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In vitro behavior of osteoblast-like cells on PLLA films with a biomimetic apatite or apatite/collagen composite coating

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Abstract To investigate the methods to improve the cell–material interaction of devices or tissue engineering scaffolds made of poly(L-lactic acid) (PLLA) polymer, apatite and apatite/collagen composite coatings were formed on PLLA films within 24 h through accelerated biomimetic processes. In vitro investigation using Saos-2 osteoblast-like cells through cell culture was conducted to assess the biological performance of these biomimetic coatings. The cell morphology on three types of surfaces, viz., PLLA film, PLLA film with the apatite coating, and PLLA film with the apatite/collagen composite coating, was studied using scanning electron microscopy (SEM). Cell viability was estimated using the MTT assay. The differentiated cell function was assessed by measuring the alkaline phosphatase (ALP) activity. The results obtained indicated that the biomimetic apatite and apatite/collagen composite coatings could significantly enhance the proliferation and differentiation of osteoblast-like cells. The apatite/collagen composite coating appears to be promising for the surface modification of PLLA-based devices with much improved interactions with osteoblastic cells.

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1 Introduction

Poly(α-hydroxyl ester) is currently used as a substrate material to support the regeneration of several tissues including articular cartilage [1] and bone [2]. This type of biodegradable polymers has the advantages of demonstrated biocompatibility, predictable resorption rate, and controllable mechanical properties. However, poly(α-hydroxy acid) is known to have poor cell–material interactions because of its hydrophobic nature and lack of cell recognition signals. In order to promote cell adhesion, surface modification of poly(α-hydroxy acid) is often attempted.

As a major component of the extracellular matrix of bone, bone apatite possesses unique properties such as good osteoconductivity [3], high affinity to living cells [4], and an ability to adsorb proteins [5]. Coatings of bioactive apatite, which is similar to bone apatite (and hence often referred to as "bone-like apatite"), have therefore been often used in bone tissue engineering. A favorable strategy to fabricate apatite coatings is to use biomimetic processes, including the classical process [6] and accelerated processes [7]. We have reported the formation of bone-like apatite on poly(L-lactic acid) (PLLA) films and scaffolds within 24 h using an accelerated biomimetic process [8].

As another major component of the extracellular matrix of bone, collagen has demonstrated good interactions with osteoblasts [9–11] because it contains specific amino acid receptors that allow them to bind directly to cell-surface receptors [12]. Collagen has been coated on various biomaterials [11, 13, 14] in order to promote cell adhesion. For bone tissue engineering, we have combined collagen with a high-strength simulated body fluid (SBF), i.e. 5SBF, and fabricated a novel composite coating containing

submicron apatite particles and collagen fibers on PLLA films and scaffolds [15].

In vitro studies using cell culture have often been used to evaluate new biomaterials. The aim of the current investigation was to evaluate the effects of two biomimetic coatings, viz., apatite coating and apatite/collagen composite coating, which were fabricated using the accelerated biomimetic processes, on cell behavior. Liu et al. incorporated bone morphogenetic protein into their biomimetic coating and found it retained biological activity [16]. In the current investigation, whether the collagen incorporated in the biomimetic coating retained its biological activity was studied. As type I collagen is the collagen in bone, it was thus used with 5SBF to form the coatings. Osteoblast-like cells were used in cell culture.

2 Materials and methods

2.1 Materials

The PLLA used in the current investigation, which had an inherent viscosity of approximately 7.11 dL/g, was commercially available (PURAC, the Netherlands). Chloroform and all chemicals of the analytical grade for making SBF were purchased from Acros (Belgium). Type-I collagen was obtained from Tsinghua University (Beijing, China). The osteoblast-like cells, Saos-2 cells, were supplied by American Type Culture Collection (USA).

2.2 Film fabrication and coating formation

PLLA films were fabricated using the solvent-casting method. PLLA granules were firstly dissolved in chloroform. The PLLA solution was then cast in Petri dishes and the solvent evaporated slowly. PLLA films with a uniform thickness ($\sim$0.1 mm) were obtained. As in our previous studies [8, 15], 5SBF was used in the accelerated biomimetic processes for coating formation. 5SBF is a high-strength SBF whose ion concentrations are five times of those in human blood plasma. To form apatite or apatite/collagen composite coatings on PLLA films, three square PLLA samples (10 mm $\times$ 10 mm $\times$ 0.1 mm) were immersed in 50 mL of 5SBF or 5SBF containing collagen (5SBFC) solution in a plastic bottle placed in a shaker waterbath set at 60 rpm, with the bath temperature being maintained at 37 °C. After 24 h of incubation, the samples were taken out of the bottles, washed carefully with distilled and deionized water, and dried in air. The chemical compositions of 5SBF and 5SBFC are shown in Table 1. The pH value of both 5SBF and 5SBFC solutions was adjusted to 6.4 using HCl and tris–hydroxymethyl aminomethane [$(CH_2OH)_3CNH_2$] before immersion of the samples.

Table 1 Compositions and ion concentrations (mM) of 5SBF and 5SBFC

	5SBF	5SBFC
Na^+	710.0	710.0
K^+	25.0	25.0
Ca^{2+}	12.7	12.7
Mg^{2+}	7.7	7.7
Cl^-	739.7	739.7
HCO_3^-	21.0	21.0
HPO_4^{2-}	5.0	5.0
SO_4^{2-}	2.5	2.5
Malonic acid	–	3 g/L
Collagen	–	1 g/L

2.3 Surface characterization

The surface of PLLA samples, without or with an apatite or composite coating, was examined using a scanning electron microscope (Stereoscan 440, LEICA, Germany) after they had been coated with a thin layer of gold. To assess the wettability of PLLA films without and with an apatite or composite coating, contact angles were measured using the sessile drop method together with an image analysis system. The water droplet (distilled water, about 1 μL) was placed on samples and its contact angles measured. For each sample, the result was the average of three measurements.

2.4 Cell culture

Saos-2 osteoblast-like cells were cultured under the condition of 37 °C in a humidified atmosphere of 5% CO_2 in air and in flasks (90 cm diameter) containing 10 mL Dulbecco's modified Eagle's medium(DMEM, Gibco), 5% fetal bovine serum(FBS; Gibco) and 1% penicillin/streptomycin. The medium was changed every third day. After 7 day culture, the Saos-2 cells were removed from the culture dish using trypsin, centrifuged and resuspended in DMEM medium to adjust the cell density to 4×10^5 cells/ml. 100 μL of the cell suspensions (about 4×10^4 cells) were placed on the samples and cultured for 4 h before 0.9 mL culture medium was added into each well. The plate was incubated at 37 °C in a 5% CO_2 atmosphere. The medium was changed every 2 days. After incubation, any non-adherent cells on the samples were removed by aspirating the medium and washing with phosphate buffered saline (PBS).

2.5 Cell morphology

The samples, after cell culture for 2 h and 6 days, respectively, were taken out of the culture plates and washed with PBS three times. Thereafter, the samples were fixed with 3% glutaraldehyde in PBS for 24 h at 4 °C. After thorough washing with PBS, samples were dehydrated sequentially in 30, 50, 70, 80, 90, 95, and 100% ethanol. Samples were dehydrated twice in each ethanol for 15 min each time. The fixed samples were freeze-dried, sputter-coated with a thin layer of gold, and examined under SEM (Stereoscan 440, LEICA, Germany).

2.6 Cell attachment and proliferating assay

In the quantitative assay, the adherent cells after being cultured for 2 h, 1 d, 3 d, 5 d, and 7 d were removed from the samples by trypsinization. The viable cells were counted using a hemocytometer. For each type of the substrates, 3 samples were used to obtain a mean value and standard deviation of the number of adherent cells.

2.7 MTT assay

The MTT assay was used as a relative measure of cell viability. After the Saos-2 cells were cultured on samples (5 mm × 5 mm) in 96-wells for 8 days, cell viability was evaluated using the MTT assay (Acros, Belgium), in which 20 μL of MTT (5 mg/L) was added to each well and incubated at 37 °C for 4 h. The blue formazan reaction product was then dissolved by adding 150 μL of DMSO. The absorbance was measured using a microplate reader.

2.8 Alkaline phosphatase assay

The alkaline phosphatase (ALP) activity was measured using an alkaline phosphatase assay kit (Zhongsheng Beikong, China). Saos-2 cells were incubated on the samples in a 24-wells plate for 8 days. After removing the culture medium, the cell layers were washed with PBS and then detached using trypsin/EDTA. After centrifugation, the cell pellets were washed with PBS and resuspended by vortexing them in 0.1 mL of deionized water with 0.1% Triton X-100. The cell pellets were disrupted via a cyclic freezing/thawing process. The prepared cell lysates were used to determine the ALP activity according the ALP assay manufacturer's instructions.

2.9 Statistical analysis

Experiments were run in triplicate per sample. All data were expressed as mean value ± standard deviation (SD) for $n = 3$. The single factor analysis of variance (ANOVA) technique was used to assess statistical significance of results obtained. $p < 0.05$ was considered to be significant.

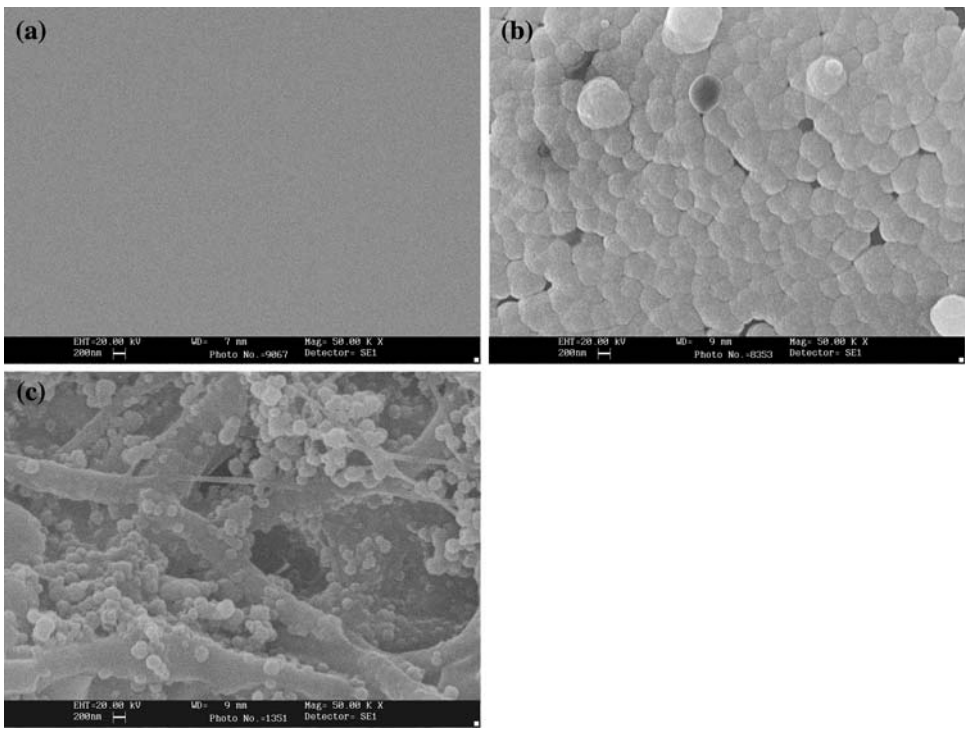

Fig. 1 SEM micrographs (magnification: ×50,000) of PLLA films before cell culture: (**a**) control without a coating, (**b**) with an apatite coating, (**c**) with an apatite/collagen composite coating

3 Results

3.1 Characterization of films

Figure 1 shows SEM micrographs of PLLA film before and after immersion in 5SBF or 5SBFC for 24 h. Before immersion (Fig. 1a), PLLA films exhibited a smooth surface morphology whereas after immersion in 5SBF for 24 h (Fig. 1b), the PLLA surface was completely covered by globular particles of apatite. After immersion in 5SBFC for 24 h (Fig. 1c), fibers and particles were observed on the PLLA surface. Previous studies showed that the coating layer formed in 5SBF was carbonate-containing apatite similar to bone apatite [8] and the coating layer formed in 5SBFC was a composite of collagen fibrils and submicron apatite particles which is similar to natural bone in composition and scale [15]. As was observed under SEM (Fig. 1), the PLLA substrate was a dense nonporous material. In contrast, the apatite layer formed on PLLA was an aggregate of tiny apatite particles with pores at their boundaries, whereas the apatite/collagen layer formed was a composite with collagen fibers and submicron apatite particles mixing randomly with each other, having larger pores on the surface.

The wettability of PLLA films without or with a coating, as represented by the contact angles determined (Table 2), was different among the three types of surfaces. PLLA films without a coating appeared to be more hydrophobic than PLLA films with an apatite coating or an apatite/collagen composite coating.

Table 2 Wettability of PLLA film with and without coating expressed as contact angle measurement

Samples	Contact angle (deg)
PLLA control films	71 ± 1
Apatite coated PLLA films	29 ± 3
Apatite/collagen coated PLLA films	22 ± 3

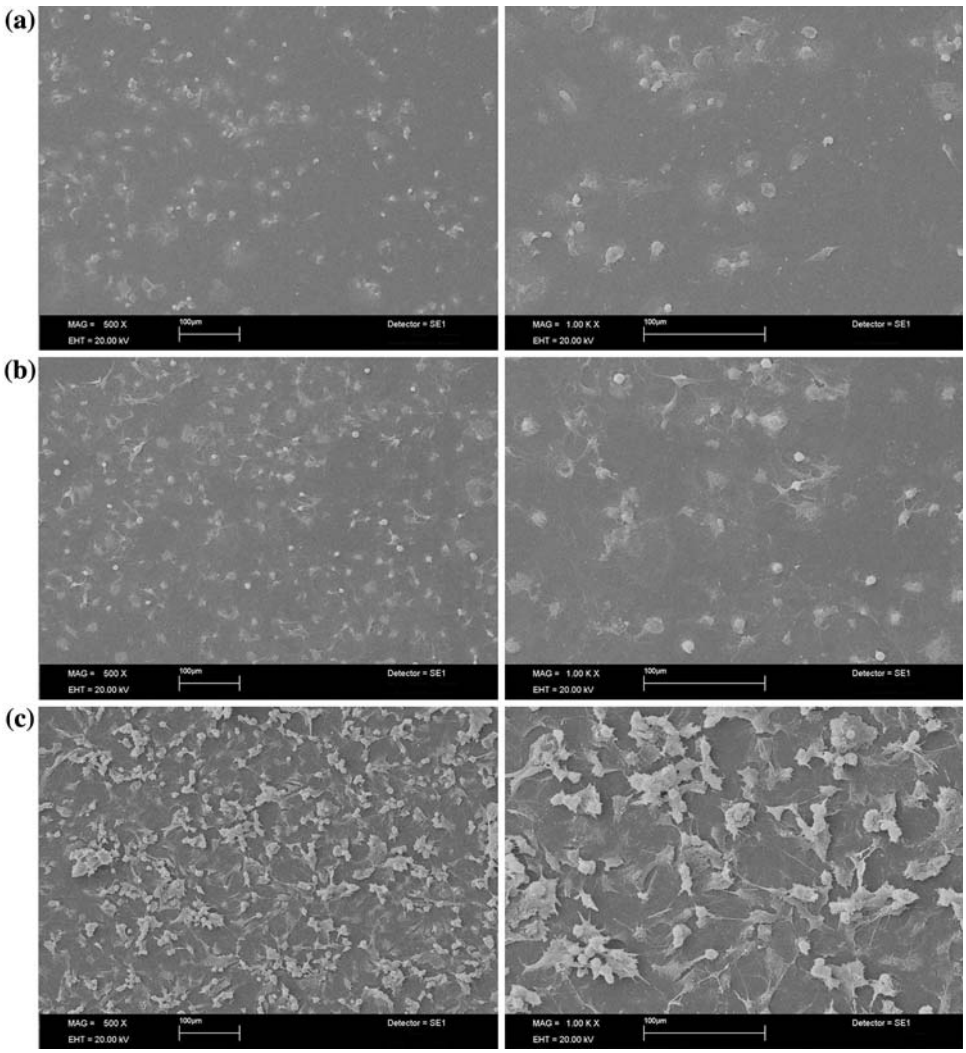

Fig. 2 SEM micrographs of Saos-2 cells after 2 h culture on PLLA films: (**a**) without a coating, (**b**) with an apatite coating, (**c**) with an apatite/collagen composite coating. (Left column of micrographs: low magnification view; right column of micrographs: high magnification view)

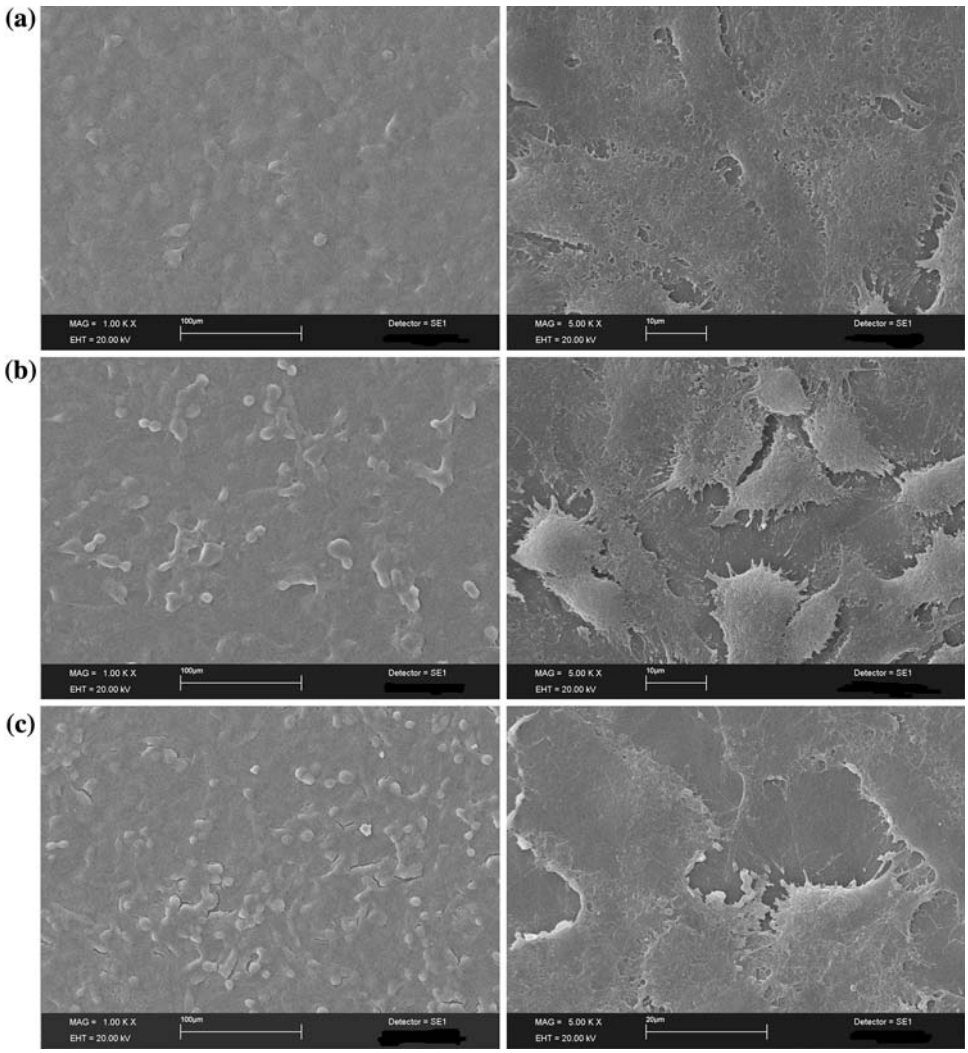

Fig. 3 SEM micrographs of Saos-2 cells after 6 day culture on PLLA films: (**a**) without a coating, (**b**) with an apatite coating, (**c**) with an apatite/collagen composite coating. (Left column of micrographs: low magnification view; right column of micrographs: high magnification view)

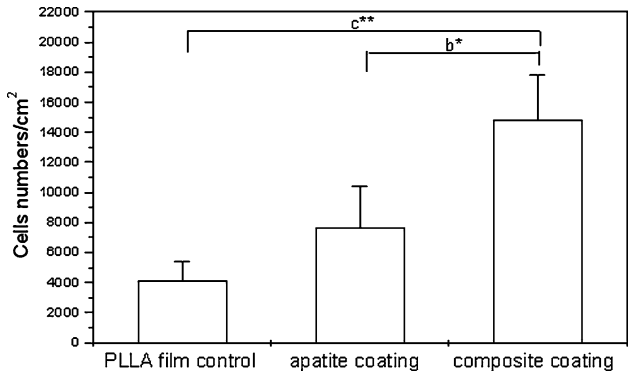

Fig. 4 The attachment of Saos-2 cells cultured on PLLA films without or with a coating. Error bars represent SD for $n = 3$. [(**b**) composite coating compared to apatite coating; (**c**) composite coating compared to PLLA surface. * $p < 0.05$; ** $p < 0.01$]

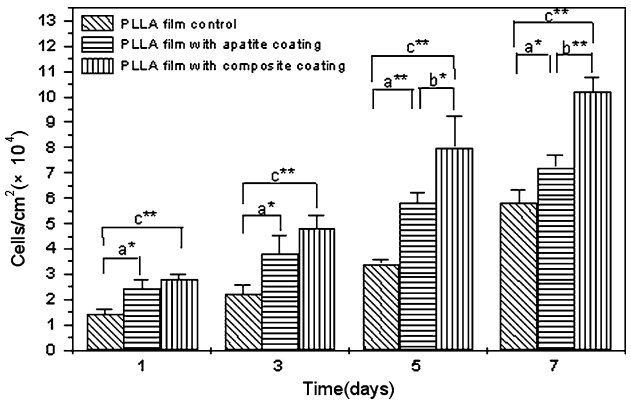

Fig. 5 Proliferation kinetics of osteoblast-like cells cultured on PLLA films without or with a coating. Error bars represent SD for $n = 3$. [(**a**) apatite coating compared to PLLA surface; (**b**) composite coating compared to apatite coating; (**c**) composite coating compared to PLLA surface. * $p < 0.05$; ** $p < 0.01$]

3.2 Cell morphology

SEM examination revealed that after 2 h of culture, more Saos-2 cells were found to attach to the composite coating and the cells had stretched their pseudopodia well, whereas fewer cells were found on the PLLA substrate and their pseudopodia were less obvious (Fig. 2). The cell number

and cell morphology on the apatite coating were somewhat between those obtained/observed on PLLA substrate and on the composite coating.

SEM observations of Saos-2 cells at day 6 of cell culture indicated that the osteoblast-like cells had proliferated and formed a 100% confluent cell layer on all substrates (Fig. 3). No obvious differences in cell morphology were observed among the cells grown on PLLA films with or without a coating. At high magnifications, the cells were found anchored to substrate surfaces by discrete filopodia exhibiting numerous microvilli.

3.3 Cell attachment

Cell attachment is the basis of cell growth, proliferation, and differentiation. After 2 h (6 h if including the 4 h before the 0.9 mL medium was added) culture, the number of Saos-2 cells attached to the apatite/collagen composite coating was significantly different in comparison with that on the PLLA control film ($p < 0.01$) and that on the apatite coating ($p < 0.05$) (Fig. 4). The number of cells attached to the apatite coating was apparently higher than that on the PLLA control film, although such difference was not statistically significant.

3.4 Cell proliferation

It was found that Saos-2 cells proliferated faster on the apatite coating ($p < 0.05$) and apatite/collagen composite coating ($p < 0.01$) than on PLLA films throughout the culture period (Fig. 5). The number of Saos-2 cells on the composite coating was higher than that on the apatite coating. The differences were significant after 5 day ($p < 0.05$) and 7 day ($p < 0.01$) cell culture.

3.5 Cell viability

The MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide] is a pale yellow substance which, when incubated with viable cells, is reduced by the cells to a dark blue Formasan product. The reduction process requires active mitochondria and hence is an accurate measure of the viability of cells in culture. After 8 days of culture (Fig. 6), the viability of Saos-2 cells on the composite coating was significantly higher than that on the PLLA surface ($p < 0.05$) and also on the apatite coating ($p < 0.05$). Although the cell viability of Saos-2 cells on the apatite coating was higher than that on the PLLA surface, the difference was not found to be significant ($p > 0.05$).

3.6 Alkaline phosphatase activity

The ALP activity, one of the markers of differentiated osteoblast functions, was assessed after 8 days of culture. Figure 7 shows the ALP activity of osteoblast-like cells cultured on PLLA films without or with a coating. The ALP activity of Saos-2 cells cultured on PLLA films with the apatite coating or the composite coating was significantly higher than that on the PLLA control films with $p < 0.05$ and $p < 0.01$, respectively. Compared to the apatite coating, the ALP activity of Saos-2 cells cultured on PLLA films with the composite coating was significantly higher ($p < 0.05$). The current investigation has therefore demonstrated that the apatite coating and the composite coating have positive effects on the growth of osteoblast-like cells and on the up-regulation of the osteoblastic

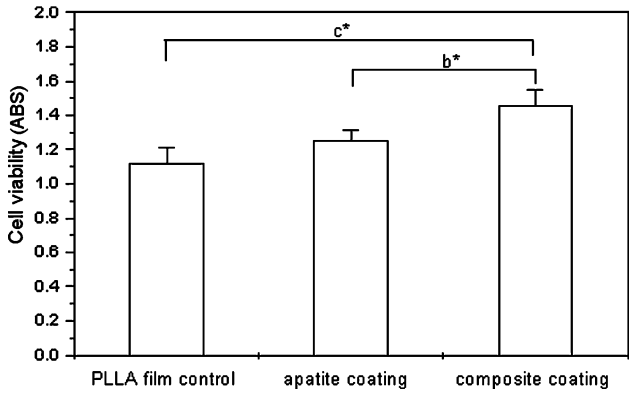

Fig. 6 MTT assay results. Formasan aborbance expressed as a measure of cell viability for osteoblast-like cells cultured on PLLA films without or with a coating. Error bars represent SD for $n = 3$. [(**b**) composite coating compared to apatite coating; (**c**) composite coating compared to PLLA control film $^*p < 0.05$]

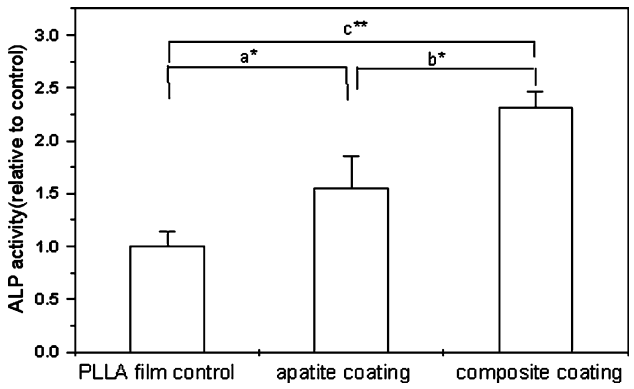

Fig. 7 ALP activity of osteoblast-like cells cultured on PLLA films without or with a coating. Error bars represent SD for $n = 3$. [(**a**) apatite coating compared to PLLA control film; (**b**) composite coating compared to apatite coating; (**c**) composite coating compared to PLLA control film. $^*p < 0.05$; $^{**}p < 0.01$]

phenotype, which was exhibited by an increased ALP activity of cultured Saos-2 cells.

4 Discussion

In the current investigation, the effects of an apatite coating or an apatite/collagen composite coating on the PLLA film, which was formed in 5SBF or 5SBFC within 24 h, on the adhesive and proliferative behavior of osteoblastic cells were analyzed. It was found that none of the three surfaces (PLLA, apatite, and apatite/collagen composite) had elicited any major deleterious or cytotoxic responses of the cells. However, PLLA films having an apatite or apatite/collagen composite coating, especially the composite coating, were obviously more suitable for osteoblast-like cells adhesion and proliferation than PLLA films without a coating.

The physical and chemical properties of biomaterial surfaces, such as topography, chemical composition, hydrophilicity, etc, could affect cell-biomaterial interactions [17, 18]. Surface properties have a major impact on the viability and functional activity of anchorage-dependent cells such as osteoblasts. In the current investigation, the wettability result, which was expressed as measured contact angles of water droplet on PLLA or coating surfaces (Table 2), suggested that apatite-coated and apatite/collagen composite-coated surfaces were more hydrophilic than PLLA bare surfaces. This improvement in hydrophilicity of the surface can be attributed to several factors. The chemical composition of the three types of surfaces, PLLA, apatite-coated, and apatite/collagen composite-coated, were different. Their surface topography was not the same (Fig. 1). However, the biomimetic apatite, which is similar to bone apatite, and the collagen in the coatings have high affinity with biological fluids and therefore have significantly improved the wettability of PLLA surface. To a lesser extent, the surface porous structure of the apatite coating and apatite/collagen coating, as shown in Fig. 1, also contributed to reducing the contact angle. The enhanced wettability of PLLA films with an apatite coating or an apatite/collagen coating obviously contributed to better cell spreading and adhesion to these surfaces.

The accelerated biomimetic processes employed in the previous [8, 15] and current investigations led to the formation of a bone-like carbonated apatite layer within 24 h. As stated in previous publications, the aim of using 5SBF and 5SBFC (and hence the accelerated biomimetic processes) was to avoid PLLA substrate degradation in an aqueous environment while forming an osteoconductive coating on PLLA surface. And indeed such coatings were satisfactorily formed on PLLA within a much shorter time than the time that is required by the classical biomimetic process. It was shown previously [8] that the apatite coating formed was similar in morphology and composition to that formed in the classical biomimetic process. In the current investigation, it was demonstrated that such a coating stimulated osteoblast-like cell proliferation and differentiation in vitro. Loty et al. [19] also reported the enhancement of osteoblastic activity on prefabricated apatite coating with osteoblasts from rat calvaria. Olmo et al. [20] cultured rat osteoblasts on bioactive glasses without and with apatite coating fabricated by soaking in SBF for 7 days. They reported that cell attachment showed a higher efficiency on the apatite layer and the biocompatibility of the glass was greatly enhanced after the formation of a surface apatite layer.

Collagen is an adhesion protein, which favours cell attachment [14, 21, 22]. Suh et al. [13] grafted collagen onto ozone oxidized PLLA surface and found that the grafted type I collagen provided a favorable matrix for cell attachment and growth. Ma et al. [22] cultured chondrocytes on collagen immobilized PLLA surfaces and found that the surfaces showed significantly improved cell spreading and growth. In the current investigation, collagen was also found to facilitate cell attachment and proliferation when it was combined with apatite particles.

The apatite/collagen composite coating was the most similar in composition to bone among the three surfaces: bare PLLA surface, PLLA surface with an apatite coating, and PLLA surface with a composite coating. This may be the main reason for the observation that the highest cell proliferation level had been obtained on the composite coating at all time points. Another reason for high cell proliferation rate on the composite coating is the surface topography. Apatite/collagen coated PLLA surfaces exhibited the most porous structure among the three types of surfaces (Fig. 1). It has been reported that cell attachment was better on rough surfaces than on smooth surfaces [23].

Saos-2 cells at day 6 of cell culture formed a 100% confluent cell layer on all substrates (Fig. 3). There was no evident difference in the morphology of the cells on different substrates. But there were greater numbers of cells on samples with an apatite coating or an apatite/collagen composite coating (Fig. 5), which would lead to better cell viability, the up-regulation of protein releases, higher integrin expression, and more adhesion plaques.

The cells used in the current investigation were the human osteosarcoma cell line Saos-2. These cells are widely used in studies on bone cell differentiation, proliferation and metabolism and are known to be capable of bone production [24, 25]. Properties associated with the osteoblast phenotype, including ALP activity, type I collagen synthesis, secretion of osteocalcin and production of a mineralized matrix, has been established [26, 27]. Among these properties, ALP is a widely recognized marker of

osteoblastic differentiation. An increase in ALP activity signifies the up-regulation of protein releases and the osteoblastic phenotype. It was found in the current investigation that the ALP activity of the osteoblast-like cells on the apatite/collagen composite coating was significantly higher than that on the apatite coating. This could be attributed to collagen in the coating which has a positive effect on the ALP activity of osteoblasts. This observation is in agreement with findings of increased ALP activity of osetoblastic cells on collagen matrices [28–31].

The bone-like apatite formed through biomimetic processes is calcium-deficient and carbonate-substituted apatite which dissolves in in vitro and in vivo environments. On the other hand, PLLA has a high degradation rate in the aqueous environment. Therefore, during the cell culture experiment, both the apatite or composite coating on PLLA and the PLLA substrate are expected to dissolve/degrade, especially when the duration of the cell culture experiment is relatively long. The emphasis of the current investigation was on studying the in vitro behaviour of osteoblast-like cells on apatite-coated PLLA. In the future, the in vitro dissolution/degradation behaviour of the coating and substrate should be carefully studied.

5 Conclusions

Apatite and apatite/collagen composite coatings formed on the PLLA substrate through accelerated biomimetic processes were found to enhance the proliferation and differentiation of osteoblast-like cells. The apatite/collagen composite coating was more effective in promoting cell–material interactions than the apatite coating, which could be attributed to the biomimetic structure of the composite coating that is similar to bone structure and also its much improved hydrophilicity. The biomimetic coatings appear to be promising for improving the biological performance of PLLA-based scaffolds for bone tissue engineering.

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