Osteoblast growth promotion by protein electrostatic self-assembly on biodegradable poly(lactide)

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Abstract—Extracellular matrix (ECM)-like coating was developed on biodegradable biomaterials based on the electrostatic self-assembly (ESA) technique to promote osteoblast growth. Poly(ethylene-imine) (PEI) was first employed to obtain a stable positively charged surface on poly(DL-lactide) (PDL-LA) substrate. Gelatin was selected as ECM-like biomacromolecule to deposit on the activated PDL-LA substrate using the ESA technique. ζ-Potential results showed alternating charge of polyelectrolytes (PEI/gelatin) layering on PDL-LA microspheres. Quartz crystal microbalance (QCM) measurement further verified the gradual deposition of PEI/gelatin on PDL-LA thin film. Osteoblast cells (MC3T3) were chosen to test the cell behavior on modified PDL-LA substrates. The osteoblast test about cell activity, intracellular total DNA content, total protein content and cell morphology by SEM investigation on ECM-like multilayer-modified PDL-LA substrate showed to promote osteoblast growth. Comparing conventional coating methods, polyelectrolyte multilayers are easy and stable to prepare. It may be a good choice for the surface modification of complex biomedical devices. These very flexible systems allow broad medical applications for drug delivery and tissue engineering.

Key words: Poly(DL-lactide); electrostatic self-assembly; biomacromolecules; osteoblast; tissue engineering.

INTRODUCTION

The development of tissue engineering in the field of orthopaedic surgery is now booming. For the repair of bone defects, the ideal biomaterial is one that has mechanical properties similar to bone, can be fabricated easily into a desired shape, supports cell attachment, contains factors to induce formation of new bone tissue, and biodegrades to permit natural bone formation and remodeling [1].

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The key role of surface for cell–materials interaction has been recognized now. For bone-anchored medical implants, the early formation of bone and an intimate contact between the formed bone tissue and the implant surface are prerequisites for a long-term successful function \textit{in vivo}. Many factors are involved in the bone formation process around an implant (e.g., bone status and patient health), among which the implanted material surface properties have been suggested to have a significant influence [2–4]. Therefore, new strategies aim at the tailoring of material’s surface only to render materials biologically active, while preserving the bulk properties of the underlying support [5].

The self-organization of polymers has been increasingly explored for the preparation of well-defined surfaces and interfaces in recent years [6–10]. Electrostatic self-assembly (ESA), which is based on the alternating physisorption of oppositely charged polyelectrolytes, represents a new, alternative solution for biomaterial coating [11]. There have been lots of attempts to build biomimetic films based on ESA in recent years; for example, with alginate/polylysine [12], chitosan/dextran sulfate [13], hyaluronic acid/poly(lysine) [14], poly(lysine)/poly(glutamic acid) [15] and collagen [16]. The principle of alternate adsorption was invented in the pioneering work of Iler [17] and Decher \textit{et al}. [18–20] introduced a related method for film assembly by means of alternate adsorption of linear polycations and polyanions, or bipolar amphiphiles. In this method the crucial feature was excessive adsorption (more than neutralization) at every stage of polycation/polyanion assembly that leads to recharging of the outermost surface at every step of film formation. The buildup is easy and the procedure can be adapted to almost any type of surface as long as surface charges are present. Moreover, the method is valid whatever the shape of the solid has been. The procedure may be carried out not only with linear polIONS and boladiions, but also with proteins [7], virus [21], ceramics [22] and charged nanoparticles [23, 24]. More recently, the activity of a protein embedded in a film and of a peptide coupled to one of the polyelectrolytes have been evidenced [25–27]. This remarkable property opens up the possibility to construct multilayer incorporating specific ligands that keep their biological activity and promote specific cell function. Most studies using the ESA technique are performed on a solid matrix, the substrates used by most investigators are gold, quartz, glass and silicon. Few of them used ‘real’ biomaterials [28–30], for example, PU, PVC or PLA, used in biomedical fields nowadays.

The current research will explore to construct extracellular matrix (ECM)-like multilayers on synthetic biodegradable polymer, poly(lactide) (PLA), with the goal of combining both the merits of the good mechanical property of PLA material and the good biocompatibility of the natural ECM-like molecules, which, therefore, can be applied to enhance the biocompatibility of PLA tissue engineering scaffold. Osteoblast cell behavior was evaluated to assess the potential of this novel surface treatment for bone implant and tissue engineering scaffold for bone reconstruction.
MATERIALS AND METHODS

Chemicals

Poly(DL-lactide) (PDL-LA) was prepared by ring-opening polymerization of DL-lactide in our laboratory. The molecular weight was $20 \times 10^4$, as determined by GPC, $M_{\text{WD}} = 1.75$. Poly(ethylenimine) (PEI) was purchased from Aldrich ($M_w = 25 \times 10^3$). The biomacromolecule used in our study was gelatin (Fluka, gelatin from porcine skin with medium gel strength). All the chemicals were used without further purification.

Multilayer preparation of the PDL-LA substrate

The PDL-LA films were prepared using the solvent-casting method in CH$_2$Cl$_2$ solution. Before the assembly, the PDL-LA substrates were ultrasonicated in 50% ethanol solution for 20 min for cleaning, then rinsed with water and finally dried under a stream of nitrogen. The PDL-LA substrates were activated by treatment with PEI at the concentration of 1 mg/ml in deionized (DI) water for 3 h, following repeated washes with water. The sequence of operations resulting in production of PEI/gelatin multilayers coating was the following. The activated PDL-LA substrate was immersed in 0.5 mg/ml gelatin (pH 7.4) for 20 min. After a 30-s wash of the substrate with DI water, the substrate was dipped into a 1 mg/ml PEI solution (pH 7.4) for 20 min. Following the same washing procedure, the substrate was exposed to gelatin solution for 20 min and rinsed with water again. Further growth of PEI/gelatin bilayers was accomplished by repeating the same cycle of immersion into the PEI solution, rinsing, immersion into the gelatin solution and rinsing. The cycle was repeated $n$ times to obtain a film of desirable thickness. After the final assembly cycle, the substrate was immersed in a 5 mg/ml glutaraldehyde solution for 20 min to fix the protein/polyion architecture. After rinsing with water, the substrate was dried with a stream of nitrogen. 8 bilayers were obtained by alternate deposition of PEI/gelatin in our experiment for the biomacromolecule multilayer.

$\zeta$-Potential measurement

PLA microspheres were prepared using an emulsification technique [31] for $\zeta$-potential measurement. The microelectrophoretic mobility of unmodified and coated PLA microspheres was measured with a Zeta potential analyzer (Zeta Plus, Brookhaven Instruments) by taking the average of 5 measurements at the stationary level. The mobilities ($\mu$) were converted to electrophoretic potentials ($\zeta$) using the Smoluchowski relation $\zeta = \mu \eta / \varepsilon$, where $\eta$ and $\varepsilon$ are the viscosity and permittivity of the solution, respectively. All measurements were performed on PLA microspheres re-dispersed in DI water.
Quartz crystal microbalance (QCM) measurements

QCM (QCM100 Quartz Crystal Microbalance, QCM25 Crystal Oscillator, SRS) was used to investigate the gradual deposition of the polyelectrolyte layers on PLA film. The QCM crystal was firstly cleaned by using cleaning solution containing 1 part 45% KOH, 39 parts ethanol and 60 parts DI water. The crystal was immersed in low concentration of PLA/acetone solution and then dried by nitrogen to get a thin film of PLA on the crystal. The crystal with the thin PLA film was activated by 1 mg/ml PEI solution and alternative deposit of PEI and gelatin, using the procedure described in the previous section, for 3 h, rinsing with DI water and drying under nitrogen. The activated substrate was then immersed in 0.5 mg/ml gelatin (pH 7.4) for 20 min. After a 30-s wash of the substrate with deionized water, the substrate was dipped into a 1 mg/ml PEI solution (pH 7.4), for 20 min. Following the same washing procedure, the substrate was exposed to gelatin solution for 20 min and again rinsed with water. Further growth of PEI/gelatin bilayers was accomplished by repeating the same cycle of immersion into the solution of PEI, rinsing, immersion into the gelatin solution and rinsing. The cycle was repeated \( n \) times to obtain a film of desirable thickness. After each step of the polyelectrolyte adsorption, the crystals were washed thoroughly with DI water and dried under nitrogen, and the QCM frequency change in air \( (f) \) was measured. The \( \Delta f \) (frequency difference before and after adsorption) was used to determine the mass adsorbed after each immersion step according to the Sauerbrey equation:

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\Delta f = -C_f \cdot \Delta m,
\]

where \( \Delta f \) is the observed frequency change (in Hz), \( \Delta m \) the change in mass per unit area (in g/cm\(^2\)) and \( C_f \) the sensitivity factor for the crystal.

The thickness of each layer can, thus, be calculated from the change in mass based on known crystal surface volume.

Cell behavior test

Osteoblast culture. Mouse MC3T3 osteoblast-like cells (cell line) were maintained in \( \alpha \)-MEM (\( \alpha \)-minimum essential media, Gibco), supplemented with 10% fetal bovine serum (FBS, Life Technologies), fungizone (2.5 \( \mu \)g/ml, Life Technologies) and gentamycin (50 \( \mu \)g/ml, Gibco) and kept at 37°C in a humidified 5% CO\(_2\) atmosphere. PDL-LA, PEI-activated PDL-LA, PEI/gelatin assembly PDL-LA films, sterilized in 75% ethanol and swollen in PBS, were placed into 96-well tissue-culture polystyrene (TCPS) plates. The wells were full covered by the substrates. Osteoblasts were then seeded in culture media to with a final density of \( 2 \times 10^4 \) cells/ml per well (200 \( \mu \)l) and incubated for due time (1 day, 5 days and 20 days) at 37°C in an atmosphere of 5% CO\(_2\) in air. Following incubation, the wells were washed twice with PBS to remove non-attached cells. The cell lysates were prepared by adding 150 \( \mu \)l 1% Triton X-100 into the wells followed by ultrasonication for 15 min. The lysates were stored in Eppendorf tubes at −20°C until use.
Cell activity test. Cell activity was determined using the MTT assay [32, 33], which is based on the mitochondrial conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Briefly, after the osteoblasts were incubated on different substrates for 5 days, 20 µl MTT solution (5 mg/ml in PBS) was added to each sample, followed by incubation at 37°C for 4 h for MTT formazan formation. The medium and MTT were replaced by DMSO solution; the samples were incubated at 37°C for an additional 5 min to dissolve the MTT formazan and also mildly shaken for 10 min to ensure the dissolution of formazan. The absorbance values was measured by using a microplate reader (Bio-Rad, model 550) at 570 nm, blanked with DMSO solution. Five replicates were read for each sample, the mean value of the five was used as the final result. Cell activity was expressed as a proportion of the absorbance value of TCPS in the same culture media.

Total DNA content. The proliferation of cells on different substrate was measured by determining the total amount of DNA using a fluorometric assay (Hoechst 33258 dye method) [34, 35]. The fluorescence was measured at 360 nm (excitation) and 460 nm (emission), and compared with a standard curve of known concentrations of standard calf thymus DNA run in parallel with experimental samples.

Total protein content. Total protein synthesized by osteoblasts cultured on the films was determined using the Pierce BCA protein assay [36]. The cell suspension (20 µl) was added to 200 µl of working reagent (sodium bicarbonate, BCA). The samples were then incubated for 60 min at 37°C and read using a spectrophotometric microplate reader at 570 nm. The absorbance for cell suspension was correlated to a standard protein curve and differences in total protein concentration were statistically compared.

Cell morphology. The morphology of cells cultured directly on substrates was examined by scanning electron microscopy. Briefly, cells were fixed in situ on the substrates with 2.5% glutaraldehyde in 0.1 M sodium cacodylate-buffered solution for 15 min. Subsequently, they were rinsed in cacodylate-buffered solution, which was followed by dehydration through a graded series of ethanol. The cells then were subjected to critical point drying and finally they were sputter-coated with gold-palladium. In control experiments, the osteoblasts were cultured on TCPS plates. Scanning electron microscopy (SEM) photos were obtained using a Jeol JSM-6301F microscope. The cell monolayers on different PDL-LA substrates were also stained with fluorescein diacetate (FDA, Sigma) for confocal laser scanning microscopy (CLSM, Bio-Rad, Radiance 2100; Zeiss Axiovert 200). FDA is an indicator of membrane integrity and cytoplasmic esterase activity [37]. Enzymatic hydrolysis of the fluorogenic ester substrate of FDA results in the intracellular accumulation of the green fluorescent product fluorescein in cells with intact plasma membranes. Stock solutions were prepared by dissolving 5.0 mg/ml FDA in
acetone. The working solution was freshly prepared by adding 5.0 µl of FDA stock solution to 5.0 ml PBS. 20 µl FDA solution was added in each well and incubated for 5 min. The substrates were then washed twice with PBS and placed on a glass slide for CLSM examination. The dye was excited at 488 nm wavelength using a laser.

RESULTS AND DISCUSSION

Construction of PEI/gelatin multilayers coating onto PLA materials

The multilayer coating onto PDL-LA films was constructed by PEI aminolysis and further alternative deposit of PEI and gelatin (Fig. 1). In our previous study, PEI was successfully used to activate the PLA film to obtain a stable positively charged surface [29]. This stable activated surface was obtained by the chemical reaction between PEI and PLA molecules based on the aminolysis of ester group. The positively-charged aminolysis surface could adsorb negative charged gelatin. The excessive adsorption (more than neutralization) at every stage of polycation/polyanion assembly that leads to recharging of the outermost surface at every step of film formation and construction of multilayers coating onto PLA substrate.

ζ-Potential measurements

ζ-Potential measurements were utilized to follow adsorption of the layers on the PLA microspheres. The microelectrophoretic mobility of the unmodified PLA microsphere, PEI-activated PLA microspheres, and subsequently polyelectrolytes (PEI/gelatin) of alternating charge, was measurement. Figure 2 shows the

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*Figure 1.* Scheme of layer-by-layer film assembly on a solid substrate by alternate adsorption of linear polycations and negatively charged proteins.
Figure 2. ζ-Potential of microcrystal as a function of polyelectrolyte layer number. The unmodified PLA microspheres does not have any charge on the surface. PEI layer shows +20 mV of ζ-potential and gelatin layer shows −19 mV of ζ-potential.

ζ-potential as a function of the polyelectrolyte coating layer number. The unmodified PLA microspheres do not have any charge on the surface, as shown in Fig. 2. It switches to positive charge after the PEI activation exhibited a value of +20 mV. Furthermore, PEI/gelatin alternate surface yielded a ζ-potential of +20 mV and –19 mV, respectively. These data confirm the alternative charging of the PLA microsphere surface through adsorption of the polyelectrolytes. The obviously alternate change of the ζ-potential verified the progressive buildup of the film by alternate deposition of the polyelectrolytes.

The adsorbed mass of polyelectrolytes on the QCM crystal can be measured via the QCM frequency shift according to the Sauerbrey equation. According to the Sauerbrey equation, the coated PLA thin film was about 4.3 nm and the activated PEI layer on PLA film was calculated as 1.2 nm. The following gradual deposition of PEI/gelatin alternate layer was calculated as approx. 0.8 and 2.85 nm, respectively (Fig. 3). The thickness of the gelatin layer obtained from QCM measurement is in close agreement with the data we obtained using the radiolabeling method (2.24 nm) [29].

Cell activity test

Figure 4 shows the cell activity of osteoblasts on various surfaces over a period of 5 days. It was found that the cell activity on PEI/gelatin assembly modified PDL-LA substrate is much higher than that on the PDL-LA virgin substrate. The activity datum of PDL-LA virgin substrate is 67.6%. As for PEI/gelatin assembly modified PDL-LA substrate, the activity datum is 101.6% comparing TCPS as 100%. It can be seen that the cell activity on PEI/gelatin multilayer modified PDL-LA substrate is higher than that of TCPS.
Figure 3. QCM monitoring of the growth of PEI/gelatin bilayers on PLA-coated QCM electrodes. The coated PLA film is about 4.3 nm. The increase of the multilayer is approx. 0.80 nm for a PEI layer and a 2.85 nm for gelatin layer.

Figure 4. Osteoblast (MC3T3) activity on different matrices determined using the MTT method. Results represent mean ± SD of triplicates from six separate experiments (P < 0.05).

**Total DNA content**

In order to assess the rate of osteoblast proliferation, total DNA content was measured for 1, 5 and 20 days of culture (Fig. 5). As expected, the ostoblasts proliferated on TCPS surfaces during the culture periods. For PDL-LA virgin, as well as PEI/gelatin assembly-modified PDL-LA substrate, a significant increase in DNA content was observed at 5 days. However, PEI/gelatin exhibit relative significant increase of cell proliferation when compared to PLA surface at that time point of culture. At day 20, this significant increase became more accentuated.

These results show that the ECM-like surface on PDL-LA substrate enhance osteoblasts proliferation.
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Figure 5. Total DNA content of osteoblasts (MC3T3) on different matrices. Results represent mean ± SD of triplicates from three separate experiments ($P < 0.05$).

Figure 6. Total protein content of osteoblasts (MC3T3) on different matrices. Results represent mean ± SD of triplicates from three separate experiments ($P < 0.05$).

**Total protein content**

Figure 6 shows the total protein content of osteoblasts on PDL-LA virgin film, PEI/gelatin assembly-modified PDL-LA film and TCPS after 1, 5 and 20 days of culture. Among the 3 substrates, the PEI/gelatin assembly-modified PDL-LA exhibits the highest total protein content of the 3 culture periods assayed.

These results show that the ECM-like surface on PDL-LA substrate can promote protein synthesis. However, further work must be performed in order to ensure its
Figure 7. SEM photos of osteoblasts on different matrices. (a) Unmodified PLA, (b) PEI/gelatin assembly-modified PLA, (c) TCPS.
biocompatibility and osteogenic effect. The use of bioactive molecules may be an interesting strategy to enhance the osteogenic potential of the PDL-LA-modified surface.

Cell morphology

Figure 7 shows that the osteoblasts can attach to different matrices. After 14 days, it can be seen that the osteoblasts cultured on the PEI/gelatin assembly-modified PDL-LA film had undergone some degree of proliferation in monolayer and covered the modified PDL-LA film completely (Fig. 7b) similar to the osteoblasts on TCPS (Fig. 7c). However, most of the osteoblasts on PDL-LA virgin film did not maintain normal spreading morphology (Fig. 7a).

Confocal laser scanning microscopy (CLSM) investigation

Figure 8 shows the CLSM images of osteoblasts attached to PDL-LA virgin substrate, PEI-activated PDL-LA substrate, gelatin assembly PDL-LA substrate and TCPS substrate. We can see a notable difference in the living osteoblasts on different substrates, because FDA can only be hydrolyzed into fluorescein that can be detected by CLSM in living cells. It showed much more osteoblast spreading on PEI-activated substrate than that on unmodified PDL-LA substrate. The osteoblasts on gelatin assembly PDL-LA substrate are spreading uniformly and completely cover the substrate surface, which is also similar with the osteoblasts on TCPS substrate.
Figure 8. CLSM images of osteoblast on different matrices. (a) PDL-LA virgin substrate, (b) PEI-activated PDL-LA substrate, (c) PEI/gelatin-multilayer-modified PDL-LA substrate, (d) tissue-culture polystyrene (TCPS).

Because of the flexibility of the ESA technique used in our study, osteo-inductive proteins, for example, the family of bone morphogenetic proteins (BMPs), can be easily employed to induce specific osteoblast functions. These very flexible systems allow broad medical applications for drug delivery and tissue engineering.

CONCLUSIONS

Electrostatic self-assembly of PEI/gelatin is successfully explored to construct an extracellular matrix-like multilayer onto the surface of PDL-LA substrate.
Potential results showed alternating charge of polyelectrolytes (PEI/gelatin) layering on PDL-LA microspheres. Quartz crystal microbalance (QCM) measurement further verified the gradual deposition of PEI/gelatin on PDL-LA thin film. The osteoblast test of cell activity, total DNA content, total protein content and SEM investigation on modified PDL-LA substrates showed that the extracellular matrix-like multilayer has a positive effect on osteoblast growth. However, further work should be performed in order to ensure its biocompatibility and osteogenic effect. It may be necessary to deposit osteoinductive proteins or other polyelectrolytes to promote specific osteoblast functions.

Compared to conventional coating methods, polyelectrolyte multilayers are easy and stable to prepare. It was easy to replace the polyelectrolyte with other proteins or polysaccharides such as collagen, alginate or chitosan. It has the potential to substitute the PLA substrate with other matrices like poly(glycolic acid) (PGA), poly(caprolactone) (PCL), poly(urethane) or poly(orthoester). Because of the flexibility of the ESA technique used in our study, osteoinductive proteins, for example, the family of bone morphogenetic proteins (BMPs), can be easily employed to induce specific osteoblast function. These very flexible systems allow broad medical applications for drug delivery and tissue engineering.

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REFERENCES
