Surface Engineering of Poly(DL-lactide) via Electrostatic Self-Assembly of Extracellular Matrix-like Molecules

Huiguang Zhu,[†] Jian Ji,[*,†] Qinggang Tan,[†] M. A. Barbosa,[‡] and Jiacong Shen[†]

Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China, and Instituto de Engenharia Biomêdica (INEB), Laboratório de Biomateriais, Porto, Portugal

Received December 24, 2002; Revised Manuscript Received January 24, 2003

We report the development of new biomacromolecule coatings on biodegradable biomaterials based on electrostatic assembly of extracellular matrix-like molecules. Poly(ethylene imine) (PEI) was employed to engineer poly(DL-lactide) (PDL-LA) substrate to obtain a stable positively charged surface. An extracellular matrix- (ECM-) like biomacromolecule, gelatin, was selected as the polyelectrolyte to deposit on the activated PDL-LA substrate via the electrostatic assemble technique. The extracellular matrix-like multilayer on the PDL-LA substrate was investigated by attenuated total reflection (ATR-FTIR), X-ray photoelectron spectrscopy (XPS), contact angle, and atomic force microscopy (AFM). The gradual buildup of the protein layer was investigated by UV−vis spectra, and it was further given a quantitative analysis of the protein layer on the PDL-LA substrate via the radioiodination technique. The stability of the protein layer under aqueous condition was also tested by the radiolabeling method. Chondrocyte was selected as the model system for testing the cell behavior and morphology on modified PDL-LA substrates. The chondrocyte test about cell attachment, proliferation, cell activity and cell morphology by SEM, and confocal laser scanning microscopy (CLSM) investigation on extracellular matrix-like multilayer modified PDL-LA substrate was shown to promote chondrocyte attachment and growth. Comparing conventional coating methods, polyelectrolyte multiplayers are easy and stable to prepare. It may be a good choice for the modification of 3-D scaffolds used in tissue engineering. These very flexible systems allow broad medical applications for drug delivery and tissue engineering.

Introduction

Tissue engineering has developed rapidly to a major field in biotechnology in recent years. New developments in biomaterial, innovative cell culture technique, and newly discovered growth factors open novel avenues to engineering vital transplantable tissues and organs for research and clinical application.[1]

The scaffold for tissue engineering can be fabricated from two classes of biomaterials: naturally extracellular matrix and synthetic biodegradable polymer. Naturally extracellular matrix molecules, such as collagen, chitosan, and gelatin, have the potential advantage of inducing specific cell interaction. However, these materials are isolated from human or animal tissue and so are typically not available in large quantities and suffer from batch-to-batch variation. In addition, naturally derived materials offer limited versatility in designing scaffold with specific properties (e.g., mechanical strength). Synthetic materials, by contrast, can be manufactured reproducibly on large scale and can also be processed into scaffold in which the macrostructure, mechanical properties, and degradation time can be readily controlled and manipulated. The greatest disadvantage of synthetic biodegradable materials is, however, the lack of cell recognition signal molecules.[2] Thus it is desirable to develop a simple approach to combine the advantages of both naturally extracellular matrix and synthetic polymer, which can also be applied to the modification of the complex 3-D tissue engineering scaffold.

The key role of surface for cell−material interaction has been recognized now. Therefore, new strategies aim at the tailoring of the material's surface only to render materials biologically active, while preserving the bulk properties of the underlying support.[3]

The self-organization of polymers has been increasingly explored for the preparation of well-defined surfaces and interfaces in recent years.[4−6] The "electrostatic self-assembly" (ESA) method, which is based on the alternating physisorption of oppositely charged polyelectrolytes, represents a new, alternative solution for biomaterial coating.[7] The principle of alternate adsorption was invented for charged colloidal particles and proteins in 1966 in the pioneering work of Iler.[8] Fromherz proposed the idea of assembling multilayers by alternate adsorption of charged proteins and linear polyions.[9] Mallouk et al.[10] developed alternate adsorption of Zr^{4+} ions and diphosphonic acid. In 1991, Decher and co-workers introduced a related method for film assembly by means of alternate adsorption of linear polycations and polyanions, or bipolar amphiphiles.[11−13] In this method the crucial feature was excessive adsorption (more than neutralization) at every stage of polycation/polyanion assembly that leads to recharg-

* To whom correspondence should be addressed. Tel: 0086-571-87951108. Fax: 0086-571-87951948. E-mail: jianji@mail.hz.zj.cn.

[†] Zhejiang University.

[‡] Instituto de Engenharia Biomédica (INEB), Laboratório de Biomateriais.

10.1021/bm025773p CCC: $25.00 © 2003 American Chemical Society
Published on Web 03/10/2003

ing 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. Now the technique is applied successfully to at least 30 different water-soluable polyions and boladiions. The procedure may be carried out not only with linear polyions and boladiions but also with proteins,[5] virus,[14] ceramics,[15] and charged nanoparticles.[16−18] Successful protein/polyion multilayer assembly provides new possibilities in the line of efforts to create a method of organizing proteins in layers and to build up such layers following "molecular architecture" plans.[5] The method was extended to build up protein superlattices, i.e., alternate arrays of different protein monolayers in predetermined orders. The investigations were mostly motivated by their biological function and biomimetics applications, e.g., for biocompatibilization of surface, enzymatic activity, sensing purpose, or molecular recognition. Though the ESA technique was widely studied on solid matrix, the substrate most investigators used was gold, quartz, glass, or silicone. Few of them used the "real" biomaterials, for example, PU, PVC, or PLA, used in biomedical fields.

The current research will explore construction of extracellular matrix-like multilayers on the 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 extracellular matrix-like molecules, which therefore can be applied to enhancing the biocompatibility of the PLA tissue engineering scaffold.

Experimental Section

Chemicals. Poly(DL-lactide) (PDL-LA) was prepared by ring-opening polymerization of DL-lactide in our laboratory; the molecular weight was 200000 obtained from GPC, MWD = 1.75. Poly(ethylene imine) (PEI) was purchased from Aldrich (M_w = 25000). The biomacromolecule used in this study was gelatin (Fluka, gelatin from porcine skin with medium gel strength). All of the chemicals were used without further purification.

Multilayer Preparation. The PDL-LA substrates were obtained by the solvent-casting method in CH_2Cl_2 solution. Before the assembly, the PDL-LA substrates were ultrasonicated in 50% ethanol solution for 20 min for the cleaning, then rinsed with water, and finally dried under a stream of nitrogen. The PDL-LA substrate was activated by treating with PEI at the concentration of 1 mg/mL in water to obtain a stable positively charged surface. The sequence of operations resulting in production of gelatin films was the following. The activated PDL-LA substrate was immersed in a 0.5 mg/mL gelatin, pH 7.4, for 20 min. After a 30 s washing 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 rinsed with water again. The further growth of PEI/gelatin bilayers was accomplished by the repetition of 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 the final assembly cycle, the substrate was immersed in a 5 mg/mL solution of glutaraldehyde for 20 min to fix the protein/polyion architecture. After being rinsed with water, the substrate was dried with a stream of nitrogen. Eight bilayers were obtained by such alternate deposition in our experiment for the biomacromolecule multiplayer.

Iodination of Gelatin. The Iodogen method[19] was used to iodinate the gelatin for the radiolabeling test of the gelatin assembly on the PDL-LA substrate surface. The procedure was as follows: add 40 μL of Iodogen solution to an Eppendorf tube and dry the solvent in a N_2 stream; add 100 μL of 0.25 M PBS to the Eppendorf, then 10 μL of radioactive iodine (100 μg/mL), and then 10 μL of gelatin (1 mg/mL) to be labeled; allow the mixture to stand on ice for 20 min, stirring occasionally; at the end of the reaction, add 250 μL of 0.05 M PBS. The free iodine was separated from the labeled gelatin in a Sephadex G-25 PD-10 column (size exclusion chromatography). Count the fractions for radioactivity in the Geiger counter as they are being eluted. This will give an idea of the retention times for the labeled gelatin and the free iodine. Recover the eluted fractions to Eppendorf tubes (~20). Withdraw a 2 μL aliquot from each fraction and transfer it (along with the tip) to a RIA tube; then count the aliquots for radioactivity in the γ-counter. The yield of the reaction was obtained by the TCA (trichloroacetic acid) method.[20] Add 45 μL of 5% BSA in PBS to an Eppendorf, 2 μL of the fraction, and 50 μL of TCA (repeat for all the fractions); let the tubes stand on ice for 20 min; centrifugate the tubes at 10000 rpm for 15 min; withdraw the supernatant and transfer it to an identified RIA tube; cut the end of the tube containing the precipitated protein and put it in another RIA tube; count the aliquots for radioactivity in the γ-counter; compute the percent of labeled protein/free iodine in each fraction.

Surface Analysis. The obtained polyelectrolyte multilayer films were dried at room temperature for 48 h and then vacuum-dried for 24 h. The surface analysis was performed with attenuated total reflection (ATR-FTIR, Bruker Vectro 22), X-ray photoelectron spectroscopy (XPS) (VG Scientific ESCA LAB 200A), atomic force microscopy (AFM) (SPA 400, Seiko Instrument Inc.), the measurement of contact angle (Kruss DSA10-MK_2) with both the sessile and captive bubble methods,[21] UV−visible spectrophotometry (Shimadzu UV-1201), and radioiodination investigation.

Cell Culture. Chondrocytes from rabbit ear were isolated according to the method of Klagsbrum.[22] The chondrocytes were grown in Ham's F12 (Gibco) supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum (FBS, Sijiqin Biotech. Co., China, lot no. 020613.2), 1.176 μg/mL $NaHCO_3$, 0.3 μg/mL L-glutamic acid, 0.05 μg/mL vitamin C, 80 units/mL penicillin, and 100 μg/mL streptomycin.

Cell Attachment and Growth Assay. PDL-LA, PEI-activated PDL-LA, and PEI/gelatin assembly PDL-LA substrates, sterilized in 75% ethanol and swollen in PBS, were placed into 96-well tissue-culturing polystyrene (TCPS) plates (Nuclon, catalog no. 167008). The wells were fully covered by the substrates. Chondrocytes were then seeded

in culture media to give a final density of 20×10^4 cells/mL per well (0.2 mL) and incubated for 12 h at 37 °C in an atmosphere of 5% CO_2 in air. Following incubation, the wells were washed twice with PBS to remove nonattached cells. The remaining cells were removed by digestion with 0.25% trypsin solution and pipetting. The number of cells attached to the polymer surfaces was determined by hemocytometric counting.[23,24] All experiments were performed at least twice, each yielding at least quadruplicate values. Cell attachment was expressed as a proportion of the number of cells attached to TCPS in the same culture media. For cell growth studies, the sample preparation was the same as for the attachment assay except that the cells were incubated for 72 h. The cell proliferation was expressed by comparing with the number of original seeding cells.

Cell Viability Investigation. Cell viability was determined by the MTT assay,[25−29] which is based on the mitochondrial conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, after the chondrocytes were incubated on different substrates for 72 h, 20 μL of MTT solution (5 mg/mL in PBS) was added to each sample, and the samples were incubated 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 wavelength 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 viability was expressed as a proportion of the absorbance value of TCPS in the same culture media.

Cell Morphology Investigation. After being seeded for 3 days, the substrates were fixed in 2.5% glutaraldehyde for 15 min and washed with PBS at least three times. In control experiments, the chondrocytes were cultured on TCPS plates. Scanning electron microscopy (SEM) photographs were obtained from Stereoscan-260 microscopy. The cell monolayers on different PDL-LA substrates were also stained with fluorescein diacetate (FDA, Sigma) for confocal laser scanning microscopy (CLSM) (CLSM, Bio-Rad, Radiance 2100; Zeiss AXIOVERT 200) investigation. FDA is an indicator of membrane integrity and cytoplasmic esterase activity.[30] 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 of PBS. A 20 μL FDA solution was added in each well, and the solution was incubated for 5 min. The substrates were then washed twice with PBS and placed on a glass slide for CLSM examination. The 488 nm wavelength of the laser was used to excite the dye.

Results and Discussion

Surface Analysis. In this study, PEI was used to activate the PDL-LA substrate in order to obtain a stable positively charged surface that can be further used to deposit biomacromolecule polyelectrolytes by the ESA technique (Figure 1). This stable activated surface was obtained by the chemical reaction between PEI and PDL-LA molecules based on the aminolysis of the ester group. Figure 2 shows the schematic illustration of activation of the PDL-LA substrate.

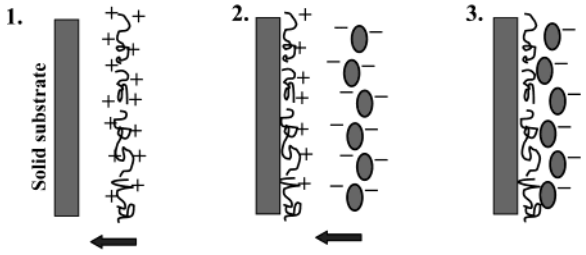

Figure 1. Scheme of electrostatic assembly on a solid substrate by alternate adsorption of linear polycations and negatively charged proteins.

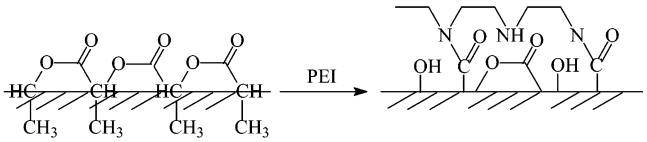

Figure 2. Schematic illustration of the modification of the PDL-LA substrate.

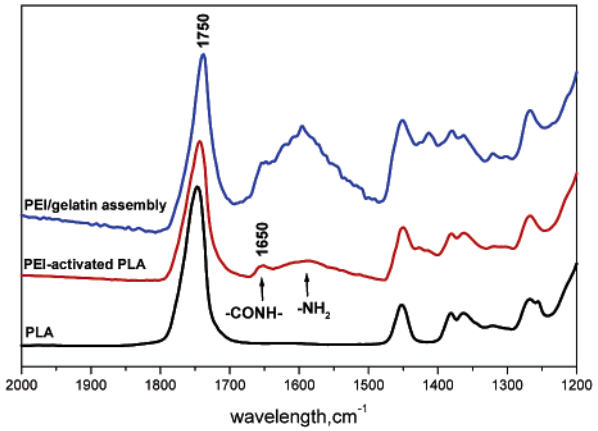

Figure 3. ATR-FTIR spectra of different PDL-LA substrates (from top to bottom): PEI/gelatin multilayer modified PDL-LA, PEI-activated PDL-LA, and PDL-LA virgin substrates.

Investigation of ATR-FTIR. The ATR-FTIR spectra for PEI/gelatin multilayer modified PDL-LA, PEI-activated PDL-LA, and PDL-LA virgin substrates are shown in Figure 3. From the result of Figure 3, the most significant peak of the PDL-LA virgin substrate is the band at about 1750 cm^{-1}. The spectra of the PEI-activated PDL-LA substrate showed a notable difference from the PDL-LA virgin substrate at the band between 1650 and 1500 cm^{-1}. The band between 1640 and 1500 cm^{-1} showed the existence of an amine group of PEI on the PDL-LA substrate. While the peak at 1650 cm^{-1} indicated "amide I" of −CO−N═ bond, it may contribute to the chemical reaction between the amine groups of PEI and the ester bond of PDL-LA which confirm the formation of a stable positively charged PEI surface on the PDL-LA substrate. The decreasing of the band of 1750 cm^{-1} and the increasing of the band between 1650 and 1500 cm^{-1} in the spectra of the PEI/gelatin multilayer modified PDL-

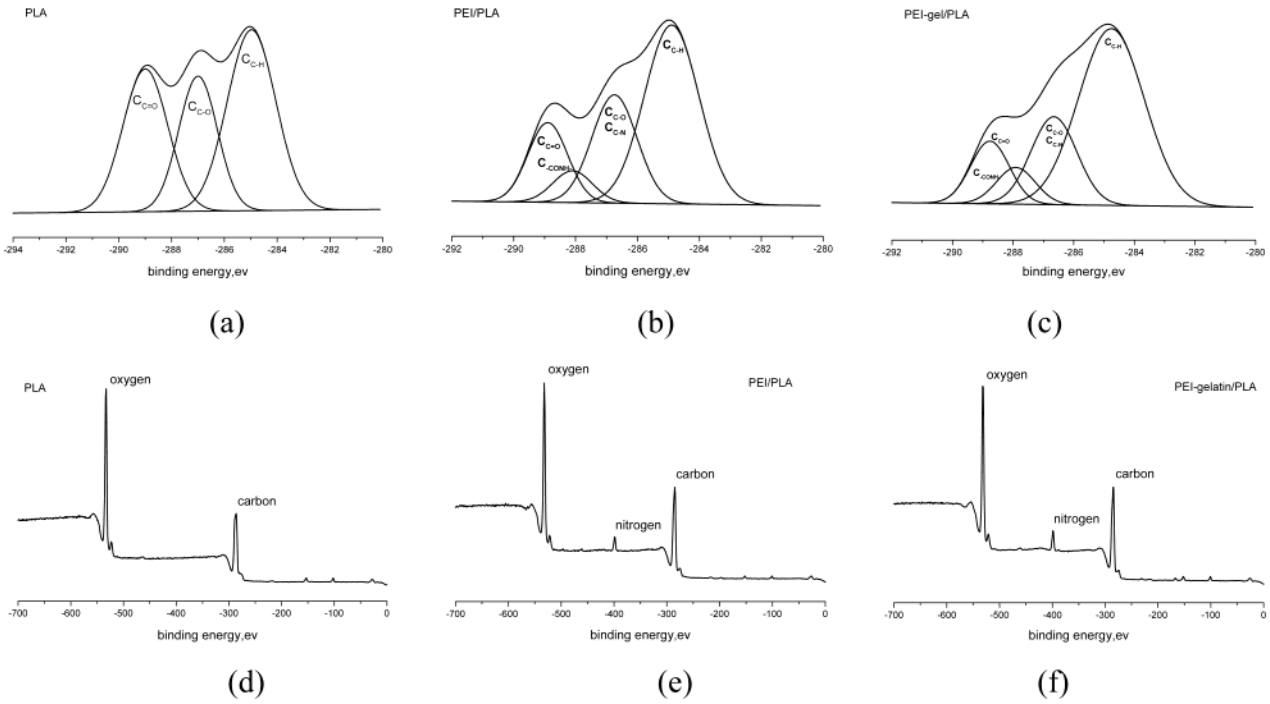

Figure 4. XPS spectra of different PDL-LA substrates: (a, d) chemical structure of PDL-LA and XPS spectra of the unmodified PDL-LA substrate; (b, e) XPS spectra of the PEI-activated PDL-LA substrate; (c, f) PEI/gelatin assembly PDL-LA substrate.

Table 1. Element Content of PEI-Activated PDL-LA Substrate by Different Tilt Angles

element content, %	PEI/PLA, 0°	PEI/PLA, 55°
C 1s	64.06	65.13
O 1s	32.06	29.78
N 1s	3.88	5.09

Table 2. Contact Angle Data (deg) of Different PDL-LA Substrates

	sessile drop	captive bubble
PDL-LA	87.7 ± 1.92	76.0 ± 0.00
PEI activated	70.2 ± 1.55	44.3 ± 1.84
PEI/gelatin	65.9 ± 2.36	31.1 ± 1.67

LA substrate indicated a thin layer of gelatin on the PEI-activated PDL-LA substrate.

XPS Analysis. Figure 4a shows the XPS spectra of the PDL-LA virgin substrate. The peaks were obtained by the "XPS peak" software affixed to the ESCA instrument. The unmodified PDL-LA surface generates the three expected peaks with almost equal peak areas, indicating that this surface contains the three carbon regions of the PDL-LA surface without notable contamination. The binding energies are 285.0 eV for C_{C-H}, 286.9 eV for C_{C-O}, and 289.0 eV for $C_{C=O}$, respectively. After the PEI activation, it showed a significant change about the carbon peaks. The peak about C_{C-H} increased notably. There appeared another carbon peak at 288.2 eV, indicating the existence of an amide bond on the surface of the PDL-LA substrate, which verified the chemical bond between PEI and PDL-LA. For the PEI/gelatin multiplayer assembled PDL-LA sample, it also showed an obvious carbon peak of amide, indicating the protein layer on the surface of the PDL-LA substrate. Besides, there are obvious nitrogen peaks on both PEI-activated and PEI/gelatin multilayer assembled PDL-LA substrates (Figure 4e,f), while there is no detectable nitrogen on the PDL-LA substrate (Figure 4d).

By changing the tilt angle, the element content about carbon, oxygen, and nitrogen was also obtained (Table 1). It was found that the nitrogen content of 0° tilt was lower than that of 55° tilt. This result indicated that the nitrogen content was higher at the outer surface of the PDL-LA substrate. That is to say, there is a decreasing distribution of the PEI from the outer to inner surface of the PDL-LA surface.

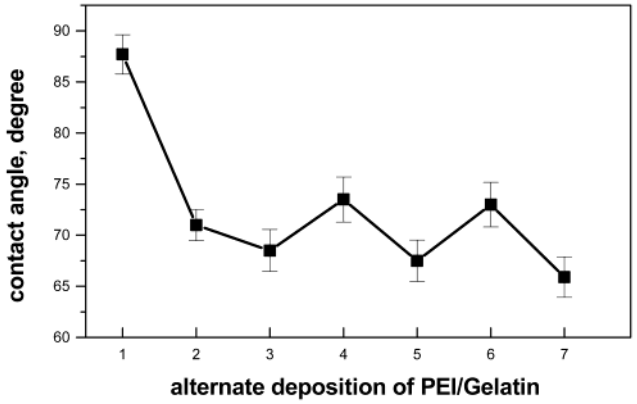

Figure 5. Contact angle data with alternate deposition of PEI/gelatin by the sessile drop method: 1, PDL-LA virgin substrate; 2, PEI-activated PDL-LA substrate; 3−7, gelatin and PEI alternate layers.

Contact Angle Measurement. Two methods for the measurement of contact angle, the sessile drop and captive bubble, were performed. The results are shown in Table 2. The results indicated that the hydrophilicity of all of the surface-treated samples was obviously enhanced.

Figure 5 shows the contact angle result of the evolution of the alternate deposition of PEI and gelatin layers. The obviously alternate change of contact angle data verified the

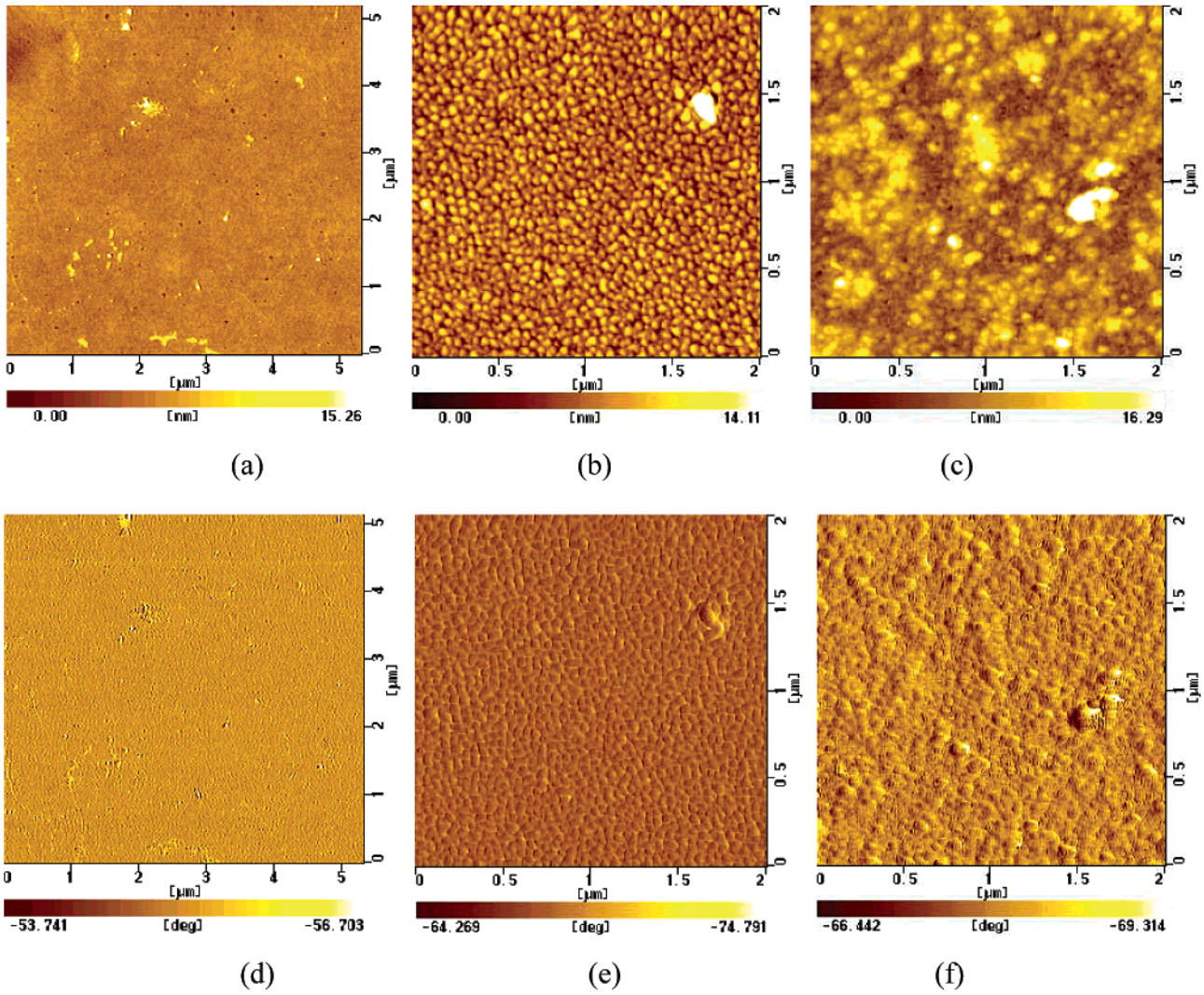

Figure 6. AFM micrographs of different PDL-LA films: topography of (a) the PDL-LA virgin substrate, (b) the PEI-activated PDL-LA substrate, and (c) the PEI/gelatin multilayer assembled PDL-LA substrate; phase micrograph of (d) the PDL-LA virgin substrate, (e) the PEI-activated PDL-LA substrate, and (f) PEI/gelatin multilayer modified PDL-LA substrate.

progressive buildup of the film by alternate deposition of the polyelectrolytes.

Atomic Force Microscopy (AFM) Investigation. The surface properties of the resulting films were characterized by atomic force microscopy (AFM). Tapping mode AFM micrographs of the film showed a notable difference between the PDL-LA virgin substrate and the modified PDL-LA substrates. The surface of the PDL-LA virgin substrate showed a smooth topography with even phase (seen at Figure 6a,d). However, the surface of the PEI-activated PDL-LA substrate was covered by a layer of granule less than 100 nm (Figure 6b,e), which may contribute to the microphase separation caused by the less compatibility of the PLA-PEI copolymer with the PLA matrix. Also, the gelatin modified PDL-LA substrate (Figure 6c,f) showed different micrographs from that of the PDL-LA virgin substrate. But the image of the granule is much more illegible than the PEI-activated PDL-LA substrate. It may be "self-healing" phenomena[3] widely observed in ESA-made film, which is presumably linked to the charge overcompensation during the adsorption step.

UV−Vis Spectral Analysis. The gradual buildup of the PEI/gelatin film on the PDL-LA matrix in the cyclic dipping

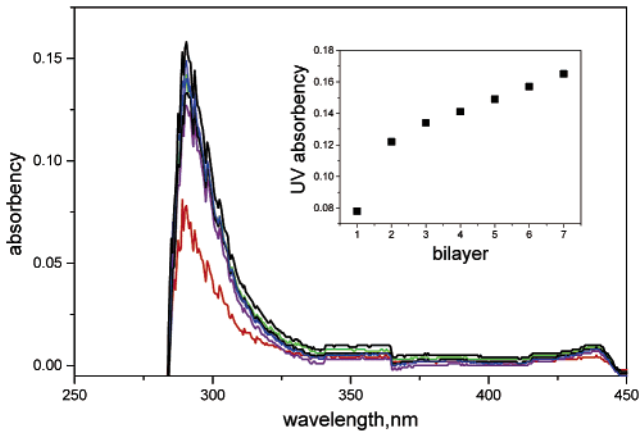

Figure 7. UV−vis absorbance of the self-assembled gelatin layer on the PDL-LA substrate.

procedure can be visualized by UV−visible spectroscopy at 290 nm (Figure 7). The sequence of the UV spectra shows a linear increase of absorbance with every new PEI/gelatin bilayer, which is typical for many layer-by-layer assembled pairs. An exception can be found at the first two or three bilayers, which showed much more gelatin adsorption than

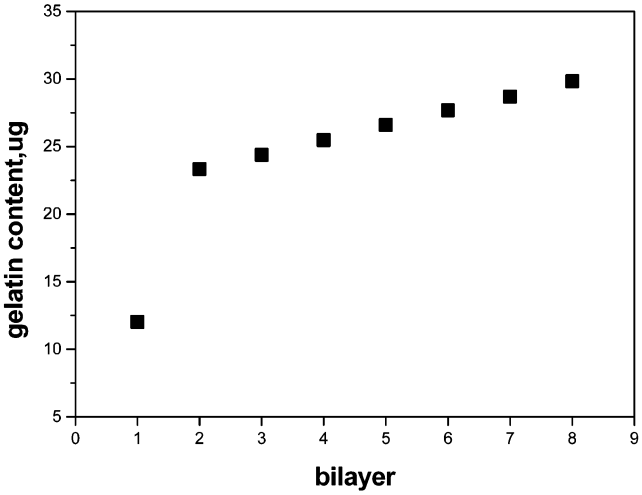

Figure 8. Relationship of gelatin content with PEI/gelatin bilayers.

those of later bilayers. It should be noticed that most of the investigation about self-assembly was focused on solid matrix, such as gold, quartz, glass, or silicone matrix, which is not real biomaterial. The PDL-LA matrix used in our experiment may have some tiny cavities or cracks on the surface because of the film processing procedure, which unavoidably results in the increasing of its real surface volume. After the cavities or cracks were compensated by two or three assemble cycles, it showed a linear increase of UV absorbance with less gelatin adsorption.

Radioiodination Study of the Gelatin Layer on the PDL-LA Substrate. Radiolabeling is one of the most useful and powerful experimental methods for the study of protein adsorption at solid−solution interfaces. It consists of incorporating a radioactive nuclide into the molecular structure of the protein to be studied and then counting the surface-bound radioactivity following contact of the material with the protein solution to which the labeled protein is added. In general, the radiolabeling technique provides data of high precision on the amount of protein adsorbed under given conditions.[31]

The Iodogen method was used in our experiment to radioiodinate the gelatin, and the yield of radioiodination of about 90% was obtained via adding the radiolabeled gelatin into 50 mL of 0.5 mg/mL gelatin solution, in which a volume of 2 mL in the RIA tube was tested by a γ-counter to get the relationship of radioactivity with gelatin content. The PDL-LA substrates used for the radioiodination investigation were cut into 8×30 mm^2 pieces, and the assemble process of PEI/gelatin was the same as that described in the Experimental Section, except that a radiolabeled gelatin solution was used. After each bilayer assembly, the substrate was placed into an RIA tube to test the radioactivity.

Figure 8 shows the result of gelatin content with PEI/gelatin bilayers. It can be seen that the result is similar to that of the UV−vis spectral analysis. There may be a cavity-compensation process during the first two or three bilayer assemblies. After that, the gelatin content increased about 0.225 μg/cm^2 for each bilayer. According to the surface volume of the PDL-LA and the density of gelatin (about 1.005), it can be calculated that the thickness of each gelatin layer is about 2.24 nm. The real thickness of the gelatin layer will be much thickner than what we calculated, because the gelatin layer does not have a critical interface with the PEI layer but entangled with the PEI layer to some degree.

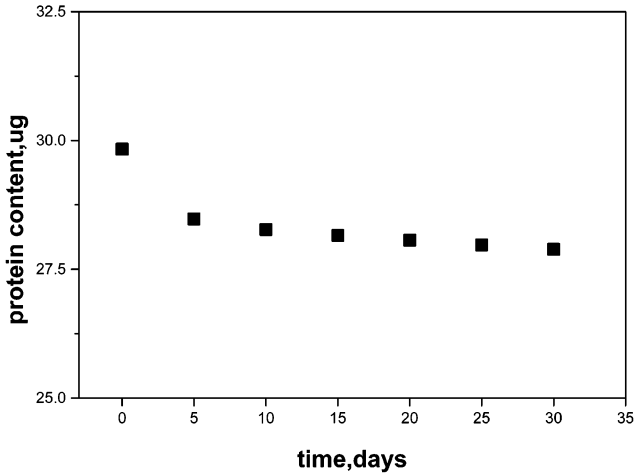

Figure 9. Stability test about the gelatin layer on the PDL-LA substrate.

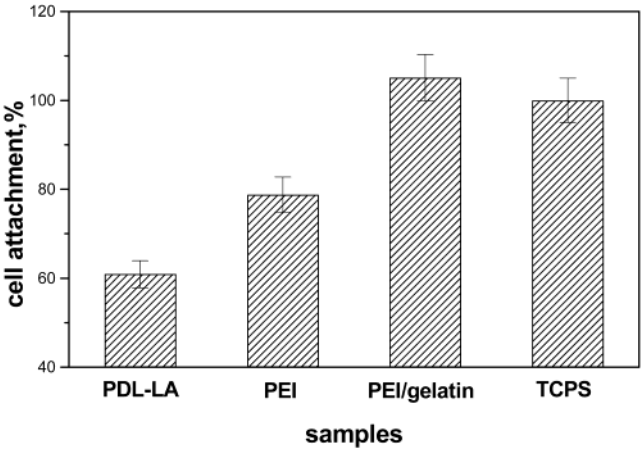

Figure 10. Attachment characteristics of chondrocytes to different PDL-LA substrates.

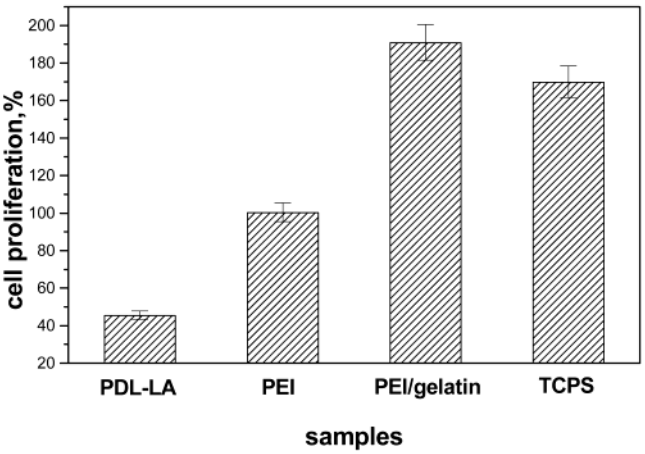

Figure 11. Proliferation characteristics of chondrocytes to different PDL-LA substrates.

Stability Test of the Protein Multilayers on the PDL-LA Substrate. Although a stable first layer on the PDL-LA substrate has been obtained in our study by the chemical covalent of PEI with PDL-LA molecules, which is much

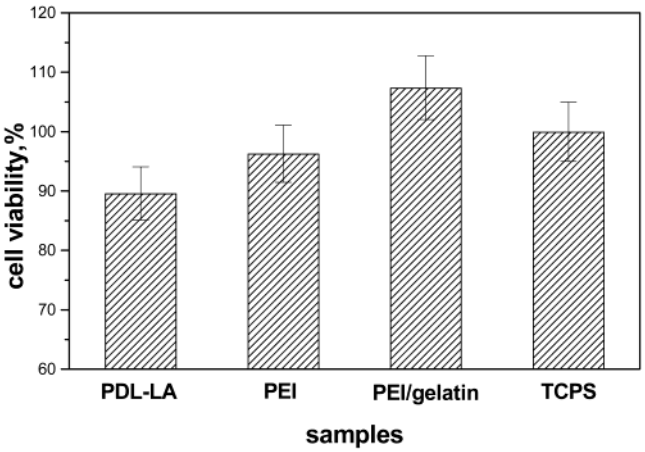

Figure 12. Cell viability of chondrocyte to different PDL-LA substrates.

more stable than mere coating method, the stability of the whole protein/PEI assemble layer is crucial to the cytomimetic surface of the PDL-LA material.

The radiolabeling technique can also be used to investigate the protein layer on the solid substrate. The radiolabeled gelatin assembly PDL-LA substrates were kept under 2 mL of PBS solution in an RIA tube. The PBS solution was extracted to test the radioactivity every 5 days. The amount of protein fall from the protein layer on the PDL-LA substrate was therefore obtained. Figure 9 shows the gelatin content on the PDL-LA substrate every 5 days. As a result, the gelatin multilayers on the PDL-LA substrate fixed by glutaraldehyde are stable to 30 days in our experiment. In comparison with the total protein amount of gelatin on the PLA substrate, the fallen protein is only about 6.5% in 30 days.

Cell Behavior Analysis. (A) Cell Attachment and Proliferation Measurement. Attachment characteristics of chondrocytes to the PDL-LA virgin substrate PEI-activated PDL-LA substrate and PEI/gelatin multilayer modified PDL-LA substrates were studied, and the results are shown in Figure 10. The values for attachment of cells were reported as a proportion of the number of cells that attached to TCPS in the same culture media. The comparison of the PDL-LA virgin substrate and ESA modified PDL-LA substrates revealed that the chondrocyte attachment on biomacromolecule multilayer modified PDL-LA substrates is better than that on PDL-LA virgin substrates. The attachment datum of the PDL-LA virgin substrate is 60.9%. As for the PEI-activated and gelatin-surface substrate, the attachment data are 78.8% and 105.1%, respectively (TCPS as 100%). Among them, PEI/gelatin multilayer modified PDL-LA

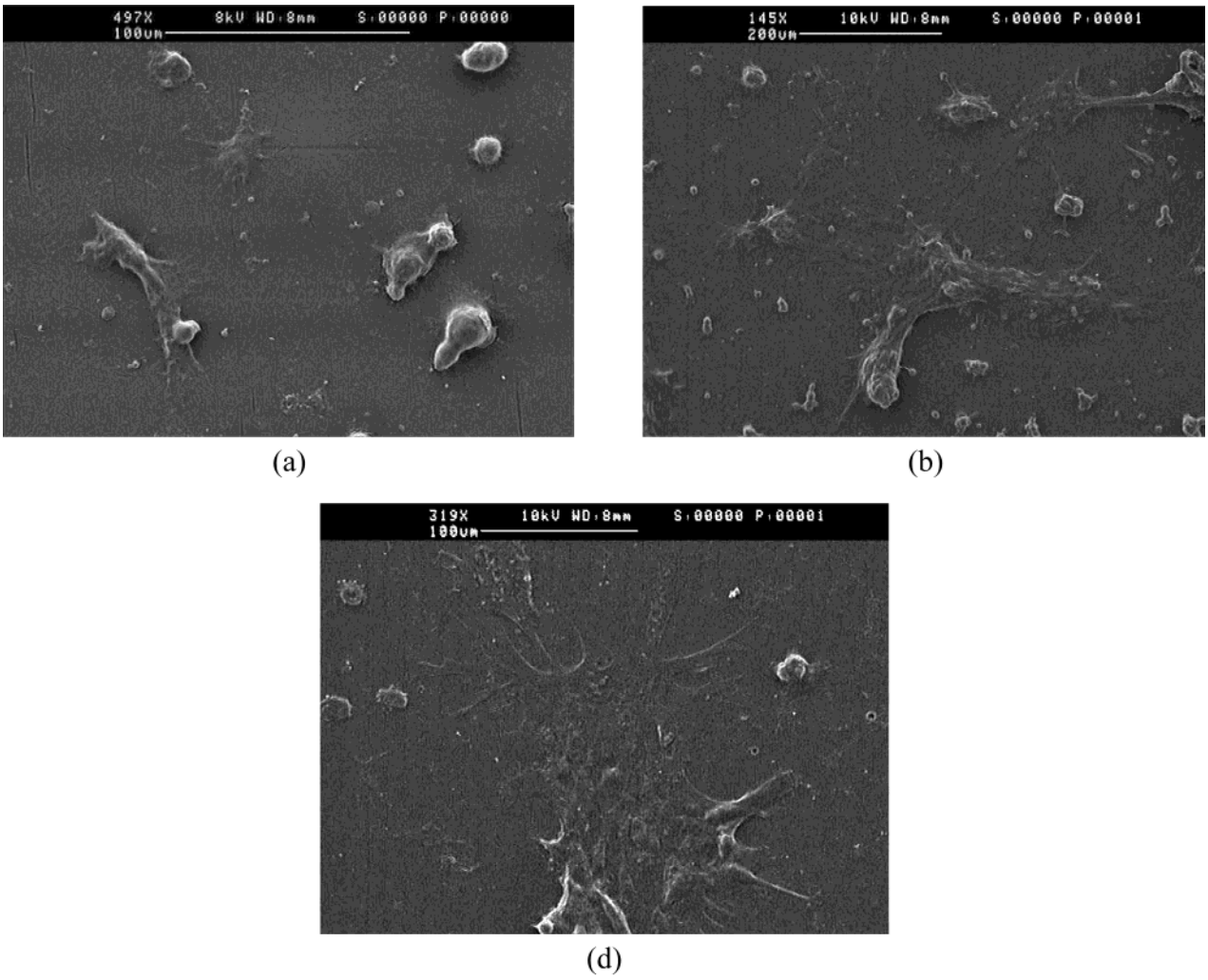

Figure 13. Morphology of chondrocytes seeded on different matrices: chondrocytes on (a) the PDL-LA virgin substrate, (b) the PEI-activated PDL-LA substrate, and (c) the PEI/gelatin multilayer modified PDL-LA substrate.

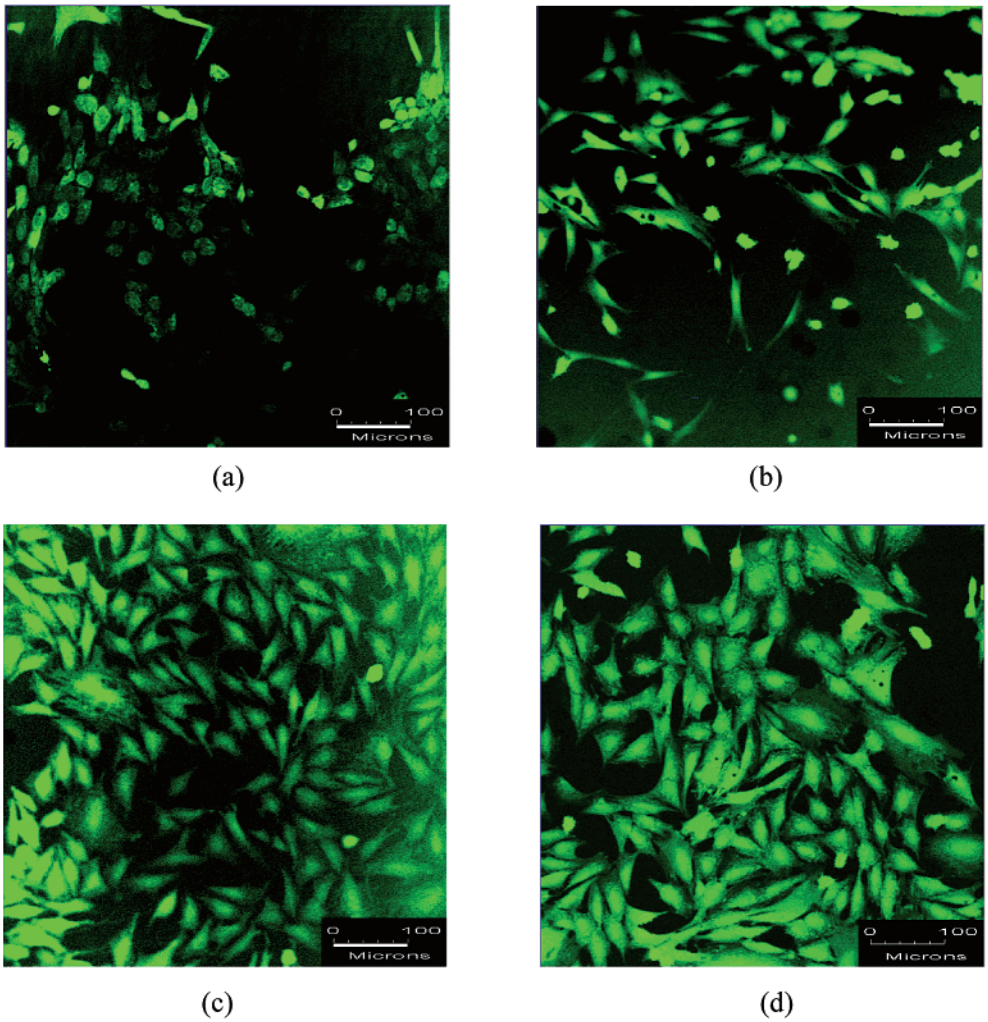

Figure 14. Confocal laser scanning microscopy (CLSM) images of chondrocytes on different matrices: chondrocytes on (a) the PDL-LA virgin substrate, (b) the PEI-activated PDL-LA substrate, (c) the PEI/gelatin multilayer modified PDL-LA substrate, and (d) tissue-culturing polystyrene (TCPS).

substrates showed the best result of chondrocyte attachment, which is also better than that of TCPS.

Figure 11 shows the growth characteristics of chondrocytes on various surfaces over a period of 72 h. It was found that the chondrocytes grow faster on the modified substrates than on the PDL-LA virgin substrate. The proliferation datum of the PDL-LA virgin substrate is 45.6%. As for the PEI-activated and gelatin-surface substrate, proliferation data are 100.5% and 191.0%, respectively, as compared to 170.0% on TCPS. Like chondrocyte attachment, the chondrocytes on the PEI/gelatin multilayer modified PDL-LA substrate also grow faster than those of TCPS.

(B) Cell Viability Measurement. Figure 12 shows the cell viability of chondrocytes on various surfaces over a period of 72 h. It was found that the cell viability on modified PDL-LA substrates is higher than that on the PDL-LA virgin substrate. The viability datum of the PDL-LA virgin substrate is 89.6%. As for PEI-activated and gelatin-surface substrate, the viability data are 96.3% and 107.4%, respectively (TCPS as 100%). It can be seen that the cell viability on PEI/gelatin multilayer modified PDL-LA substrates is higher than that of TCPS, similarly to cell attachment and proliferation.

Investigation of Cell Morphology. Figure 13 shows that the chondrocytes attach to different matrices. After 3 days, it can be seen that all of the chondrocytes cultured on the modified matrices had undergone some degree of proliferation in the monolayer. The chondrocytes on the biomacromolecule multilayer modified PDL-LA substrate surface maintain normal spreading morphology (seen from Figure 13c). However, most of the chondrocytes on the PDL-LA virgin substrate have not spread completely (Figure 13a). The chondrocyte on the PEI-activated PDL-LA substrate (Figure 13b) seems also to have a good spreading morphology. It may contribute to the readsorption of proteins in culture medium on the PEI surface. However, it was an uncontrollable procedure since there are lots of proteins in the culture medium or blood and, therefore, is not recommended for the modification of a biomedical device.

Confocal Laser Scanning Microscopy (CLSM) Investigation. Figure 14 shows the confocal laser scanning microscopy (CLSM) images of chondrocyte attaching to the PDL-LA virgin substrate, PEI-activated PDL-LA substrate, gelatin assembly PDL-LA substrate, and TCPS substrate. We can see a notable difference about the living chondrocyte on different substrates because the FDA can only be hydrolyzed into fluorescein that can be detected by CLSM in living cells. It showed much more chondrocyte spreading on the PEI-activated substrate than that on unmodified PDL-

LA substrate. The chondrocyte on the gelatin assembly PDL-LA substrate is spreading uniformly and completely covered the substrate surface, which is also similar with the chondrocyte on the TCPS substrate.

From the results of chondrocyte attachment, proliferation, viability assays, and cell morphology investigation, we can conclude that the biomacromolecule multilayer modified PDL-LA substrates can obviously promote chondrocyte attachment and growth.

Conclusion

Electrostatic self-assembly of PEI/gelatin was explored to construct an extracellular matrix-like multilayer onto the PDL-LA substrate. The ATR-FTIR and XPS spectra study, contact angle measurement, and AFM analysis indicated that those biomacromolecules were successfully immobilized onto the PDL-LA surface. The gradual buildup of the assemble layer was verified by contact angle measurement, UV−vis analysis, and the radiolabeling technique. Further investigation gave a quantitative result of the protein layer on the PDL-LA substrate by the radiolabeling technique, and also the stability of the protein layer on the PDL-LA substrate was verified. The result of the chondrocyte culture test on those modified PDL-LA substrates showed that the extracellular matrix-like mutilayer has positive effect on the attachment, growth, and viability of the chondrocyte. The SEM and CLSM photographs of chondrocytes on the modified PDL-LA substrate also showed much normal spreading morphology than that of the PDL-LA virgin substrate.

All of the above results demonstrate that electrostatic self-assembly of PEI and gelatin can construct an extracellular matrix-like mutilayer on the synthetic biodegradable polymer PDL-LA and might have potentials for drug delivery, tissue engineering, and other biomedical applications.

Acknowledgment. This research was financially supported by the Major State Basic Research Foundation of China (Grant G1999054305) and the Natural Science Foundation of China (NSFC-20174035).

References and Notes

(1) Sittinger, M.; Perka, C.; Schutz, O.; Haupl, T.; Burmester, G. R. *Z. Rheumatol.* **1999**, *58*, 130−135.
(2) Kim, B. S.; Mooney, D. J. *Trends Biotechnol.* **1998**, *16*, 224.
(3) Bertrand, P.; Jonas, A.; Laschewsky, A.; Legras, R. *Macromol. Rapid Commun.* **2000**, *21*, 319.
(4) Ulman, A. *Chem. Rev.* **1996**, *96*, 1533.
(5) Sano, M.; Lvov, Y.; Kunitake, T. *Annu. Rev. Mater. Sci.* **1996**, *26*, 153−187.
(6) Laschewsky, A. *Eur. Chem. Chronicle ECC* **1997**, *2*, 13−24.
(7) Chluba, J.; Voegel, J. C.; Decher, G.; Erbacher, P.; Schaaf, P.; Ogier, J. *Biomacromolecules*. **2001**, *2*, 800−805.
(8) Iler, R. *J. Colloid Interface Sci.* **1966**, *21*, 569.
(9) Fromhertz, P. In *Electron Microscopy at Molecular Dimension*; Baumeister, W., Ed.; Springer-Verlag: Berlin, **1980**; p 338.
(10) Lee, H.; Kepley, L.; Hong, H.-G.; Mallouk, T. *J. Am. Chem. Soc.* **1988**, *110*, 618.
(11) Decher, G.; Hong, J.-D. *Ber. Bunsen-Ges.* **1991**, *95*, 1430.
(12) Decher, G. *Science* **1997**, *227*, 1232.
(13) Lvov, Y.; Decher, G.; Mohwald, H. *Langmuir* **1993**, *9*, 481.
(14) Lvov, Y.; Haas, H.; Decher, G.; Mohwald, H.; Mikhailov, A.; Mtchedlishvily, B.; Morgunova, E.; Vainshtein, B. *Langmuir* **1994**, *10*, 4232.
(15) Kleinfeld, E.; Ferguson, G. *Science* **1994**, *265*, 370.
(16) Hong, J.-D.; Lowack, K.; Schmitt, J.; Decher, G. *Prog. Colloid Polym. Sci.* **1993**, *93*, 98.
(17) Schmitt, J.; Grunewald, T.; Krajer, K.; Pershan, P.; Decher, G.; Loshe, M. *Macromolecules* **1993**, *26*, 7058.
(18) Korneev, D.; Lvov, Y.; Decher, G.; Schmitt, J.; Yarodaikin, S. *Physica B* **1995**, *214*, 954.
(19) *Iodine-125, a Guide to Radioiodination Techniques*; Amersham Life Science, 1993; p 64.
(20) Hwan, M. K.; Sang, B. H.; Moon, S. K.; Jong, S. K.; Goo, T. O.; Dong, H. H. *J. Pharmacol. Toxicol. Methods* **1996**, *36*, 163−169.
(21) Adamson, A. W.; Gast, A. P. *Physical Chemsitry of Surfaces*, John Wiley & Sons: New York, 1997; pp 27−28.
(22) Klagsbrum, M. *Methods Enzymol.* **1979**, *58*, 560.
(23) Blum, R. N.; Wright, R. A. *J. Gen. Intern. Med.* **1992**, *7*, 140−144.
(24) Okuda, M.; Miura, I.; Juji, F.; Takashima, H. *Int. Arch. Allergy Immunol.* **1994**, *104* (Suppl. 1 (1)), 6−8.
(25) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.
(26) Zund, G.; Ye, Q.; Hoerstrup, S. P.; Schoeberlein, A.; Schmid, A. C.; Grunenfelder, J.; Vogt, P.; Turina, M. *Eur. J. Cardio-thoracic Surg.* **1999**, *15*, 519−524.
(27) Risbud, M. V.; Bhonde, M. R.; Bhonde, R. R. *J. Biomed. Mater. Res.* **2001**, *57*, 300−305.
(28) Strand, B. L.; Ryan, T. L. In't Veld, P.; Kulseng, B.; Rokstad, A. M.; Skjak-Brek, G.; Espevik, T. *Cell Transplant.* **2001**, *10*, 263−275.
(29) De Ceuninck, F.; Gaufillier, S.; Bonnaud, A.; Sabatini, M.; Lesur, C.; Pastoureau, P. *Biochem. Biophys. Res. Commun.* **2001**, *285*, 926−931.
(30) Bancel, S.; Hu, W. S. *Biotechnol. Prog.* **1996**, *12*, 398−402.
(31) Davids, S. *Test procedures for the blood compatibility of biomaterials*; Kluwer Academic Publishers: Dordrecht, 1993; pp 287−330.

BM025773P