Gelatin Multilayers Assembled on Poly(L-lactic acid) Surface for Better Cytocompatibility

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ABSTRACT: Gelatin multilayers were assembled on PLLA substrate at pH 3, 5, and 7, which was below, around, and above the isoelectric point of the amphoteric polymer, using the layer-by-layer assembly technique. The multilayer deposition on the PLLA substrate was monitored by X-ray photoelectron spectroscopy (XPS) and water contact angle measurement. The XPS, water contact angle, and atomic force microscopy data indicated that the layer thickness, surface hydrophilicity, and surface morphology of the gelatin multilayers assembled strongly depended on the pH at which the layers were deposited. Chondrocyte culture was used to test the cell attachment, cell morphology, and cell viability on the PLLAs modified with gelatin multilayers. All these modified PLLAs exhibited dramatically improved cytocompatibility compared with the virgin PLLA, and even better performance than that of tissue culture polystyrene (TCPS). The best cell compatibility was observed for the gelatin multilayers assembled at pH = 5. These results indicate that the cell compatibility of biodegradable polyesters can be effectively and facilely improved by assembly of gelatin multilayers, and the performance can be controlled by adjusting the solution pH at which the multilayers are deposited. This may help the design and fabrication of better scaffolds for tissue engineering. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 109: 530–536, 2008

Key words: biocompatibility; self-assembly; surfaces; poly(L-lactic acid); gelatin

INTRODUCTION

Tissue engineering has become a rapidly developing field in recent years because of its potential in the treatment of lost or damaged human tissue and organs. Scaffolds are very important in tissue engineering, serving as substrates, and physical supports. Among the synthetic and natural materials for this application, poly(L-lactic acid) (PLLA) has attracted particular attention because of its mechanical properties, processability, and biodegradability, which can be tuned to meet particular needs. As a result, PLLA has already been employed for many clinical purposes. However, the hydrophobicity and poor cytocompatibility of the PLLA surface restrict its use as a friendly interface with living cells, and much effort has been devoted to modifying the surface of PLLA to improve its cytocompatibility without altering its bulk properties.[1] The modification approaches reported in the literature include hydrolysis,[2] ozone oxidization,[3] plasma treatment,[4,5] grafting technique,[6] electrostatic self-assembly,[7,8] entrapment of a polymer,[9] coating of natural polymers,[10] and combinations of these techniques.[11,12]

One of the natural polymers often used for this purpose is gelatin. For example, it has been reported that 3T3 fibroblasts showed better proliferation on the PLLA treated directly with an alkaline solution of gelatin.[13] In another study, Zhu et al. assembled multiple bilayers of poly(ethylene imine) (PEI)/gelatin on PLLA surface and reported improved chondrocyte attachment and growth.[14] It is well known that gelatin can adsorb readily to many surfaces under a broad range of conditions, and a large body of literature is available on the adsorption of gelatin.[15–19] The adsorption of gelatin is particularly interesting because the structure of the adsorbed layer varies with pH, temperature, ionic strength and solution concentration.[20] In our previous study, we took advantage of the ease and flexibility of the electrostatic self-assembly method and the amphoteric natural of the gelatin, and adsorbed a single layer of gelatin as a polycation and polyanion to PLLA surface at pH 3.4 and 7.4, respectively, and found that the incorporation of a gelatin layer to the PLLA substrates significantly improved their

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cytocompatibility, with the gelatin treatment at pH = 3.4 being most effective.[21]

In this study, we attempted to assemble multiple layers of gelatin on charged PLLA substrates without using another polyelectrolyte as an interlayer by repetition of the adsorption and subsequent drying process,[22] which affords structurally regulated ultrathin gelatin films with controllable thicknesses. In addition, by controlling the pH of the gelatin solution, we were able to build stable films on PLLA substrates and studied the cytocompatibility of these surfaces.

EXPERIMENTAL

Materials

Poly(L-lactic acid) (PLLA) (M_v = 1.17 × 10^5 g/mol) was synthesized in our laboratory following the procedure reported previously.[23] PLLA powder was hot pressed at ~ 180°C under 10 MPa pressure (QLB-D Shenyang, China) into films of ~ 100 μm thickness, which were cut into 1 × 1 cm^2 pieces prior to use. Poly(allylamine hydrochloride) (PAH) (M_w = 7 × 10^4 g/mol) and poly(sodium 4-styrenesulfonate) (PSS) (M_w = 7 × 10^4 g/mol) were purchased from Aldrich (USA). The gelatin used in this study from bovine skin was purchased from Sigma (USA). All these chemicals were used as received without further purification. Water was purified using a Millipore Milli-Q system (18.2 MΩ).

Gelatin adsorption to the modified PLLA surfaces

The PLLA films coated with two PAH/PSS bilayers (PSS as the outermost layer) were prepared following the procedure reported in our previous article.[21] The modified films were then immersed in a gelatin aqueous solution at pH = 3, 5, and 7, respectively, for 20 min to allow the gelatin to adsorb, and then the films were removed, rinsed with water for 30 s and dried with a stream of nitrogen. The gelatin deposition was repeated until the desired number of layers of gelatin was obtained. The gelatin-modified PLLA films were dried under vacuum at room temperature for 24 h before analysis. To evaluate the stability of the gelatin multilayers, the PLLA substrates with gelatin layers assembled (eight layers for pH 3 and 7, and 2 layers for pH 5) were immersed in PBS (pH = 7.4) buffer for 24 h. The films were removed from the buffer, rinsed with sufficient water and dried with a stream of nitrogen. They were then vacuum-dried for 24 h before XPS measurement.

Surface analysis

X-ray photoelectron spectra (XPS) were recorded on a Thermo ESCALAB 250 spectrometer using Al Kα excitation (1486.6 eV) at a take-off angle of 90° between the plane of the sample surface and the entrance lens of the detector optics. The atomic concentrations were determined using sensitivity factors obtained from standard samples (C_{1s} : 0.25; O_{1s} : 0.66; N_{1s} : 0.42; S_{2p} : 0.54). The surface morphology was detected using an atomic force microscope (AFM) (SPA300, Seiko) operating in the tapping mode. Water contact angles were measured using a sessile drop technique on a KRUSS DSA1 v 1.80 Drop Shape Analyzer at room temperature with Milli-Q water as the probe fluid.

Cell culture

Human chondrocytes from human fetal articular cartilage were isolated and cultured following the method of Hong.[24] Full thickness articular cartilage from aborted fetuses (average gestational age 20 weeks) were collected from the First Hospital of Jilin University and used for cell isolation from the femoral head and patellar groove. The research was approved before the initiation of this study by the local Ethical Committee in compliance with Chinese laws. The chondrocytes were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% FBS (GIBCO), 1.0 × 10^5 U/L penicillin (Sigma), 50 mg/L L-ascorbic acid (Sigma), 100 mg/L Streptomycin (Sigma), and 10 m*M* HEPES (Sigma).

Cell attachment and cell morphology

Cell attachment and cell morphology on three groups, each corresponding to the adsorption pH of 3, 5, and 7, respectively, of 24-well tissue culture plates (NUNC) with modified and virgin PLLA films were studied. Each group contained four replicates of six samples: virgin PLLA, and PLLA with one, two, four, six, and eight adsorbed gelatin layers. These samples were sterilized under ultraviolet (UV) radiation for 30 min. The bottom surface of the well was fully covered by the sample. The human chondrocytes of second passage were harvested and seeded in the wells. Each well was inoculated with a total of 1 mL medium containing 2.5 × 10^4 cells. The cells were allowed to attach to the films undisturbed in a humidified incubator (37°C, 5% CO_2). After 24 h culture, unattached cells were removed by washing thoroughly with PBS and the attached cells were fixed for 10 min in 3% glutaraldehyde at room temperature. Samples were rinsed in PBS and then the adherent cells were dyed with one drop of Giemsa stain (Sigma) for 30 min, and washed with distilled water. Cell attachment and cell morphology were examined by a inverted microscope (TE2000-U, Nikon) under bright field working mode. For each

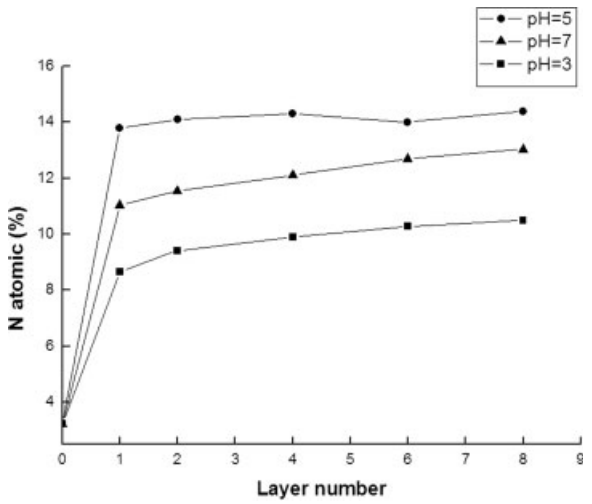

Figure 1 Nitrogen content measured by XPS of the gelatin multilayers assembled at pH = 3, 5, and 7. (Note the films assembled at pH = 3 and 7 are thinner than at pH = 5, as discussed in the text.)

specimen, nine pictures were taken with a Nikon DXM1200F digital camera. The cell number in each picture was counted, and the mean value of the nine counts for each specimen was presented. All experiments were done at least twice and the data were analyzed in the software Origin (version 7.0) to obtain mean values and standard deviation.

Cell viability

The viability of cells cultured on substrates was assessed by 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method,[25] which was used in our study to quantitatively assess the number of viable cells attached and grown on the substrate surfaces. Discs of samples were placed on the bottom of the wells of 96-well tissue culture plates (NUNC) sterilized under UV radiation for 30 min. Four parallel measurements were averaged for each sample. Briefly, after the chondrocytes (0.5×10^4 cells in 200 μL medium) were incubated in the 96-well tissue culture at 37°C and 5% CO_2 for 68 h, 20 μL of MTT (5 mg/mL in PBS) were added to each well, and the chondrocytes were incubated for additional 4 h. After removal of the medium, the converted dye was dissolved with 0.2 mL of 0.04*M* HCl in isopropanol. The solution (150 μL) in each well was mixed and transferred to another 96-well plate, and the absorbance values of the converted dye were measured at wavelength of 540 nm using a Thermo Electron MK3 μm, and the mean value of the four readings for each sample was used as the final result. The relative cell viability was defined as the ratio of the absorbance from the sample to that of the tissue culture polystyrene (TCPS) in the same culture medium.

RESULTS AND DISCUSSION

Gelatin adsorption to the modified PLLA films

Gelatin is a protein molecule that carries both negatively and positively charged groups, and its isoelectric point (IEP) is ~ 5.0. We have investigated the assembly of gelatin multilayers at pH 3, 5, and 7.[26] At pH 3, which is below the IEP of the gelatin, the gelatin molecules carry positive net charges, and are expected to strongly adsorb to the negatively charged substrate. On the other hand, at pH 7, which is above the IEP, the net charges the gelatin molecules carry are negative, and even though both the molecules and the substrate carry net charges of the same sign, there is still considerable adsorption. This probably is due to the interactions between the local positive charges on the gelatin molecules with the negative charges on the substrate, and the chains adopt more stretched conformation. At pH 5, the positive and negative charges each gelatin molecule carries are balanced, and the interactions between the charges of the gelatin molecule and the substrate result in the maximal adsorption at pH 5. The process is repeated, and continuous growth of gelatin multilayers is possible.[26] In the present work, we attempted to improve the cytocompatibility of PLLA using the gelatin multilayers. The PLLA films were first coated with two PAH/PSS bilayers so that the surface of the film carried negative charges.[21] This gelatin assembly process was repeated and the linear growth of the multilayers on the PLLA films was obtained when the pH of the gelatin solution was 3, 5, and 7, respectively. Figure 1 shows the surface nitrogen content obtained by XPS at 90° take-off angle. It can be seen that the substrate surface contained ~ 3% of nitrogen contributed by the PAH deposited on the PLLA. After the assembly of the first gelatin layer, the nitrogen content jumps to 9, 11,

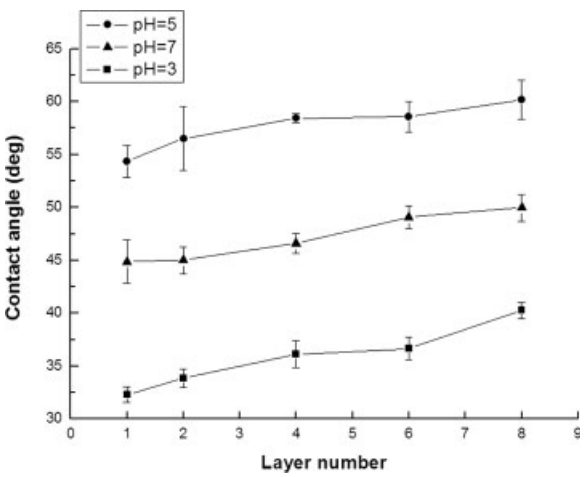

Figure 2 Water contact angle (sessile drop) on the different gelatin multilayers assembled on the PLLA substrates.

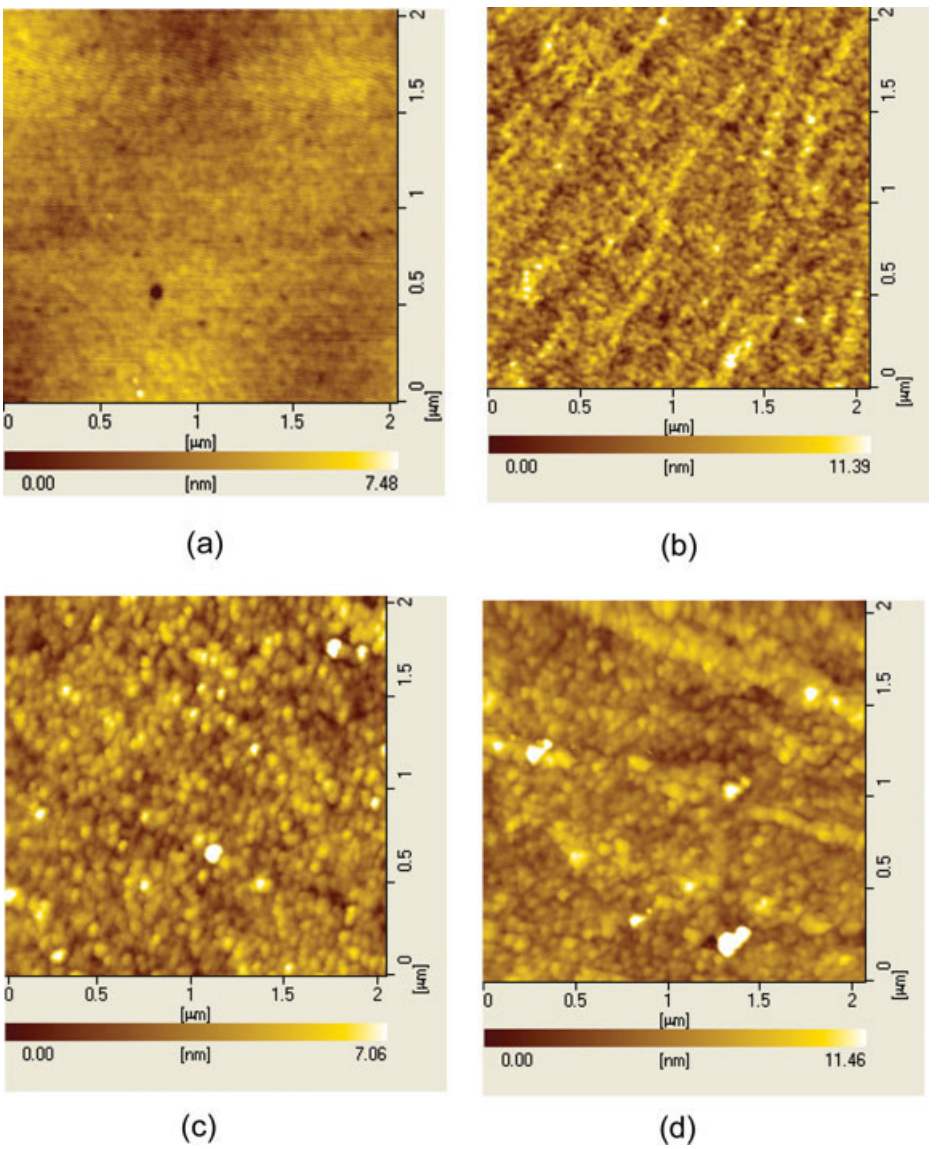

Figure 3 AFM height images of the surfaces of virgin PLLA (a), and gelatin multilayers assembled on the PLLA at pH = 3 (four layers) (b), pH = 5 (two layers) (c), and pH = 7 (four layers) (d). The rms roughness of the films a, b, c, and d is 0.93, 1.83, 1.31, and 2.02 nm, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and 14% for pH 3, 7, and 5, respectively, due to the rich nitrogen content in the gelatin molecules. This also indicates that the amount of gelatin assembled strongly depends on the pH of the gelatin solution, with the least of which at pH 3 and the most at pH 5, consistent with our results obtained by quartz crystal microbalance (QCM) technique.[26] The surface nitrogen content continues to increase with more gelatin layers assembled, with the exception at pH 5, where it remains roughly the same at $\sim$ 14%, which matches the nitrogen content in the gelatin examined by XPS.[21] This is because at pH 5 the amount of gelatin assembled in each cycle is high, and the thickness of the first layer is greater than the sampling depth of XPS at 90° take-off angle, which is $\sim$ 4 nm,[27,28] so that the contribution from the substrate to the XPS signal is negligible after the first gelatin layer is deposited. At pH 3 and 7, each gelatin layer assembled is much thinner,[26] and the XPS signal due to the substrate gradually decreases and the contribution from the gelatin increases with more layers deposited on top of the substrate.

Contact angle measurement is a more surface selective technique and can indicate the properties of the outermost layer. Figure 2 displays the water contact angles on the various gelatin-modified PLLA surfaces. It is clear that the water contact angle in all these cases is significantly lower than that of virgin PLLA, which is $\sim$ 73°,[21] indicating that the assembly of gelatin multilayers on PLLA substrate

TABLE I
Stability of the Gelatin Multilayers Assembled on the PLLA in the PBS Monitored by the Surface Nitrogen Content

pH at which gelatin was assembled	Surface N% of the film	Surface N% of the film after incubating in PBS (pH = 7.4) for 24 h
3	10.49	9.13
5	14.10	13.80
7	13.03	13.12

can significantly improve its hydrophilicity. More interestingly, the contact angle is dependent on the pH at which the gelatin multilayers is assembled. It is known that gelatin molecule is comprised of hydrophobic amino acids (such as glycine, praline, and alanine) as well as hydrophilic amino acids (hydroxyproline and serine for example).[29] Because of the different interactions involved at different pH in the assembly step, the adsorbed gelatin molecules adopt different conformation at the surface, and during the drying step of the assembly process, various amino acid residues may also rearrange near the surface to minimize the interfacial free energy,[22] resulting in very different surface hydrophilicity and contact angle.

In addition, the surface morphology of the gelatin multilayers was studied using atomic force microscopy (AFM), and the AFM topography images of virgin PLLA and representative gelatin-modified PLLA films are included in Figure 3. The surface of the virgin PLLA exhibits a smooth topography [Fig. 3(a)] and the surface roughness (root mean square, RMS) is 0.93 ± 0.08 nm. Five locations were tested in one image to obtain the RMS data. After gelatin layers are assembled on the PLLA substrate, the roughness of the film surface increases. For gelatin multilayers assembled at pH 3, 5, and 7, the RMS roughness is 1.83 ± 0.03, 1.31 ± 0.02, and 2.02 ± 0.02 nm, respectively. At pH 5 the gelatin molecules cover the substrate completely and more uniform, so it shows a relatively smooth surface [Fig. 3(c)] compared with the other two gelatin multilayer surfaces [Fig. 3(b,d)], as indicated by the lower RMS roughness.

Before testing the cytocompatibility of the gelatin-modified PLLA surface, the stability of the gelatin layers on the modified PLLA surfaces was evaluated. Table I lists the surface N% of the modified PLLA films after being exposed to PBS buffer at pH 7.4 for 24 h, the conditions to be used for cell attachment study. It can be seen that for the gelatin layers assembled at pH 5 and 7, the surface nitrogen content remain practically the same after exposure to the buffer solution, indicating that the multilayers are stable and intact. However for the layers deposited at pH 3, the surface N% decreases from 10.5% to 9.1%, showing that some of the gelatin molecules have desorbed. This supports our above argument on the contact angle data that the conformation of the gelatin molecules in the multilayer depend on the solution pH at which they are deposited. Apparently exposure of the gelatin layers to a solution with a pH very different from the one at which they were assembled induces changes in the conformation and charge distribution of the gelatin molecules adsorbed, resulting in the change in the interactions between the molecules and the substrate and hence partial desorption.[29,30] On the other hand, for the gelatin multilayers assembled at pH 3, even though some desorption had occurred in the buffer, the surface was still covered with gelatin layers based on the N%. Overall the XPS data in Table I show that the gelatin multilayers assembled without crosslinking treatment is stable for cell attachment study. This result is also consistent with Serizawa's finding on the stability of collagen layers assembled on QCM electrodes.[22]

Cell behavior analysis

Cell attachment and viability

The chondrocytes cell attachment results are reported as proportions of the number of cells attached to TCPS in the same culture medium. Figure 4 shows the chondrocytes cell attachment on the PLLA films modified with one, two, four, six, and eight layers of gelatin. It can be observed that for the PLLA modified at pH 3 and 7, the number of the cell attached increases with the number of the gelatin layers assembled, and reaches a plateau at four layers of gelatin. For the PLLA modified at pH 5, the cell attachment number reaches a plateau at two layers, which is 123%, higher than the plateau values for pH 3 and 7, which are about 110 and 118%,

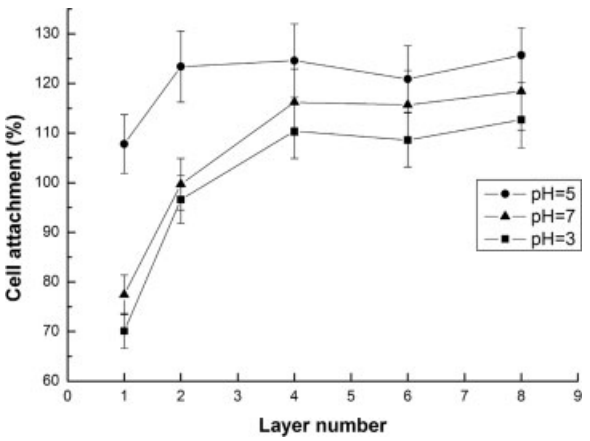

Figure 4 Attachment characteristics of chondrocytes on different substrates at 24 h with a seeding density of 2.5 × 10^4 cells/well.

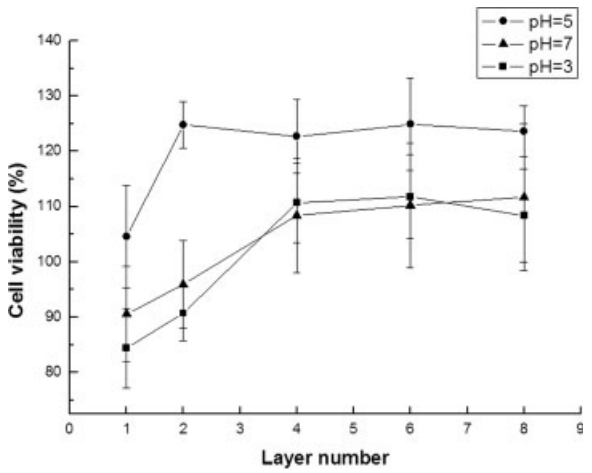

Figure 5 Cell viability of chondrocytes on different substrates at 72 h with a seeding density of 0.5×10^4 cells/well.

respectively. Figure 5 plots the cell viability of chondrocytes on the different samples over a period of 72 h (TCPS as 100%). Similar trends as that of the cell attachment are found. The cell viability reaches a plateau of 125% on the PLLA surface modified with two gelatin layers at pH 5, while the plateau values for the PLLA surface with four gelatin layers assembled at pH 3 and 7 are 111 and 108%, respectively. The cell attachment and cell viability numbers for the PLLA modified with gelatin multilayers were much higher than the corresponding numbers for virgin PLLA, which were 53 and 75%, respectively,[21] indicating that the deposition of gelatin multilayers on the PLLA surface can dramatically improve the cell compatibility of the PLLA. The numbers were even significantly higher than that for TCPS, showing that the gelatin multilayers were better substrate for the cells than TCPS. The results discussed earlier shows that the introduction of gelatin to the modified PLLA surfaces significantly increases the cell attachment and the cell viability, and the numbers are even better than that for TCPS.

Cell morphology

Figure 6 shows the optical micrographs of the chondrocytes on the different substrates. On the virgin PLLA substrate [Fig. 6(a)], the chondrocytes are

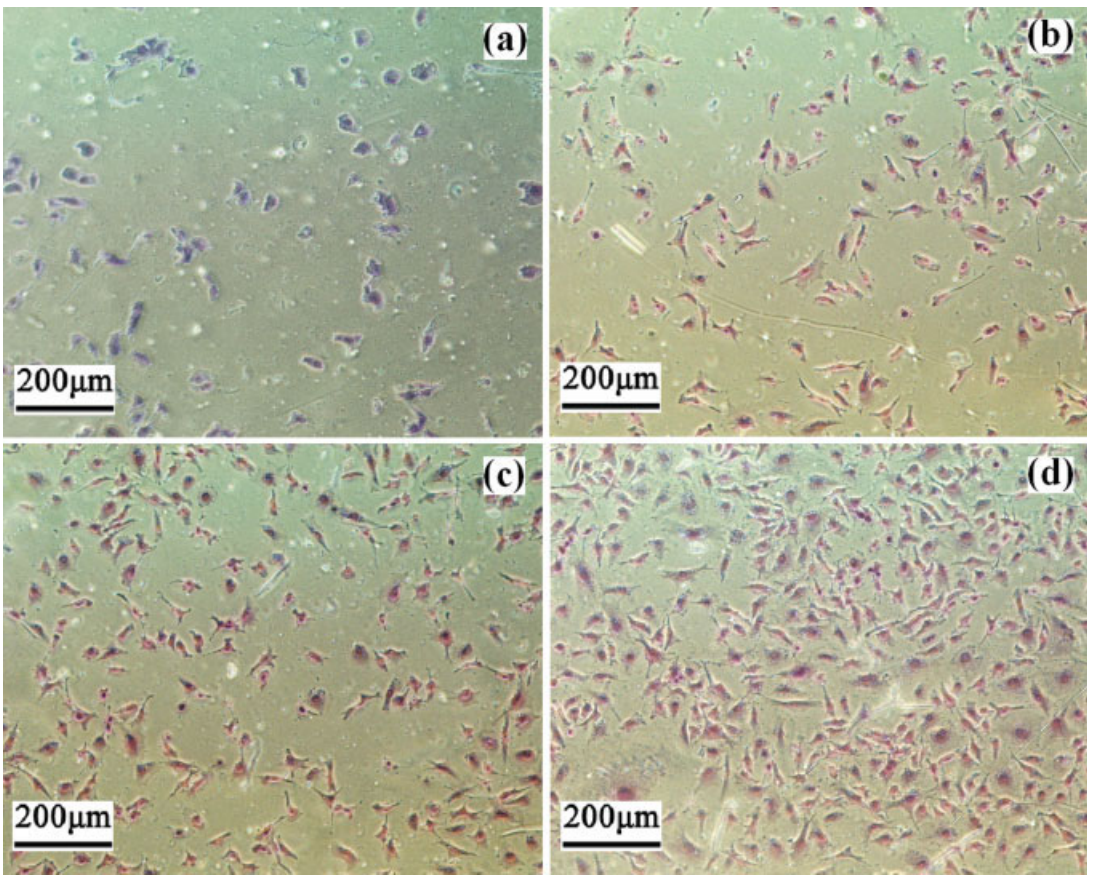

Figure 6 Chondrocyte morphology on the surfaces of virgin PLLA (a), and gelatin multilayers assembled on the PLLA at pH = 3 (four layers) (b), pH = 7 (four layer) (c), and pH = 5 (two layers) (d). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

small and round-shaped, and accumulates with each other to form cell clusters. With gelatin multilayers deposited on the PLLA surface, the chondrocytes exhibit good spreading morphology. Especially on the PLLA substrate with gelatin multilayers assembled at pH 5, the chondrocytes attach not only in large number but also distribute evenly. As discussed earlier, when assembled at pH = 5, the gelatin molecules not only cover the substrate completely but also the molecules distribute evenly. Furthermore, the gelatin is a natural extracellular matrix molecules that may be recognized by cells,[31,32] therefore the PLLA surface covered with gelatin multilayers exhibits improved cell compatibility as we observed experimentally, exceeding that of TCPS.

CONCLUSIONS

In this work we used the layer-by-layer deposition technique to assemble gelatin multilayers at different pH on PLLA surface. Because of the amphoteric nature of gelatin, its assembly behavior is strongly dependent on the pH of the gelatin solution. The assembly can be carried out at pH below, around, or above the IEP of the gelatin to yield multilayers. The layer thickness, the surface hydrophilicity, and the surface morphology of the gelatin multilayers can be controlled by adjusting the solution pH at which the multilayers are assembled. Since gelatin is a natural extracellular matrix molecules that may be recognized by cells, the modification of PLLA surface by assembly of gelatin multilayers can dramatically improve its cytocompatibility. Regardless of the pH at which the gelatin multilayers are deposited, the cell attachment and cell viability on the modified PLLA are much greater than that of virgin PLLA, and even significantly exceed that of TCPS. The cell compatibility properties of these gelatin-modified PLLAs strongly depend on the pH at which the multilayers are assembled. The multilayers deposited at around the IEP of the gelatin exhibit the best cytocompatibility performance, where the chondrocytes attach not only in large numbers but also distribute evenly in good spreading morphology. The cell compatibility performance of the gelatin-modified PLLA may be attributed to the characteristics of the multilayers controlled by the solution pH at which they are assembled. These findings indicate that gelatin multilayers can be assembled to modify the surface of hydrophobic biodegradable polyesters to improve their cell compatibility, and their performance can be controlled by adjusting the assembly pH. This may help the design and fabrication of better scaffolds for tissue engineering.

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