A novel technique to introduce free amino groups onto polyester scaffolds via aminolyzing the ester groups with diamine has been developed recently. Positively charged chitosan was then deposited onto the aminolyzed poly(1-lactic acid) (PLLA) membrane surface in a layer-by-layer assembly manner using poly(styrene sulfonate, sodium salt) (PSS) as a negatively charged polyelectrolyte. The layer-by-layer deposition process of PSS and chitosan was monitored by UV-vis absorbance spectroscopy, energy transfer by fluorescence spectroscopy, and advancing contact angle measurements. The existed chitosan obviously improved the cytocompatibility of PLLA to human endothelial cells. The cell attachment, activity, and proliferation on the PLLA membranes assembled with three or five bilayers of PSS/chitosan with chitosan as the outermost layer were better than those with one bilayer of PSS/chitosan or the control PLLA. The cells also showed morphology of an elongated shape with abundant cytoplasm, and a confluent cell layer was reached after being cultured for 4 days. Measurement of von Willebrand factor secreted by these endothelial cells (ECs) verified the endothelial function. Hence, better ECs compatible PLLA were produced.

Introduction

Biodegradable poly(lactic acid) has been one of the most attractive biomaterials being used as scaffolds in tissue engineering and has already been employed for clinical purposes because it degrades to natural metabolites and may be tailored to particular applications by manipulation of shape, porosity, degradation time, etc. However, the hydrophobicity and the poor cytocompatibility of the polylactides lead to the inefficiency of the scaffold in constructing a friendly interface with living cells. It is the surface of a biomaterial that first comes into contact with a living body; hence, the initial response of cells to the biomaterial mostly depends on the surface properties. Therefore, surface modification of polylactides to improve their cytocompatibility is necessary. Many works have been devoted to the surface modification of poly(1-lactic acid) (PLLA) toward improving its cytocompatibility, such as grafting polymerization, ozone oxidation, plasma modification, entrapment, coating of natural polymers, etc.1-5

Recently, we have developed a novel technique to introduce free amino groups onto polyester scaffold surfaces such as PLLA, polycaprolactone, poly(ester urethane), etc., via aminolyzing the ester groups with diamine. Cytocompatible components such as gelatin, chitosan, or collagen were further covalently immobilized through glutaraldehyde bridging. Improved cytocompatibility to human endothelial cells has been achieved.6,7 The introduction of the free amino groups on these polyester surfaces provides also the possibility to modify the polymer surface in a simpler manner, for example, layer-by-layer (LBL) assembly of charged species, because these aminolyzed polyesters can be used as polycationic substrata. This technique is more practical and important for scaffolds with irregular shape and inner structure, where traditional methods are generally unavailable. By this technique, many charged biopolymers such as negatively charged heparin, glycosaminoglycan (GAG), bovine serum albumin (BSA), chondroitin sulfate (CS), alginate sodium, hemoglobin as well as positively charged collagen, chitosan, gelatin, poly(1-lysine) and poly(ethylene imide) (PEI) can be adsorbed alternatively on the polyester surface (Scheme 1).

The technique of LBL assembly discovered by Decher and co-workers,8 has been employed in wide areas, such as in biosensors, separation or dialysis membranes, nonlinear optical devices, surface modification, etc., due to its simplic-
ity and versatility. The basic process of this technique consists of oppositely charged polyelectrolytes on the alternate adsorption. Sequential adsorptions of anionic and cationic polyelectrolytes allow the construction of multilayer films on the substrate. The process has many important advantages over other techniques for preparing multilayer thin films; for example, the assembly is based on spontaneous adsorptions and no stoichiometric control is necessary to maintain surface functionality, the assembled molecular films exhibit a much larger thermal and mechanical stability and can be prepared up to hundreds of layers, and so on. Most of all, the technique is not restricted to substrate and can be used for coating arbitrarily shaped objects, which is most important for surface modification of scaffolds in tissue engineering.

Herein we describe the surface modification of the biodegradable PLLA using LBL assembly technique after PLLA was aminolyzed with diamine. Poly(styrene sulfonate, sodium salt) (PSS) and chitosan were chosen as polyelectrolytes for alternate deposition. Chitosan, derived from chitin which is the main structural element of the cuticles of crab and shrimp, has been exploited for wide biomedical applications such as wound dressing, drug delivery system, and tissue engineering owing to nontoxicity, nonimmunogenicity, biocompatibility, and biodegradability. The cytocompatibility of chitosan to human umbilical vein endothelial cells (HUVECs) has been verified previously. Moreover, it is positively charged in solution below pH 7 and its charge density is high. The high charge density allows chitosan to be used as a polycation in a LBL assembly system, on which various polyanions can be adsorbed. In addition, PSS is a negatively charged polyelectrolyte which has been used widely in a LBL assembly system. Therefore, chitosan and PSS were chosen as the polymer combination because of both the cytocompatibility and the layer stability in culture medium (pH 7.4). This work concerns the construction of assembly films with PSS and chitosan on the aminolyzed PLLA membrane surface via LBL deposition. The culture of HUVECs in vitro showed that the cytocompatibility of the modified PLLA was improved obviously.

Experimental Section

Materials. Poly(styrene sulfonate, sodium salt) (PSS) ($M_w = 700000$) and chitosan (medium molecular weigh) were obtained from Aldrich and used as received. Poly(allylamine hydrochloride) (PAH, Aldrich) was labeled with fluorescein isothiocyanate (FITC, Sigma) at 4 °C for 48 h and then dialyzed with water for 4 weeks. Chitosan was labeled with rhodamine isothiocyanate (Rd) as follows: 0.5% (w/v) chitosan solution was stained by 2 mg/mL rhodamine B isothiocyanate at 4 °C for 48 h and then dialyzed with 0.05 M acetic acid for 4 weeks. PLLA was synthesized using the method described previously. All the water used in this paper was triple distilled.

Preparation of Aminolyzed PLLA Membrane. Aminolyzed PLLA membrane was fabricated via an aminolysis reaction on a PLLA surface, which has been described previously. In brief, the PLLA membrane was first prepared by dissolving 3 g of PLLA in 100 mL of distilled 1,4-dioxane and then cast onto a stainless steel plate. The solvent was evaporated at 35 °C for 24 h. The membrane was further dried under reduced pressure for another 24 h at 30 °C. The translucent PLLA membrane with a thickness of ~80 μm was yielded. The membrane was immersed in alcohol/water (1/1, v/v) solution for 2–3 h to clean oily dirt, rinsed with a large amount of water, and dried under reduced pressure for 24 h at 30 °C. The membrane was subsequently immersed in distilled 1,6-hexanediamine/propanol solution with a concentration of 0.06 g/mL for 4 min at 58 °C then was incubated in water for 24 h at room temperature to rinse off free 1,6-hexanediamine, and dried under reduced pressure at 30 °C to obtain the aminolyzed PLLA membrane.

LBL Deposition of PSS/Chitosan on the Aminolyzed PLLA Surface. The aminolyzed PLLA membrane was treated in 0.012 M HCl solution for 15 min at room temperature and washed with large amount of water. The membrane was subsequently incubated in PSS solution (1.0 mg/mL) for 20 min to adsorb a layer of PSS and then rinsed with water three times. In the next step, the surface charge of the membrane was reversed by the adsorption of a layer of chitosan (1.0 mg/mL, pH 3.8), followed by rinsing with 0.3% acetic solution at first and then rinsing with water. After the desired number of layers had been deposited, the membranes were dried at 30 °C under reduced pressure.

Samples for Energy Transfer Study by Fluorescence Spectroscopy. After the first layer of PSS was adsorbed, the aminolyzed PLLA membrane was immersed in 0.5 mg/mL FITC–PAH for 20 min, followed by washing with a large amount of water. Then n bilayers of PSS and chitosan were adsorbed, keeping Rd–chitosan as the outermost layer for measurement of the energy transfer from FITC–PAH to Rd–chitosan by fluorescence spectroscopy.

Human Umbilical Vein Endothelial Cell Culture. The endothelial cells were isolated from human umbilical cord veins of a newborn baby with 1.0 mg/mL collagenase (type I, Sigma)/phosphate buffer solution (PBS, pH 7.4) for 20–25 min at room temperature. The isolated HUVECs were routinely seeded on the beds prelaid with control or modified PLLA membranes as well as on tissue culture polystyrene (TCP) (Nunc, Denmark) as control. The HUVECs were incubated in a culture medium consisting of 20% v/v fetal calf serum (FCS, Sijiqing Biotech. Co., China) and 80% RPMI1640 (Gibcobrl Co.) supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin in humidified air containing 5% CO₂ at 37 °C. After incubation for 24 h, the culture medium was changed and then changed every 2 days. The HUVECs were fixed with 2.5% glutaraldehyde for 30 min for observation of cell morphology under scanning electron microscopy (SEM) after being cultured for 4 days. The cell attachment and proliferation ratio were averaged from five parallel measurements at 12 h and 4 days, respectively, by trypsinization of the HUVECs and counting the cell number under a hemocytometer. The cell proliferation ratio was defined as ($N_f - N_i$)/$N_i$, where $N_i$ and $N_f$ represent the cell number per well at 12 h and 4 days, respectively. The cell activity was measured using meth-
ylthiazolotetrazolium (MTT) method according to refs 21 and 22. The absorbance is recorded at a wavelength of 490 nm.

**Measurement of Released von Willebrand Factor.** Secretion of the von Willebrand factor (vWF) was measured in the supernatant of the cultured HUVECs by vWF enzyme linked immunosorbent assay (ELISA, Sun Diagnostics, Shanghai, China). Cell cultures were washed with the culture medium without serum after the HUVECs were cultured for 72 h, followed by 2 h of incubation in culture medium with 20% FCS. Subsequently, the supernatant medium was collected and centrifuged (10 min, 400g) for vWF measurement using vWF ELISA. Spectrophotometric readings were performed at 490 nm. Final results were obtained by comparison with a standard curve constructed using dilutions of normal plasma. One milliliter of normal plasma was assumed to contain \( \sim 10 \mu g \) of vWF (1 unit).

**Characterization.** A UV–vis spectrum was measured on a UV–visible spectrophotometer (CARY 100 BIO, America). Water contact angle was measured at room temperature using a sessile drop technique on a DSA10-MK2 contact angle measuring system from Krüss. Advancing contact angle was measured to reveal the surface wettability. Before the measurement, all the samples were dried at 30 °C under reduced pressure to a constant weight and stored in ambient air for 1 day. Fluorescence spectroscopy was recorded on a Fluorolog Spex device. Excitation was set at 488 nm for the energy transfer. The scanning force microscopy (SFM) image was obtained on a scanning probe microscope (SPA400, Seiko) in a tapping mode. The cell morphology was observed under SEM (Stereoscan 260, Cambridge).

**Results and Discussion**

**Monitoring the LBL Deposition Process.** Exploiting Rd–chitosan as the positively charged component, UV–vis spectroscopy was used to monitor the layer growth of PSS/chitosan films (Figure 1a). The aminolyzed PLLA membrane was first acidified with HCl solution and then alternately immersed in PSS and Rd–chitosan solutions to fabricate PSS/chitosan multilayers. The spectra of the multilayers exhibited a maximum absorbance at \( \sim 564 \text{ nm} \) arising from the rhodamine groups. The intensity of the absorbance increased linearly with the growth of PSS/chitosan bilayers until four bilayers. After that, the increase of absorbance became slow (Figure 1b).

The surface wettability of the sequentially layered PSS and chitosan adsorbed PLLA membrane confirmed also the occurrence of LBL assembly. The measurement of contact angle has been widely employed to diagnose the surface chemistry of synthesized surfaces such as a self-assembled monolayer. The surface wettability measured by an advancing contact angle is controlled primarily by the polymer’s outermost surface layer if the layer is uniform. Thus, it is possible to monitor the deposition process by determining how surface wettability changes in a LBL manner. In our case, samples having 1–20 layers of the PSS/chitosan were built up. Samples with an odd number of layers have PSS as the outermost layer, whereas samples with an even number of layers have chitosan as the outermost layer. Figure 2 shows the alteration of advancing contact angle with the change of the outermost layer between PSS and chitosan on the aminolyzed PLLA membrane. The advancing contact angle of the uncoated PLLA membrane was determined to be 85.3 ± 2°. The contact angles displayed a distinct oscillation as the outermost layer changed from PSS to chitosan, demonstrating the more hydrophilicity of samples containing PSS.
as the outermost layer than that with chitosan as the outermost layer. However, the same outermost layer did not present the same contact angle. There was some fluctuation in a range of about 3–5°. It may be caused by the layer interpenetration between neighboring PSS and chitosan films where the segments of the underlying layer are able to influence the surface hydrophilicity. This is likely the main reason for the slow growth of layer thickness after assembling some layers due to the weakening of surface charge. It is interesting that the contact angle decreased from 85.3 ± 2° (the aminolyzed PLLA) to 69.9 ± 1.5° even when just one PSS layer was deposited on the aminolyzed PLLA membrane; i.e., a single layer of these adsorbed polyelectrolytes can greatly influence the surface hydrophilicity.

It is known that the surface morphology of biomaterials has also a big influence on cell attachment, proliferation, and function in addition to the surface chemistry. SFM measurements displayed that the PLLA membrane surface became rougher and had some pores with a size of several tens to hundreds of nanometers after it was aminolyzed with 1,6-hexanediamine (Figure 3b). It is understood that the assembly films grow homogeneously when the size of the roughness and the pores is far larger than the molecular size of PSS or chitosan. Figure 3c showed that one bilayer of PSS/chitosan (chitosan as the outermost layer) has little influence on the surface morphology of PLLA. After four bilayers are deposited, the surface became a little bit smoother (Figure 3d). This result was consistent with the previous conclusion that the deposition of polyelectrolyte layers has the ability to smooth-out a rough surface. Moreover, the pores on the surface became smaller as well. It is worth noting that this smoothing effect occurred in a scale of micrometers. Deposition of PSS/chitosan multilayers had less influence on the morphology in a nanometer scale as shown in Figure 4. Here, PSS/chitosan multilayers were assembled on a freshly cleaved mica (negatively charged) instead of PLLA. The SFM images indicated that some aggregates of chitosan appeared on the first layer. After seven layers were deposited, almost the same surface roughness (rms) was found, i.e., 0.51 and 0.50 nm for one and seven layers, respectively. This proves again that PSS and chitosan can be deposited alternately in a LBL assembly manner. The images also reveal that there existed defects on a one PSS layer deposited surface, which influence both the surface wettability and the cytocompatibility (see below).

Decher considered that polyelectrolytes can be assembled to form multilayers on planar solid supports, and the multilayers can be controlled at least in one dimension along
the layer normal. Therefore, fluorescence energy transfer measurement has been widely employed to study the distance between the donor layer and the acceptor layer. Figure 5 displays the energy transfer from FITC-PAH to Rd-chitosan. The energy transfer process is one type of the photophysical processes where the electronic energy transfer takes place from the excited donor molecules to the acceptor molecules. Because the maximum emission of FITC, i.e., 540 nm (Figure 5a), is in the range of the excitation of rhodamine, FITC-PAH and Rd-chitosan were assembled on the aminolyzed PLLA surface with PSS/chitosan combinations in between. The red shift of the FITC emission can be attributed to its binding with PAH. Parts b-d of Figure 5 reveal the efficient energy transfer from the donor (FITC-PAH) to the acceptor (Rd-chitosan). The donor fluorescence was quenched completely, and the acceptor fluorescence was sensitized in return by excitation energy transfer. The transfer efficiency reached the maximum when the assembly films between the donor and the acceptor had three layers and then decreased. There are several reasons that may lead to the decrease of energy transfer, for example, the distance increase between the donor and the acceptor, and the smaller amount of Rd-chitosan being adsorbed after assembly of some layers (Figure 1b). From the strong energy transfer, one can also deduce that the layer thickness increment should be very minimal at the assembled pH value and the absence of salt in the assembly procedure. It should be noted that the layer thickness of the outermost layer differs from the inner layers. Material release somewhat occurs from the top layer when a next layer is assembled. Together with nonlinear increment of layer thickness, one can explain why the fluorescence intensity from Rd-chitosan, the outermost layer, increased at the first several layers.

In conclusion, PSS and chitosan was successfully assembled on the aminolyzed PLLA membrane surface in a layer-by-layer manner. The alteration of the surface chemistry had a big influence on its properties, thus intriguing the cytocompatibility of the PLLA membranes.

The Cell Compatibility of the Modified PLLA Membranes. The chitosan steadily existing on PLLA membrane surface provides the possibility of improving the membrane’s cytocompatibility. The endothelial cell culture results showed that the cell attachment ratio of the modified PLLA membranes with one, three, or five bilayers (chitosan as the outermost layer) was not obviously changed compared with the control PLLA (Figure 6). However, the cell activity and the proliferation ratio were improved obviously. The cell activity on PLLA assembled with three bilayers of PSS and chitosan was even better than that on TCPS. Observed under SEM after being cultured for 4 days, the cells on the PLLA coated with one bilayer did not spread well and accumulated to form a cluster. There were also scarcely cells on the control PLLA membrane. The cells, however, on the PLLA coated with three or five bilayers spread very well, displayed the morphology of an elongated shape with abundant cytoplasm, and reached confluence (Figure 7). Hence, one can conclude that the endothelial cells presented higher activity and proliferation and better morphology on the PLLA modified with three or five bilayers of PSS/chitosan than those on the one bilayer modified PLLA with the same outermost layer (chitosan). On the other hand, deposition of only one PSS layer on the aminolyzed PLLA resulted in lower cell activity and proliferation compared with one.
bilayer of PSS/chitosan. Relative activity of 85.2% and proliferation ratio of 14.6% were measured for the one PSS layer only, while 100% and 18.2 were obtained for the one PSS/chitosan bilayer, respectively. These results demonstrate...
that the negatively charged PSS is not effective as chitosan in enhancement of cytocompatibility due to either the charge repulsion or disadvantageous surface chemistry. As pointed out in the LBL deposition part, there may exist defects on the one bilayer-modified PLLA surface that were not fully covered by the next chitosan layer because of the thin layer thickness. This would result in an overall decrease of the cytocompatibility compared with three or five bilayer modified PLLA, where the assembly defects can be largely diminished. It is worth noting that the existence of assembly defects in the first several layers is quite normal for the LBL technique.

Von Willebrand factor (vWF) is an adhesive glycoprotein synthesized exclusively in endothelial cells and megakaryocytes. Endothelial vWF affects the platelet adhesion and aggregation, blood coagulation, and fibrinolysis. Therefore, one can investigate the HUVECs function through the measurement of the vWF secretion by the cells growing on those modified PLLA and on TCPS. Figure 6 (last column, open bar) shows that all cells seeded on TCPS or the modified PLLA membranes secreted vWF and maintained the endothelial function after HUVECs were cultured in vitro for 72 h. Compared with the control PLLA, the HUVECs grown on PLLA coated with three or five bilayers of PSS and chitosan secreted more vWF. Considering the comprehensive results of cell attachment, activity, proliferation, morphology, and vWF secretion, the PLLA membranes deposited with three or five bilayers of PSS and chitosan with chitosan as the outermost layer has better cytocompatibility.

Conclusion

We have shown here that the biocompatible chitosan has been successfully deposited onto the aminolysed PLLA membrane surface in a layer-by-layer assembly manner. The cytocompatibility of the chitosan deposited PLLA membrane to human endothelial cells was obviously improved. Considering the comprehensive results of cell attachment, activity, proliferation, morphology, and vWF secretion, the PLLA membranes deposited with three or five bilayers of PSS and chitosan with chitosan as the outermost layer has better cytocompatibility. In conclusion, herein we provided a practical and simple technique, i.e., the aminolysis and the following layer-by-layer assembly, through which a cytocompatible polymeric material can be easily fabricated. Assembly of other charged biocompatible components such as cell growth factors and differentiation factors to further modulate cell growth and functionality can be also expected.

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References and Notes

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