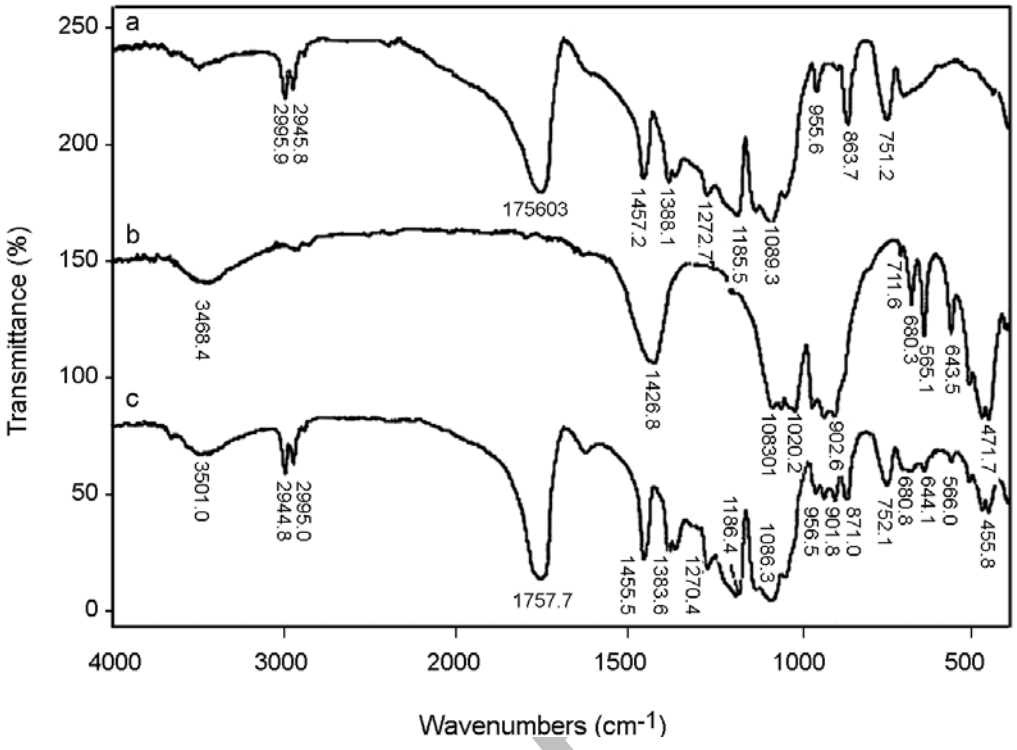


Figure 1. FTIR Spectra of (a) PDLLA, (b) wollastonite, and (c) PDLLA/wollastonite composite.

pipetted into a 96-well plate and the absorbance was determined at 490 nm according to microplate reader (BIO-RAD, model 550).

Fluorescent Staining and Observation

After each time point, the discs of pure PDLLA and composites were stained with 0.01% AO (acridine orange) for 5 min, and then examined in a fluorescence microscope (OLYMPUS B×60) after being washed with PBS. According to fluorescence observation, living cells may be clearly found on the surface of the composites with cell nucleus in bright green and cytoplasm in orange after blue light excitation, and dead cells would be stained in red.

RESULTS AND DISCUSSION

FTIR Studies of Composite Microstructure

Figure 1 is the FTIR spectra of PDLLA, wollastonite, and PDLLA/wollastonite composite. Compared with the spectrums of PDLLA and wollastonite, the PDLLA/wollastonite composite did not display obvious new absorption bands. That means there was no chemical reaction between such two materials.

Testing of Mechanical Properties

Mechanical properties of the PDLLA and PDLLA/wollastonite composites were evaluated by tensile and bending strength tests. Figure 2 shows the result of tensile strength changing with the composition of the composites. When the composite contained 5% (w/w) of wollastonite, the tensile strength of the composite decreased significantly in

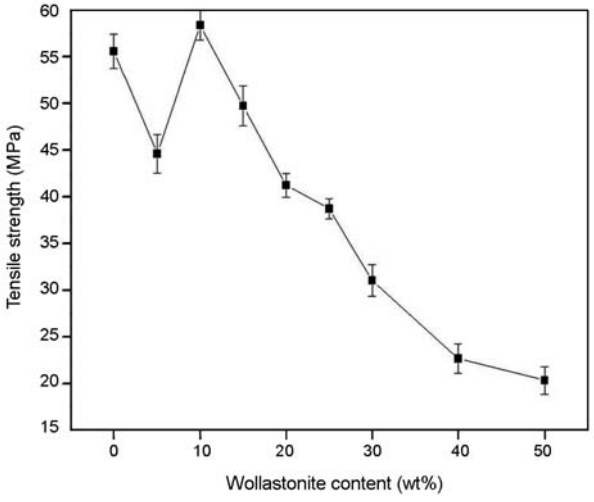


Figure 2. Plots of the tensile-strengths of the composites vs. filler contents.

comparison with pure PDLLA. And when the content of wollastonite reached 10%, the tensile strength was at its maximum, which is even higher than that of PDLLA. Afterwards, with the increase of wollastonite mass in the composite their strength showed downward trends. There was also further indication that no significant increase in the tensile strength was observed after wollastonite was interfused in PDLLA, due to absence of chemical bond produced between the two materials and PDLLA molecules entangled intramolecularly, thus the wollastonite particles could not strongly tether to PDLLA matrix in the composites.

Figure 3 shows the dependency of the bending strength and the bending modulus on the filler contents. In the bending strength (curve A in Figure 3) all the composites showed lower values relative to that of pure PDLLA. At the lower content of wollastonite ranging 5-20%, the bending strength increased with the increase of wollastonite mass in the composite. However, the bending strength began to decrease almost in a linear fashion when the mass of wollastonite surpassed 25 wt%.

In Figure 2 and curve A in Figure 3, when the composite contained 5% (w/w) of wollastonite, the tensile strength and the bending strength of the composite displayed naturally lower values which were probably due to the content of the wollastonite in the composite being too small to form a continuous phase to endure the stress. The wollastonite acted as a defect to decrease the mechanical properties of the composite.

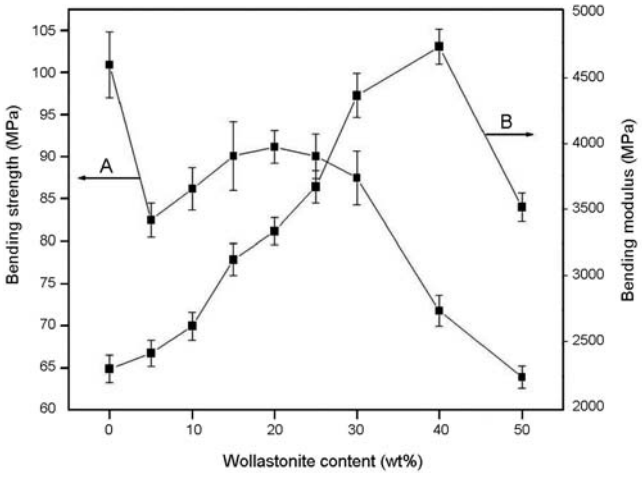
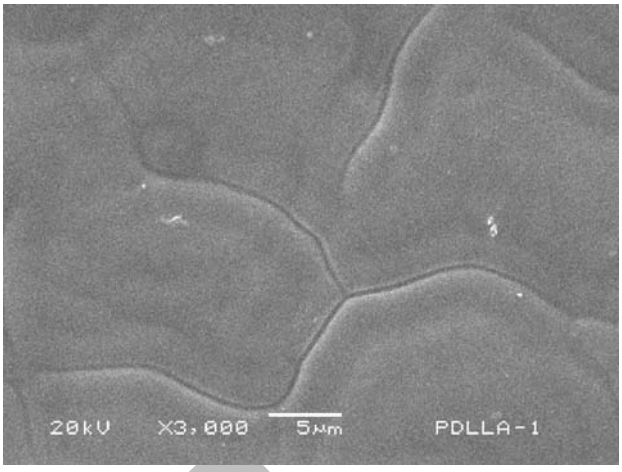
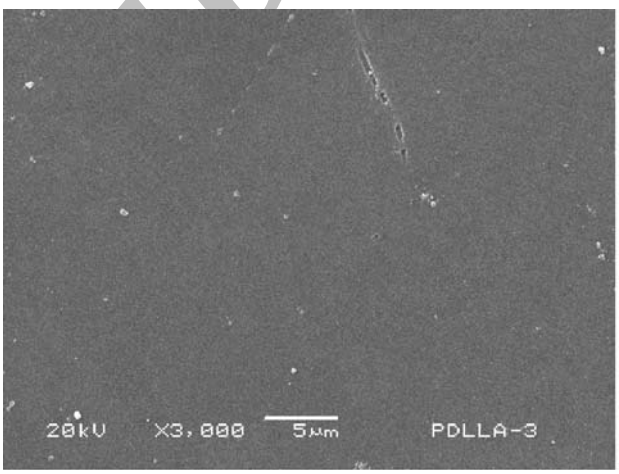


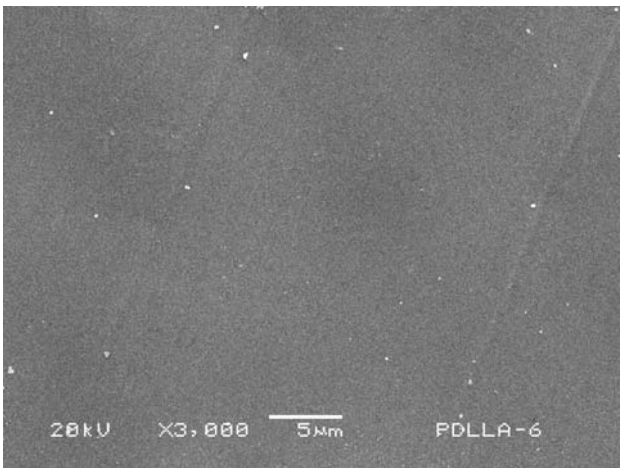
Figure 3. Plots of the bending-strength (A) and bending modulus (B) of the composites vs. filler contents.



(a)



(b)



(c)

Figure 4. SEM Morphologies of the surface of PDLLA films immersed in SBF at (a) 1 day (PDLLA-1), (b) 3 days (PDLLA-3), and (c) 6 days (PDLLA-6).

Apart from the strength results, the bending moduli of the composites were significantly increased compared to PDLLA (Figure 3, curve B). When the content of wollastonite was in the range of 0-40 wt%, the bending modulus increased remarkably with the increasing wollastonite contents. However, the excess wollastonite in the composite led to lowering modulus values. The results have indicated that the wollastonite could obviously improve bending modulus of the composition which could diminish the distortion of the composition when it is under stress.

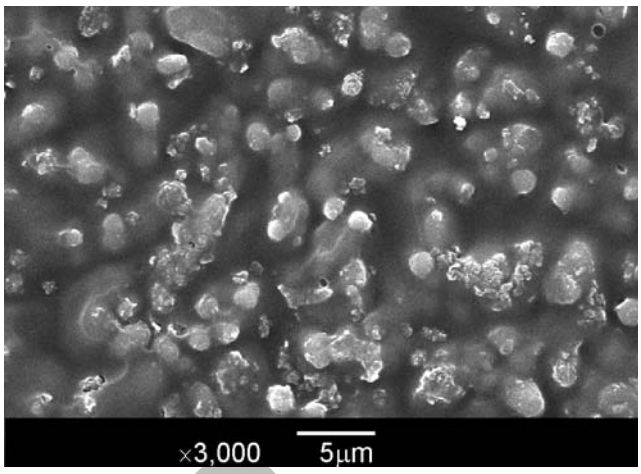
Deposition of HCA

In order to investigate the dispersion homogeneity of wollastonite particles in the PDLLA matrix and the deposition on the composition surface immersed in SBF, the films were investigated by SEM.

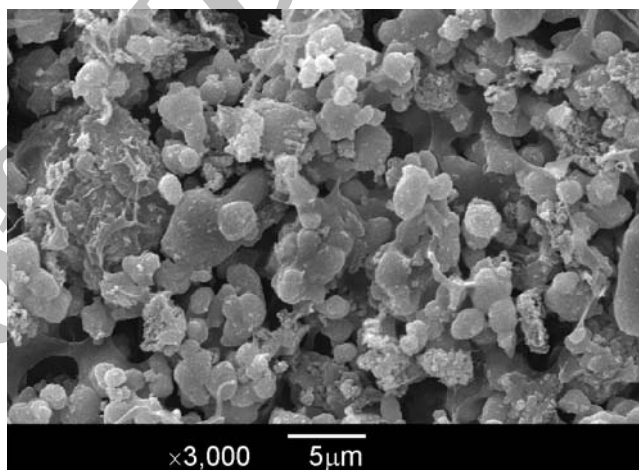
The surface morphologies of the PDLLA films immersed in SBF on the 1, 3, and 6 days (PDLLA, PDLLA-3, and PDLLA-6) measured by SEM are shown in Figures 4a-4c. It is apparent that no deposition was formed as a result of contact of pure PDLLA film with SBF. This was also proved by the result of XPS investigation.

Figures 5a-5c shows the morphologies of the surface of the films with different proportional PDLLA/wollastonite, viz. PW-10%, PW-30%, and PW-50% unimmersed in SBF. It could be seen that wollastonite particles were uniformly distributed throughout the PDLLA matrix. There was not any obvious aggregation of wollastonite particles in the composites. With the increase of wollastonite content, the packing of the particles grew denser. These results indicated the excellent equality between the PDLLA and the wollastonite particles.

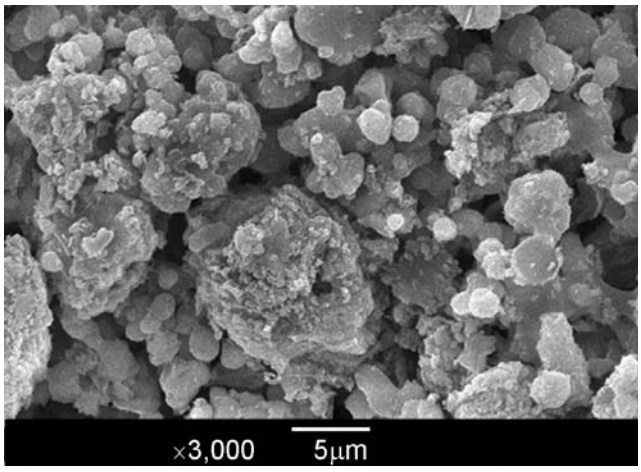
The morphologies of the surface of the composition films with different wollastonite content immersed in SBF for 1, 3, and 6 days depicted by SEM are shown in Figures 6a-6i. The results were compared to those of Figures 5a-5c, which were not immersed in SBF. It is apparent that there is an obvious spherical deposition on the surface of all composite films in Figure 6. It shows the gradual development of a deposition layer, as confirmed by FTIR analyses on the surface of the composite samples as a result of longer immersion in SBF after 1, 3, and 6 days. In addition, the spherical deposition increased



(a)



(b)



(c)

Figure 5. SEM Morphologies of the surface of the PDLLA/wollastonite composite films with different proportions of (a) 10%, (b) 30% , and (c) 50% wollastonite.

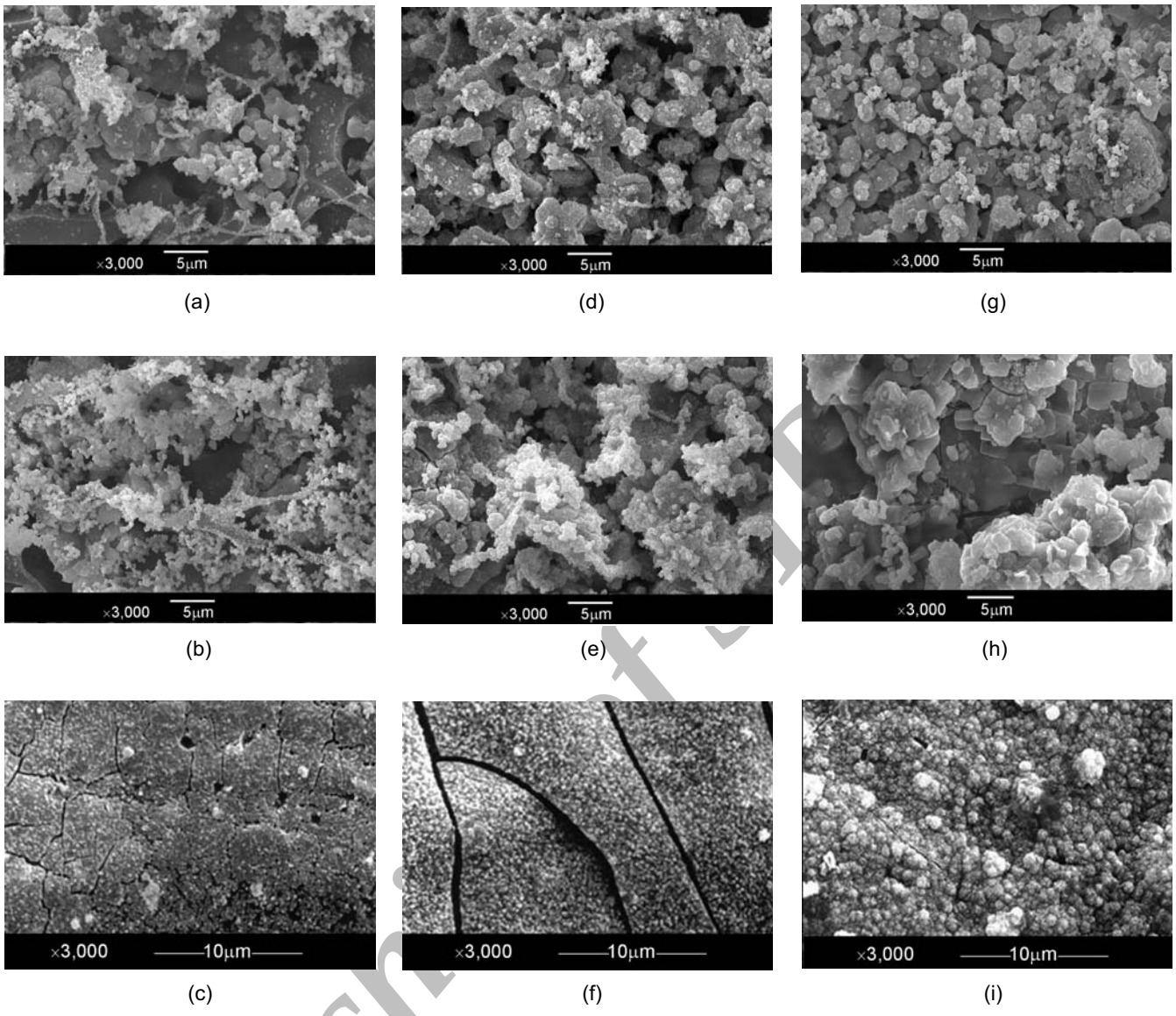


Figure 6. SEM Morphologies of the surface of the PDLLA/wollastonite composite films with different proportional wollastonite immersed in SBF at different days: (a) 10% wollastonite, 1 day (10%-1); (b) 10% wollastonite, 3 days (10%-3); (c) 10% wollastonite, 6 days (10%-6); (d) 30% wollastonite, 1 days (30%-1); (e) 30% wollastonite, 3 days (30%-3); (f) 30% wollastonite, 6 days (30%-6); (g) 50% wollastonite, 1 days (50%-1), (h) 50% wollastonite, 3 days (50%-3), (i) 50% wollastonite, 6 days (50%-6).

with the increasing of the wollastonite content.

For the composite samples, the spherical deposition formed on the surface of the composition films was investigated using FTIR, XPS, and TF-XRD, as shown in Figures 7, 8, and 9.

Figure 7 shows the FTIR spectra of (a) the PDLLA/wollastonite composition and the surface deposition of the composition immersed in SBF, (b) at 1 day, (c) at 3 days, and (d) at 6 days. FTIR confirmed that deposition formation took place on the composition surface after immersion in SBF and the deposition is hydroxyapatite (HA). Compared to the spectrum of PDLLA/wollastonite composition particles (a), the deposition of the composition (b), (c), and (d) displayed new absorption bands at wavenumbers 563, 602, 1039, and 1084 cm^{-1}. And the absorption bands have developed bigger and more acute with time. These absorption bands are associated with the characteristics of PO_4^{3-}. In the composition spectrum, the absorptions of carboxylic carbonyl band at

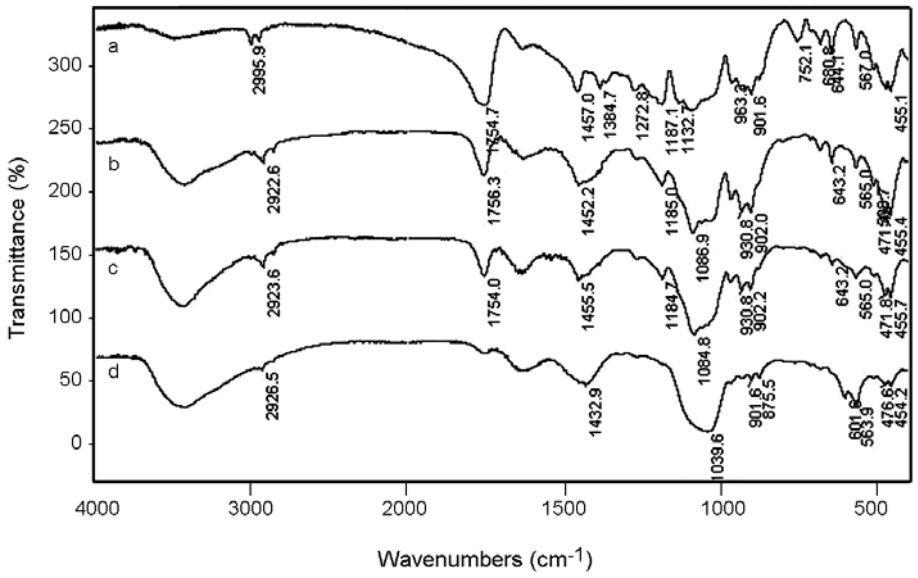


Figure 7. FTIR Spectra of the (a) PDLLA/wollastonite composition and the surface deposition of the composition immersed in SBF at (b) 1 day, (c) 3 days, and (d) 6 days.

1756 cm^{-1}, and the absorption of saturated C–H band at 2996 and 2944 cm^{-1} are characteristics of PDLLA structure. The absorption bands at wave numbers of 455 and 471 cm^{-1} are associated with the characteristics of SiO_4^{2-}. The absorption bands associated with the characteristics of PDLLA and SiO_4^{2-} fade away. It is concluded from these results that the surface of the specimen was covered with a layer deposition of HA.

Figure 8 shows XPS spectra of the crystals formed on the surface of the composite, containing 30 wt% wollastonite after soaking in SBF for 6 days. In Figure 8a one peak at 132.391 eV ascribed to P of PO_4^{3-} is observed. In Figure 8b two peaks at 347.388 and 351.003 eV ascribed to Ca^{2+} are observed. The results confirm that the surface deposition comprises Ca^{2+} and PO_4^{3-}. The Ca and P peaks were detected and the atomic ratio can be calculated through the area of the XPS spectra and the atomic ratio between Ca and P was 1.92, which was slightly higher than the Ca/P ratio for the HAP (1.67). This is probably because the

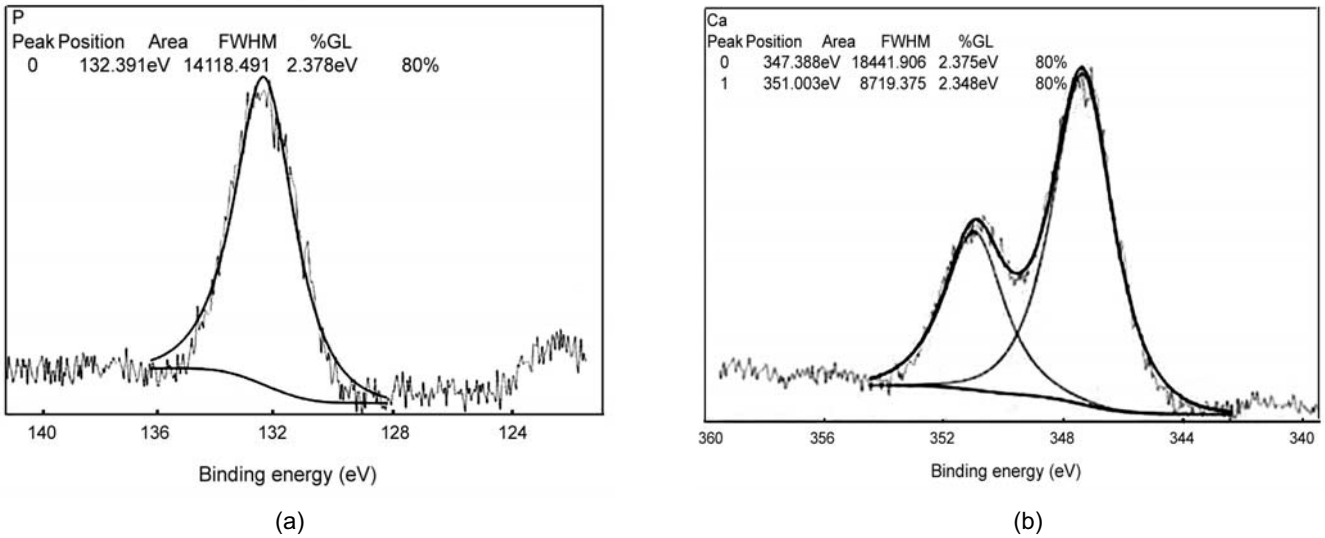


Figure 8. XPS Spectra of (a) P and (b) Ca of the surface deposition of the composition (PW-30) immersed in SBF for 6 days.

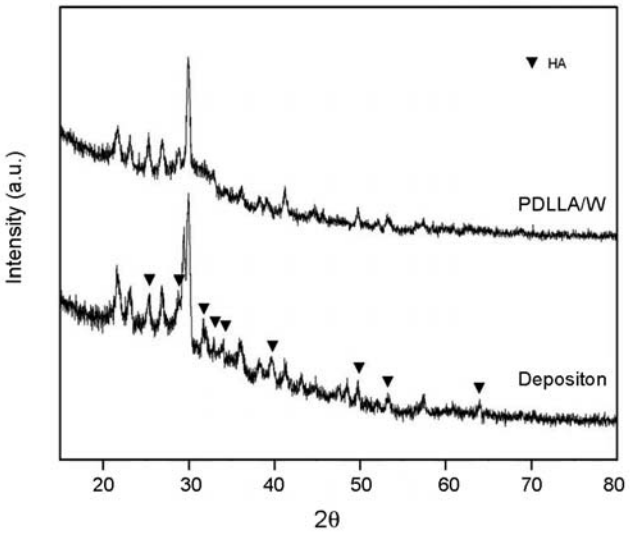


Figure 9. TF-XRD Graphs of PDLLA/wallastonite (70/30) composite before and after immersion in SBF.

composition could not cover the film completely. And the Ca/P ratio is higher than the theoretical ratio which is ascribed to the Ca of the wollastonite.

No HAP crystals were deposited on the surface of the pure PDLLA after soaking in SBF for 6 days (data not shown). It is obvious from the results described above that wollastonite/PDLLA composite could induce deposition of a HAp layer on the surface after exposed to SBF for 6 days, which indicated good bioactivity after filled with wollastonite particles. These results suggest that incorporation of wollastonite into polymer is a useful approach to prepare bioactive composite.

Figure 9 illustrates TF-XRD graphs of composite scaffolds with 30 wt% wollastonite before and after immersion in SBF. Compared to the XRD patterns of the composite scaffold without being immersed in SBF, the apatite diffraction peaks at about 25.6°, 28.9°, 31.8°, 32.9°, 34°, 39.8°, 49.6°, 53.2°, and 64° (2θ) were observed in the XRD pattern of the composite scaffold with 30 wt% wollastonite after soaking for 6 days.

The above in vitro studies were successful in confirming the high potential for HA formation on the composite surfaces, which is a measure of the considerable bioactivity of the material [8]. It was confirmed that an HA layer could form within few days of immersion in SBF. As Figure 6 shows, the structure and morphology of the HA layer changed during immersion in SBF. Small HA crystals deposited and developed into a continuous HA layer after several days of immersion and HA forming ability of the specimens is improved by increasing wollastonite proportion. This is likely to occur by the mechanisms similar to that of bioglass [30,31]. When the specimens were immersed in SBF, the Ca^{2+} and Si^{4+} in wollastonite were dissolved and released into SBF. The release of Ca^{2+} and Si^{4+} made the solution around the specimens highly supersaturated, and since the release of Ca^{2+} is faster than that of Si^{4+}, a Si^{4+} rich layer was formed on the surface of the films which functioned as initiator of hydroxyapatite nucleation and the hydroxyapatite layer spontaneously grew in SBF by consuming Ca^{2+} and PO_4^{3-} in the solution, as SBF is highly supersaturated with respect to hydroxyapatite at the films [32,33].

Biocompatibility of PDLLA/Wollastonite in Vitro

Osteoblast cells were co-cultured with samples for 1, 3, and 6 days and then assayed for cell viability (Figure 11) and proliferation (Figure 10). According to fluorescenct microscope observation, osteoblast cells had grown to be confluent nearly on day 3, and cells overlapped each other after cultured for 6 days on the surface of the composite (PW-15), while less cells were observed on the surface of the pure PDLLA films. MTT results have indicated that there are

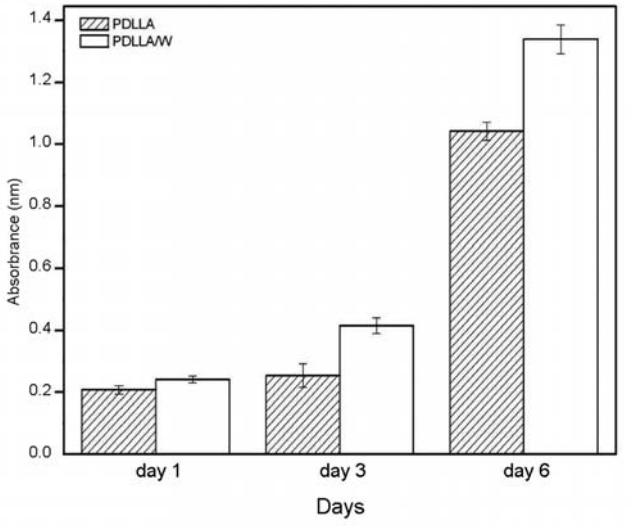


Figure 10. Cell proliferation assay (MTT). Cells seeded at a density of 1×10^4 cell/well for (a)1 day, (b) 3 days, and (c) 6 days. Mean±SD of triplicate independent samples.

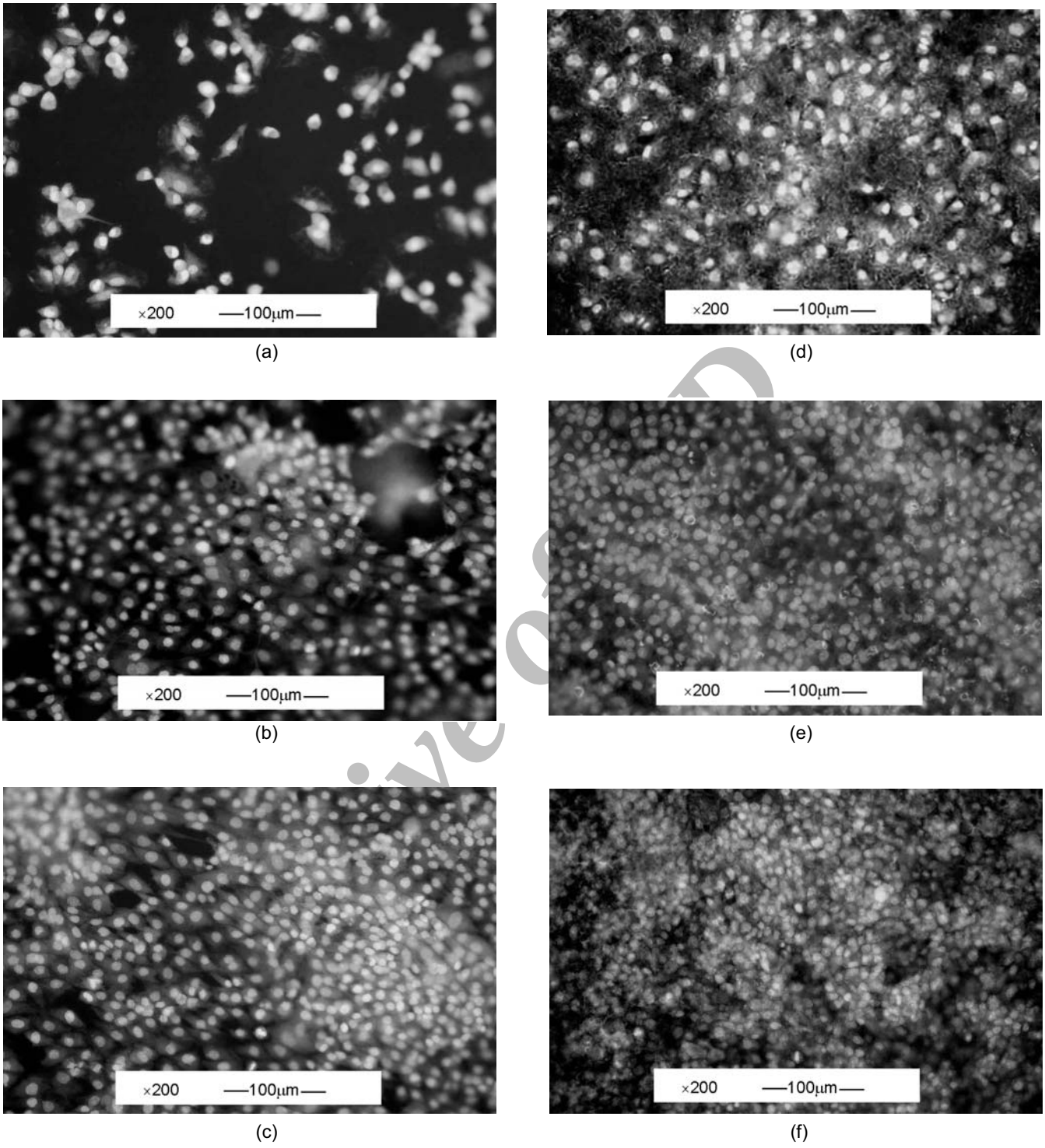


Figure 11. Osteoblast cells cultured on the surface of the PDLLA films for (a) 1 day, (b) 3 days, (c) 6 days and on the surface of the PDLLA/wollastonite composite films with the wollastonite content of 30 wt% for (d) 1 day, (e) 3 days, and (f) 6 days.

significant ($P<0.05$) increases in cell numbers on both PDLLA and the composite (PW-15) at 1, 3, and 6 days. However, more proliferations in cell numbers were observed on composite (PW-15) compared to those on PDLLA films with the same time intervals, especially on 6th day.

CONCLUSION

Uniform PDLLA/wollastonite composites were successfully prepared and exhibited similar mechanical properties compared to pure PDLLA. These composite films were bioactive, confirmed by the formation of the HAP layer on the surface of the composites after immersing in SBF. The in vitro cell culture experiment proved that the PDLLA/wollastonite composite shows good biocompatibility for the growth of osteoblast cells. All of these results indicate that the PDLLA/wollastonite composites may have promising medical applications in bone repair and bone tissue engineering.

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