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Polysaccharide surface engineering of poly(D, L-lactic acid) via electrostatic self-assembly technique and its effects on osteoblast growth behaviours

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Abstract The objective of this study was to surface modify the poly (D, L-lactic acid) (PDLLA) films and assess the effects of the modified surfaces on the functions of osteoblasts cultured in vitro. A layer-by-layer (LBL) self assembly technique, was used leading to the formation of multilayers on the PDLLA film surfaces. Chitosan (Chi) and poly (styrene sulfonate, sodium salt) (PSS) were utilized as polycation and polyanion in this study, respectively. The layer structure was investigated by using X-ray photoelectron spectroscopy (XPS) and water contact angle measurement, respectively. XPS analysis displayed the presence of chitosan on PDLLA surface. A full coverage of coating with PSS/Chi layers was achieved on the PDLLA surface only after the deposition layers of PEI/$(PSS/Chi)_2$. These results showed that PDLLA films could be modified with PSS/Chi pairs which may affect the biocompatibility of the modified PDLLA films. To confirm this hypothesis, cell proliferation, cell viability as well as alkaline phosphtase activity of osteoblasts on layer-by-layer modified PDLLA films as well as control samples were investigated in vitro. The proliferation of osteoblasts on modified PDLLA films was found to be greater than that on control ($p < 0.05$ and $p < 0.01$) after 1, 4 and 7 days culture, respectively. Cell viability measurement showed that the PSS/Chi modified PDLLA films have higher cell viability ($p < 0.01$) than control. Osteoblast differentiation function (ALP) on LBL-modified PDLLA film was found significantly higher ($p < 0.01$) than that of virgin PDLLA films. These data suggests that PSS/Chi pair was successfully employed to surface modify PDLLA film via a layer-by-layer technique, and enhanced its cell biocompatibility.

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1. Introduction

Tissue engineering has developed rapidly in recent years. Biodegradable poly (lactic acid) is currently being investigated as an attractive material for regeneration of several tissues. Transplantation of biodegradable poly (α-hydroxyl ester) scaffold in which seeded with isolated cells has been developed as general approach to regenerate, replace or enhance tissue function. When biomaterial implants come into contact with host tissues, the initial response of cells mostly depends on the surface properties of implanted materials [1]. Although poly (lactic acid) is widely used in tissue engineering, be lack of tissue compatibility and resistance to biological environment are problems that still remain. The biomaterial surface has played key role in cell-biomaterial interactions, which has been recognized now. Thus, new strategies aim at tailoring surface of a biomaterial only to render material biologically active, while keeping the bulk properties of the underlying substrate was developed [2]. Many methods for surface engineering have been carried out, and many different biologically active molecules have been chemically or physically immobilized on polymeric matrixes e.g. covalently immobilizing Arginine-Glycine-Aspartic (RGD) containing peptide [3], and grafting the ozone oxidized poly (L-lactic acid) membrane with collagen [4].

We present here another approach, a layer-by-layer self-assembly, to surface engineer PDLLA films. This technique is based on the consecutive adsorption of polyanions and polycations via electrostatic interactions. The

electrostatic attraction between positively and negatively charged molecules seems to be a good choice as a driving force for multilayer build up [5]. One important feature of this method is the adsorption at every step of polyanion/polycation assembly, which results in recharging of the outermost layer during the film fabrication process. The layer-by-layer self-assembly of polyanions and polycations into multilayered coatings has emerged as a versatile, inexpensive yet efficient technique to "build" biologically active surfaces [6, 7] in recent years. Layer-by-layer self-assembled multicomposites make it possible to combine two or more desirable properties of biomaterials (e.g. bioactivity, biodegrability...) to surface engineer materials. Successful polycation/polyanion multilayer assembly opens new possibilities in an effort to create a technique to organize layers following a "molecular architecture" principle [8, 9].

In this study, layer-by-layer self-assembly of PSS and chitosan was employed to fabricate bioactive coatings on PDLLA films. The sequential multilayer coating process was initiated by adsorption of a PEI layer onto PDLLA substrates. It leads to a positively charged surface with high charge density. Subsequently this surface was kept in contact, in turn, with a solution of polyanion PSS and a solution of polycation chitosan.

The rationale to employ chitosan to surface engineer of PDLLA is that chitosan is the analogue of glycosaminoglycans (GAGs). Glycosaminoglycans and hyaluronic acid as well as collagen are important components in extracellular matrix, which play an important role in cell growth behaviour [10]. Structurally, chitosan is a linear polysaccharide consisting of β (1→4) linked D-glucosamine residues with a variable number of randomly located N-acetyl-glucosamine groups. It thus shares some characteristics with various glycosaminoglycans and hyaluronic acid present in articular cartilage [11]. Since GAGs properties include many specific interactions with growth factors, receptors and adhesion proteins, suggests that the analogous structure in chitosan may also have related bioactivities. Chitosan displays several advantages over other synthetic polycations. It is obtained from N-deactylation of chitin by alkaline treatment, chitin being the second most abundant naturally occurring polysaccharide [12]. Chitosan was extensively studied for its applications in tissue engineering [13–16] and drug delivery systems [17, 18] in recent years.

The objective of the present study was to fabricate and characterize surface modified PDLLA films with PSS/Chi via a layer-by-layer technique. We hypothesized that this PSS/Chi pair coating would be helpful for improving osteoblast growth on PDLLA substrates. Therefore, the influence of such surface modification of PDLLA substrate on osteoblast growth behaviour was investigated in vitro as well.

2. Materials and methods

2.1. Materials

Poly (ethylene imine) (PEI) ($M_w = 75000$) was purchased from Aldrich (Shang Hai, China). Chitosan (Chi, medium molecular weight, deactylation degree >85%) were obtained from Sigma-Aldrich China inc. (Shang Hai, China). Poly (styrene sulfonate, sodium salt) (PSS, $M_w = 70000$) was obtained from Aldrich (Shang Hai, China). Poly(D,L-lactic acid) was provided by Chengdu Institute of Organic Chemistry, Chinese Academy of Science, with a weight average molecular weight (Mw) of 2.5×10^5.

2.2. Fabrication of multilayer

1 g PDLLA sample was dissolved in 20 ml chloroform, then transferring this solution to a 90-mm diameter glass plate to form film by evaporation.

Chitosan (Chi) was dissolved in 2 vol.% acetic acid with a concentration of 5 mg/ml. Poly (styrene sulfonate, sodium salt) (PSS) solution of 5 mg/ml was prepared by dissolving in distilled water as polyanion. PEI solution with a concentration of 5 mg/ml was prepared with distilled water. The prepared PDLLA films were immersed in PEI solution for 20 min, thus obtaining a precursor layer with a stable positive charge to initiate the layer-by-layer self-assembly process. The multilayer construction was accomplished by successively dipping the PDLLA substrates in the poly (styrene sulfonate, sodium salt) and polysaccharide (Chi) solutions (alternating between PSS and Chi), followed by 10 min adsorption. After PSS adsorption, distilled water was used to rinse two times each for 1 min. After chitosan adsorption, 0.3% acetic acid solution was first used and then distilled water to rinse the deposited PDLLA films. Eleven bilayers, terminated with a layer of chitosan, i.e. PDLLA/PEI/$(PSS/Chi)_5$, were obtained by such alternative deposition in this study for further cell culture investigations.

2.3. Characterization of surface

As an indication of surface energy of the modified polymer surface, water contact angles were determined at room temperature using a sessile drop technique on a DSA10-MK2 system from Krüss. Four samples were measured in each group. Three different areas were measured for each sample. Characterization of the modified PDLLA surface is typically performed using methods such as X-ray photoelectron spectroscopy. XPS analysis was therefore performed using a Perkin Elmer 5600 ESCA system. The hydrocarbon peak maximum in the C1s spectra was set to 285.0 eV referenced the binding energy scales for the samples.

2.4. Cell culture

Osteoblasts were isolated via sequential collagenase digestions of neonatal rat calvaria according to established protocol [19]. They were cultured at 37 in a humidified atmosphere of a 5% CO_2 in air, in 50 cm^2 flasks containing 5 mL Dulbecco's Modified Eagle Medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco). The medium was changed every third day and for sub-culture; the cell monolayer was washed twice with phosphate-buffered saline (PBS) and incubated with trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA; Gibco) for 10 min at 37 to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature, and the cells were washed twice by centrifugation and resuspended in complete medium for re-seeding and growing in new culture flasks. Osteoblasts at population numbers 2-3 were used in the present experiments.

2.5. Cell proliferation

Osteoblasts were seeded onto modified films and the original PDLLA films as well as tissue culture polystyrene (TCPS) at a density of 30000 cells/cm^2 in a 24-well plate. The cells were maintained in complete medium that was refreshed every 3 days. Assay was performed at 1, 4 and 7 days, respectively. At each time interval, plates were rinsed twice with PBS solution and then the cells were detached from the substrate by 0.25% trypsin in phosphate buffered saline (PBS) solution. The number of adhered cells was counted with a Z1 Coulter Counter (Beckman Coulter Inc. Beijing, China) and the measured particle size range was set between 6 μm and 25 μm.

2.6. Cell viability

The MTT assay was used as a measure of relative cell viability. MTT is a pale yellow substrate (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrasodium bromide), which is reduced by living cells to a dark blue formazan. This process requires active mitochondria and is thus an accurate measure of the viability of cells in a culture. After the osteoblasts were cultured in 24 well for 7 days, the cell viability was evaluated using the MTT assay (MTT; Sigma). 100 μL of MTT(5 mg/ml) was added to each well and incubated at 37°C for 4 h. At the end of the assay, the blue formazan reaction product was dissolved by adding 0.5 ml DMSO and transferred to a 96 well plate. The absorbance was measured at 570 nm using a Bio-Rad 550 spectrophotometric microplate reader.

2.7. Total intracellular protein content

This procedure is performed according to Webster [20] in 6 well Plate. Osteoblasts (30000 cells/cm^2) were seeded onto the modified PDLLA films and control one, and cultured in DMEM with 10% fetal bovine serum under cell culture conditions for 7 days. At the end of the prescribed time interval, osteoblats were lysed using distilled water, and was followed by three freeze-thaw cycles. Total protein content in the cell lysates was determined spectrophotometrically using a commercially available kit. Light absorbance of these samples was measured at 570 nm on a Bio-Rad 550 spectrophotometric microplate reader. Total intracellular protein (expressed as mg) synthesized by osteoblasts cultured on the films was determined from a standard curve of absorbance versus known concentration of albumin run in parallel with experimental samples.

2.8. Alkaline phosphatase assay

In this section, osteoblasts were cultured in 6 well Plate, which covered with the modified PDLLA films and control one. After removal of the culture medium, the cell layer was washed twice with PBS, and harvested in 1.0 mL double-distilled water with a disposable cell scrapper (Costar), sonicated in ice bath for 10 min, and then centrifuged for 10 min at 2000 rpm. The supernatant was used for determining the ALP activity, according to Otto [21] with paranitrophenyl phosphate (Sigma) as substrate. The absorbance at 405 nm was measured via a spectrophotometer in a 96-well microplate reader (Bio-Rad 550). The ALP activity (expressed as (μmols of converted p-nitrophenol/min) was normalized by total intracellular protein synthesis (determined as described in the total intracellular protein content section) and thus expressed as μmols p-nitrophenol/min/mg protein [20]. ALP activity of osteoblats cultured on untreated PDLLA films served as controls.

2.9. Statistical analysis

All data were expressed as means $\pm$ standard deviation (SD) for $n = 4$. Single factor analysis of variance (ANOVA) technique was used to assess the statistical significance of results between groups. The statistical analysis was performed with the software OriginPro (version 6.1) at a confidence level of 95% and 99%.

3. Results and discussion

3.1. Characterization of films

XPS is a surface sensitive analysis technique, which is capable of providing information about the presence of different elements at the surface. The change in chemical structure of the film surface was investigated by XPS. The control PDLLA film surface shows carbon (binding energy of

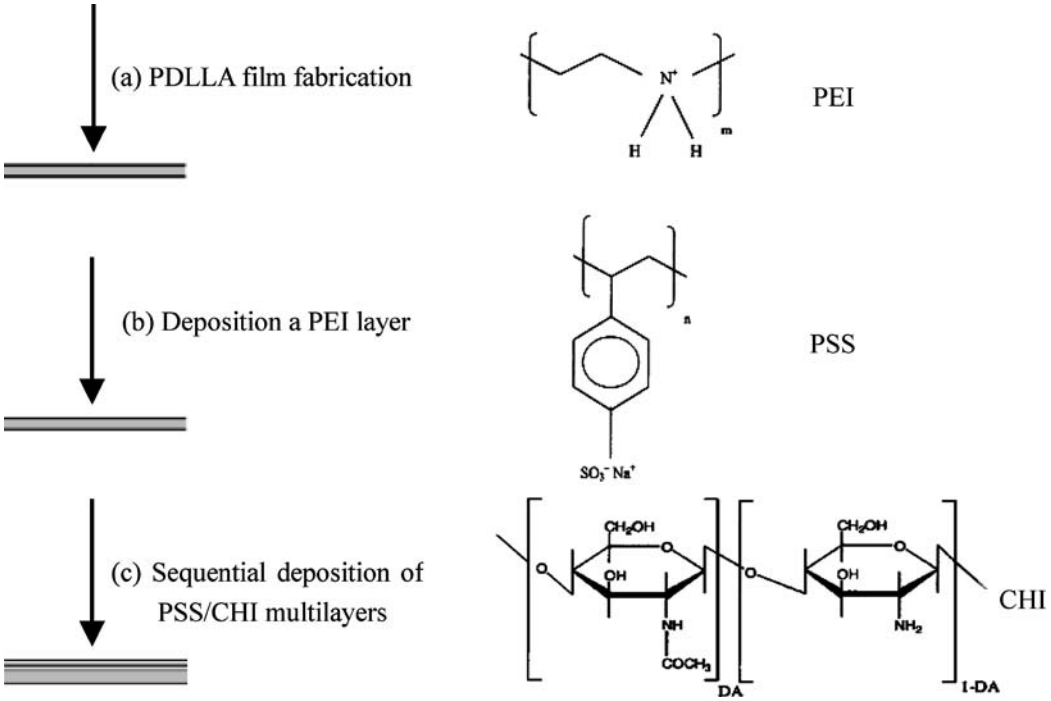


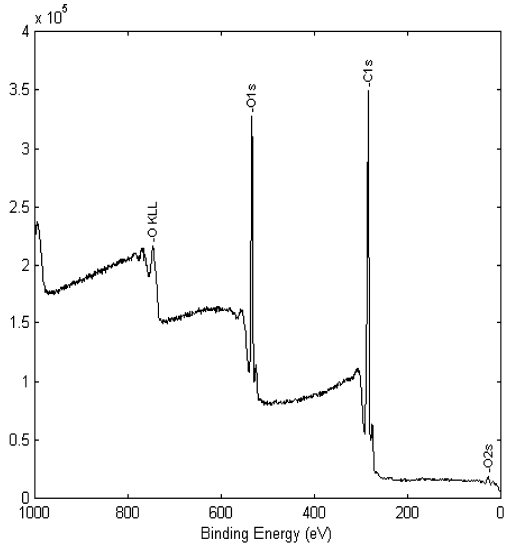
Fig. 1 Schematic diagram of the deposition of Chi/PSS self assembly multilayer on PDLLA films: (a) Preparation of PDLLA films; (b) Adsorption of a PEI layer; (c) Sequential adsorption of the PSS and Chi polyelectrolytes. The molecular structures of the polyelectrolytes are presented. DA is the degree of acetylation of chitosan.

285 eV) and oxygen (binding energy of 532 eV) peaks, as expected in Fig. 2 (a). However, for the LBL-modified modified films, an additional peak corresponding to nitrogen (binding energy of 400 eV) was observed, the result of XPS analysis showed carbon, oxygen and nitrogen peaks presented in Fig. 2 (b). The chitosan (PEI/(PSS/Chi)$_5$) coated PDLLA film surface was enriched with nitrogen atoms, which was derived from the amine group (-NH_2) and amide group (-CONH-) of chitosan. Since the outermost surface of biomaterials is immediately located at the interface with the tissues and/or cells, the biocompatibility of the LBL-modified PDLLA films will be different from that of the control. Therefore, the behavior of osteoblasts on control and modified films were studied to evaluate the effect of LBL-modified PDLLA films on the culture of osteoblasts in vitro.

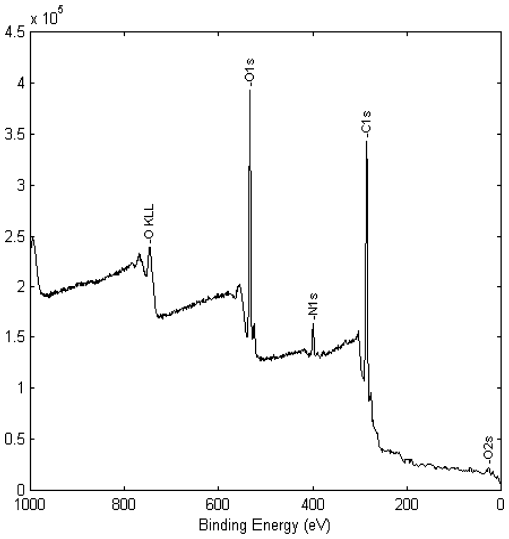
Measurement of contact angles on the virgin PDLLA surfaces and LBL-modified PDLLA films gave an indication of the relative hydrophobicity/hydrophilicity of these surfaces before and after the modification procedure. Figure 3 shows the change of water contact angle of coated PDLLA surfaces as a function of the number of coating layers. For the initial four coating layers, a gradual drop of the water contact angle was observed with decreasing the coating layer number. From the five-layers coating of PDLLA/PEI/(PSS/Chi)$_2$, the contact angle began to change alternatively between 72° and 63° depending on the outmost layer component. With further increasing the deposition cycles, both values relating to the respective outmost layer component remained constantly. This alternative change in contact angle was also reported in other literature [22, 23], indicating that fully covered and layered coatings are well developed on PDLLA films after the deposition cycles PEI/(PSS/Chi)$_2$. The wettability of fully coated PDLLA films is dominantly controlled by the outmost coating component, i.e., PSS and Chi.

3.2. Cell proliferation

Figure 4 shows osteoblast proliferation on virgin PDLLA and LBL-modified PDLLA films using PSS/Chi pair after 1, 4, and 7 days culture. Cells adhered to LBL-modified PDLLA films were significantly higher ($p < 0.05$) than those on virgin PDLLA samples after 1 day in culture. After 4 and 7 days culture, it was noted that statistical significantly higher ($p < 0.01$) cell numbers on chitosan deposited PDLLA films than that of on PDLLA films. By 7th day, cell numbers were $(7.48 \pm 0.87) \times 10^4$ and $(9.67 \pm 0.38) \times 10^4$ cells/well on virgin PDLLA films and LBL-modified PDLLA films, respectively. The chitosan terminating polyelectrolyte layer in this study improved the osteoblast proliferation behaviour, which indicated that chitosan is beneficial to cell proliferation. Considering cell proliferation as the basis of forming tissue, the result suggests that PSS/chitosan coating was helpful for improving cell growth and in turn tissue formation.



(a)



(b)

Fig. 2 XPS spectra of original PDLLA film and LBL-coated PDLLA films: (a) original PDLLA film and (b) PEI/(PSS/Chi)$_5$ coated PDLLA film.

3.3. Cell viability

MTT reagent is a pale yellow substance that is reduced to a dark blue formosan product when incubating with viable cells by mitochondrial succinate dehydrogenase in complex II, which plays a critical role in both oxidative phosphorylation and the tricarboxylic acid cycle [24, 25]. Therefore, the production of formosan can reflect the level of cell viability. Figure 5 shows the absorbance of formosan produced by viable cells attached on LBL-modified PDLLA films, uncoated PDLLA films and TCPS after 7 days culture, respectively. Cells adhered to LBL-modified PDLLA films showed a significant difference ($p < 0.01$) compared to the control sample. The osteoblasts cultured in TCPS showed higher

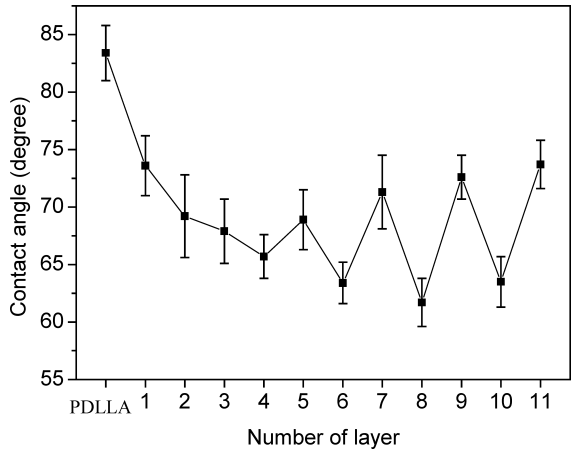


Fig. 3 Water contact angles as a function of the number of coating layers. The even layer numbers correspond to PSS as the outermost layer; the odd layer numbers correspond to chitosan as the outermost layer except for the first layer of PEI.

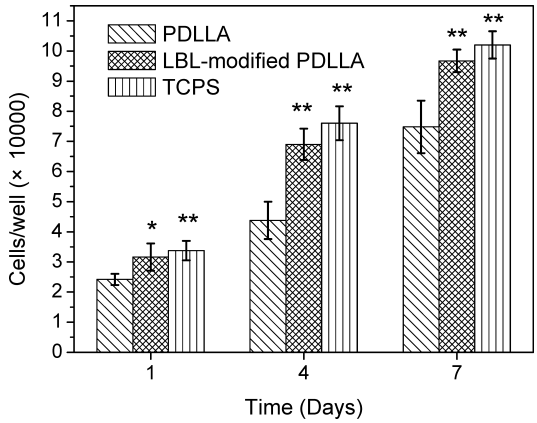


Fig. 4 Proliferation kinetics of osteoblasts cultured on LBL-modified PDLLA films (PEI/(PSS/Chi)$_5$) and original PDLLA films. The initial seeding density was 30000 cells/cm^2. Error bars represent means ± SD for $n = 4$. $^{*}p < 0.05$; $^{**}p < 0.01$ (compared to control).

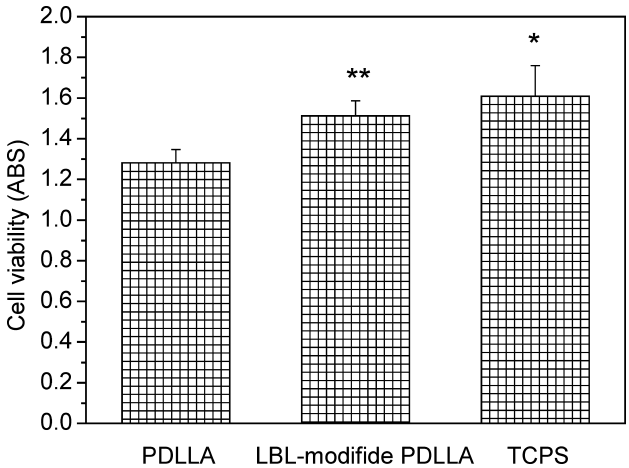


Fig. 5 MTT assay, Formosan absorbance expressed as a measure of cell viability from osteoblasts seeded onto LBL-modified PDLLA films (PEI/(PSS/Chi)$_5$) and control. The initial seeding density was 30000 cells/cm^2. Error bars represent means ± SD for $n = 4$. $^{*}p < 0.05$; $^{**}p < 0.01$ (compared to control).

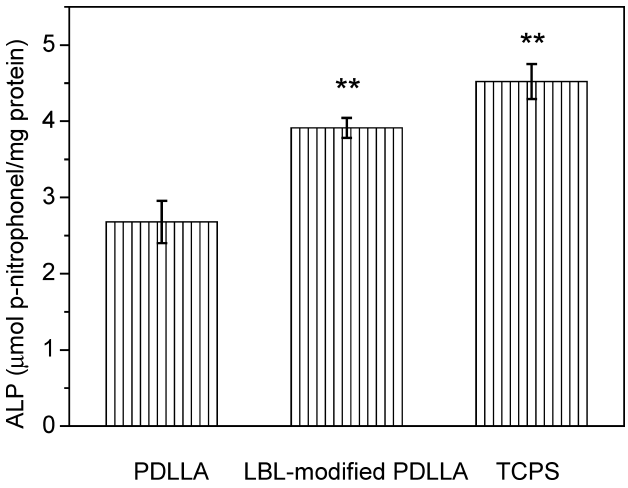


Fig. 6 Alkaline phosphatase activity of osteoblasts seeded on LBL-modified PDLLA films (PEI/(PSS/Chi)$_5$) and control after 7 days culture. The initial seeding density was 30000 cells/cm^2. Error bars represent means ± SD for $n = 4$. $^{*}p < 0.05$; $^{**}p < 0.01$ (compared to control).

viability ($p < 0.05$) comparable to that of uncoated PDLLA film in this study. The result in this study indicates that chitosan as terminating layer in polyelectrolyte multilayer films is biocompatible and non-toxic for surface engineering.

3.4. Alkaline phosphatase (ALP) activity

Differentiated function of rat osteoblasts was assessed by monitoring their ALP activity. The ALP assay was carried out after 7 days proliferation with an initial seeding density of 30000 cells/cm^2. Figure 6 showed the ALP activity of osteoblasts cultured on LBL-modified PDLLA films and control films. A significant difference on chitosan LBL-modified sample ($p < 0.01$) was observed when compared to that of control. On the other hand, the ALP activity of cell cultured in plastic well was little higher than that found on LBL-modified PDLLA films. The results suggested that the differentiation of osteoblasts depended markedly on the surface properties of substrates. Alkaline phosphatase (ALP) is one of the most widely measured osteoblast marker, a ubiquitous enzyme, which catalyzes the hydrolysis of phosphate esters at an alkaline pH condition [26]. Several studies have demonstrated the importance of ALP in bone matrix mineralization [27]. Thus, the result of the present study indicates that chitosan LBL modification is helpful for bone formation.

4. Conclusions

In this study, chitosan and PSS, were employed to surface modify poly (D, L-lactic acid) films by layer-by-layer technique aim at improving the biocompatibility of PDLLA. The success of coating was confirmed by combined techniques of XPS and contact angle measurements. Contact angle measurement supported a full coverage of coating developed only after five layers deposition PEI/(PSS/Chi)$_2$. For these fully coated surfaces, the surface wettablity was dominated by the outmost coating layer. LBL assembly using PSS/Chi as the coating pair has proved to be a successful way to generate bioactive and fully-covered chitosan terminating layer on the hydrophobic PDLLA surface. In vitro investigation was also performed on PDLLA surface with fully covered chitosan-terminating layer. Proliferation, MTT assay and alkaline phosphtase activity tests demonstrated the consistent results that such surface engineering was beneficial to improve the cell biocompatibility of PDLLA substrates.

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