

Improved Biological Characteristics of Poly(L-Lactic Acid) Electrospun Membrane by Incorporation of Multiwalled Carbon Nanotubes/Hydroxyapatite Nanoparticles

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Significant effort has been devoted to fabricating various biomaterials to satisfy specific clinical requirements. In this study, we developed a new type of guided tissue regeneration (GTR) membrane by electrospinning a suspension consisting of poly(L-lactic acid), multiwalled carbon nanotubes, and hydroxyapatite (PLLA/MWNTs/HA). MWNTs/HA nanoparticles were uniformly dispersed in the membranes, and the degradation characteristics were far improved. Cytologic research revealed that the PLLA/MWNTs/HA membrane enhanced the adhesion and proliferation of periodontal ligament cells (PDLs) by 30% and inhibited the adhesion and proliferation of gingival epithelial cells by 30% also, compared with the control group. After PDLs were seeded into the PLLA/MWNTs/HA membrane, cell/membrane composites were implanted into the leg muscle pouches of immunodeficient mice. Histologic examinations showed that PDLs attached on the membranes functioned well in vivo. This new type of membrane shows excellent dual biological functions and satisfied the requirement of the GTR technique successfully in spite of a monolayer structure. Compared with other GTR membranes on sale or in research, the membrane can simplify the manufacturing process, reduce the fabrication cost, and avoid possible mistakes in clinical application. Moreover, it does not need to be taken out after surgery. PLLA/MWNTs/HA membranes have shown great potential for GTR and tissue engineering.

1. Introduction

Biomedical materials play a key role in modern medicine. Material scientists have tried various techniques and different components to fabricate biomedical materials according to specific clinical requirements. For example, guided tissue regeneration (GTR) membranes have recently attracted extensive interest because a considerable number of teeth could be preserved from extraction for periodontal diseases through GTR therapy.^{1–8} According to the classical studies of Nyman et al.,⁹ only periodontal ligament cells (PDLs) have potential for the regeneration of periodontium. The GTR membrane functions as a barrier to deflect the gingival tissue away from the root surface and create a protected space over the bone defect that allows the remaining PDLs to proliferate on the root surface. Hence, the ideal GTR membrane should have selectivity for adhesion and proliferation of different cells. In consideration of this selectivity, GTR membranes were designed as a two-layer or three-layer structure, or even as a compositional gradient in previous studies.^{2–8} The layer that faced the defect would accelerate the periodontal tissue regeneration, while the other layer inhibits the epithelium down growth. However, there are two main disadvantages in such designs: (1) significantly increasing the difficulty and complexity of manufacturing

procedures, which means an increase in costs, time, and errors, and (2) introducing possible mistakes by clinicians that could confuse the surfaces of the membrane. So, we have to find out the proper technique and ingredients for the fabrication of a new type of membrane which is composed of a monolayer but possesses dual biological functions.

The electrospinning technique was invented in 1934 and could produce polymer fibers from nanometer to micrometer size in diameter.¹⁰ Recently, it has been introduced to tissue engineering.¹¹ Numerous materials have been spun into nanofibers, by static electric forces, which possess a three-dimensional porous structure microscopically and are shown as membranes macroscopically. It was reported that such membranes showed good biocompatibility.^{12–17}

Poly(L-lactic acid) (PLLA) has been widely investigated in tissue engineering because of its good biocompatibility.^{18–21} However, several disadvantages were also recognized.²² The local acidic environment during hydrolysis was known to be toxic for cells. Also, the mechanical properties of highly porous scaffolds made of the PLLA membrane were poor, which limits their use in bone–tissue regeneration. Some scaffolds were quickly broken down even before the formation of new bone was finished due to too high of a degradation rate, which affects the clinical results.

Recently, carbon nanotubes (CNTs) have become well-known to have a high potential in biological applications due in part to their unique mechanical, physical, and chemical properties.^{23,24} In vivo studies have confirmed good biocompatibilities of multiwalled carbon nanotubes (MWNTs) with various cells, especially for osteoblast cells.^{25–28} Researchers have discovered

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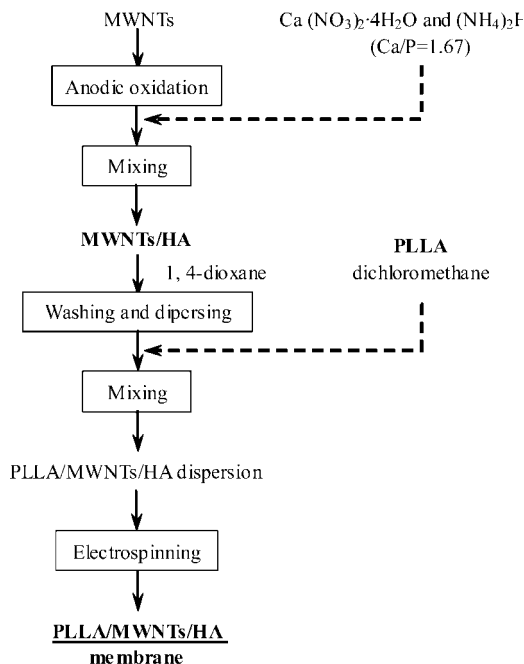


Figure 1. Schematic diagram of PLLA/MWNTs/HA membrane fabrication process.

that hydroxyapatite (HA) improved the biological properties of PLLA, and nanoscaled HA crystallites were well combined on the MWNTs' surface.^{29,30} Moreover, it is remarkable that Webster's group^{27,31} suggested that the higher surface energy and nanostructured surface would enhance the adhesion of osteoblast cells and inhibit the adhesion of osteoblast competitive cells.

Therefore, we employed an electrospinning technique in the incorporation of multiwalled carbon nanotubes/hydroxyapatite (MWNTs/HA) nanoparticles into PLLA and the fabrication of a composite membrane to satisfy the specific requirements of GTR. To our knowledge, this is the first trial on the fabrication of a biomedical membrane which is composed of a monolayer but possesses dual biological functions. The characteristics of this new type of membrane were investigated using scanning electron microscopy (SEM), energy dispersive X-ray (EDX), Raman spectra, and *in vitro* degradation tests. The adhesion and proliferation of PDLCs and gingival epithelial cells (GECs) on this membrane were examined by morphology observation, cell counting, and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Animal experiments were carried out to test the function of PDLCs attached on the membrane *in vivo*.

2. Experimental Section

Fabrication of PLLA, PLLA/HA, and PLLA/MWNTs/HA Membranes. PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes were fabricated using the electrospinning technique described in previous literature.^{32,33} Figure 1 shows a schematic diagram of the fabrication progress of the PLLA/MWNTs/HA membrane. The semicrystalline PLLA was purchased from Shandong Key Laboratory of Medical Polymer Materials, having an average molecular weight of 2.62×10^5 . MWNTs (purity, >95%; diameter, 20–40 nm) was purchased from Shenzhen Nanotechnologies Co. Ltd. MWNTs were first modified by anodic oxidation; then, MWNTs/HA nanoparticles (3 wt % MWNTs) were *in situ* synthesized by a wet method with $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and $(\text{NH}_4)_2\text{HPO}_4$ (Ca/P = 1.67) by using an ultrasonic sonicator. The nanoparticles prepared were washed with 1,4-dioxane

repeatedly to remove the water and dispersed again in 1,4-dioxane to form a suspension. Dichloromethane and PLLA particles were added to the suspension until a weight ratio of MWNTs/HA to PLLA of 1:9 was achieved. The suspension was kept in a 50 °C oven for 12 h to get a mixed solution. Before electrospinning, ultrasonic stirring of the solution was maintained for 1 h.

After investigation of the processing parameters which have influenced the electrospinning process, the optimum electrospinning parameters were fixed as follows: voltage = 10 kV, injection rate = 1.0 mL h^{-1} , PLLA concentration = 6 wt %, distance = 100 mm, inner diameter of spinneret = 0.7 mm.

Characteristics Examination of Electrospun Membranes. The electrospun PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes were gold-coated using sputter coating to observe the surface morphology by SEM (Hitachi S-4700 FEG-SEM). An EDX analysis (Oxford INCA) was carried out to confirm the existence of P and Ca elements in the membranes. A Raman spectrum analysis (RM2000, Renishaw, U.K.; 632.8 nm, He–Ne Laser machines) was conducted to confirm the existence of MWNTs in the PLLA/MWNTs/HA composite membrane. Electrospun membranes were cut into rectangles ($20 \times 20 \times 0.05 \text{ mm}$) for an *in vitro* degradation test. The rectangles were measured for their initial weight (W_0), placed in closed bottles containing 15 mL of a phosphate buffer solution (PBS, pH 7.4), and incubated *in vitro* at 37 °C for 1–7 weeks. Then, the weights of the degraded rectangles (W_t) were measured. The following characteristics were investigated: (a) the mass loss percentage (W_L) of the rectangles was calculated using $W_L \% = (W_0 - W_t)/W_0 \times 100\%$, and (b) the pH value at the end of each incubation period was measured using a pH meter (LIDA PHS-3C, China).

Human PDLCs Culture on Membranes. PDLCs were obtained from healthy teeth extracted for orthodontic reasons. Informed consent was obtained from the patients before the extractions. The midmost of the periodontal ligament attached to the root surface was removed with a curet, cut into small pieces, and cultured in a tissue culture medium. An α -modification of Eagle's medium (α -MEM; GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum and antibiotics was used to culture the cells. After reaching 80% confluence, the cells were passaged with 0.25% trypsin/0.02% ethylene diaminetetraacetic acid (EDTA). The cells between the third and the fifth passages were used in the following studies. PDLCs were harvested with 0.25% trypsin/0.02% EDTA and transferred to an osteogenic differentiation medium, α -MEM containing 10% fetal bovine serum and antibiotics supplemented with 10 nM dexamethasone, 10 mM β -glycero-phosphate, and 50 mg L^{-1} of ascorbic acid. After 7 days of culturing, PDLCs were transfected by the recombinant adenovirus containing green fluorescent protein at a ratio of 1:20 for 2 h. A flow cytometer was used to determine the ratio of transfection. Membranes were cut into a round shape, 10 mm in diameter, to fully cover the bottom of wells of tissue culture plates, and then were fixed. PDLCs were seeded into three kinds of membranes and tissue culture polystyrene (TCPS; control group) at a density of 5000 cells/well and cultured in an osteogenic differentiation medium. After 1, 3, 5, and 7 days of culturing, cells were detached with 0.25% trypsin/0.02% EDTA at 37 °C for 10 min, repeatedly pipetted, centrifuged, and resuspended. The number of suspended cells was counted using a hemocytometer under an inverted fluorescence microscope. Five random fields were counted per substrate. The number of viable cells was also determined with a MTT assay, which has previously been described.^{34,35} After PDLCs were cultured on membranes for 7 days, the specimens were observed with a confocal laser microscope and SEM.

Human GECs Culture on Membranes. Cells were obtained from the gingival tissue of systemic healthy individuals removed during periodontal surgery. Informed consent was obtained from the patients before the surgeries. The explants were treated with 6 mg mL^{-1} of Dispase (Sigma Chemical, MO) in HEPES buffered saline at 4 °C to separate the epithelium from the underlying fibrous connective tissue. The epithelium was then removed and incubated at 37 °C in 0.25%

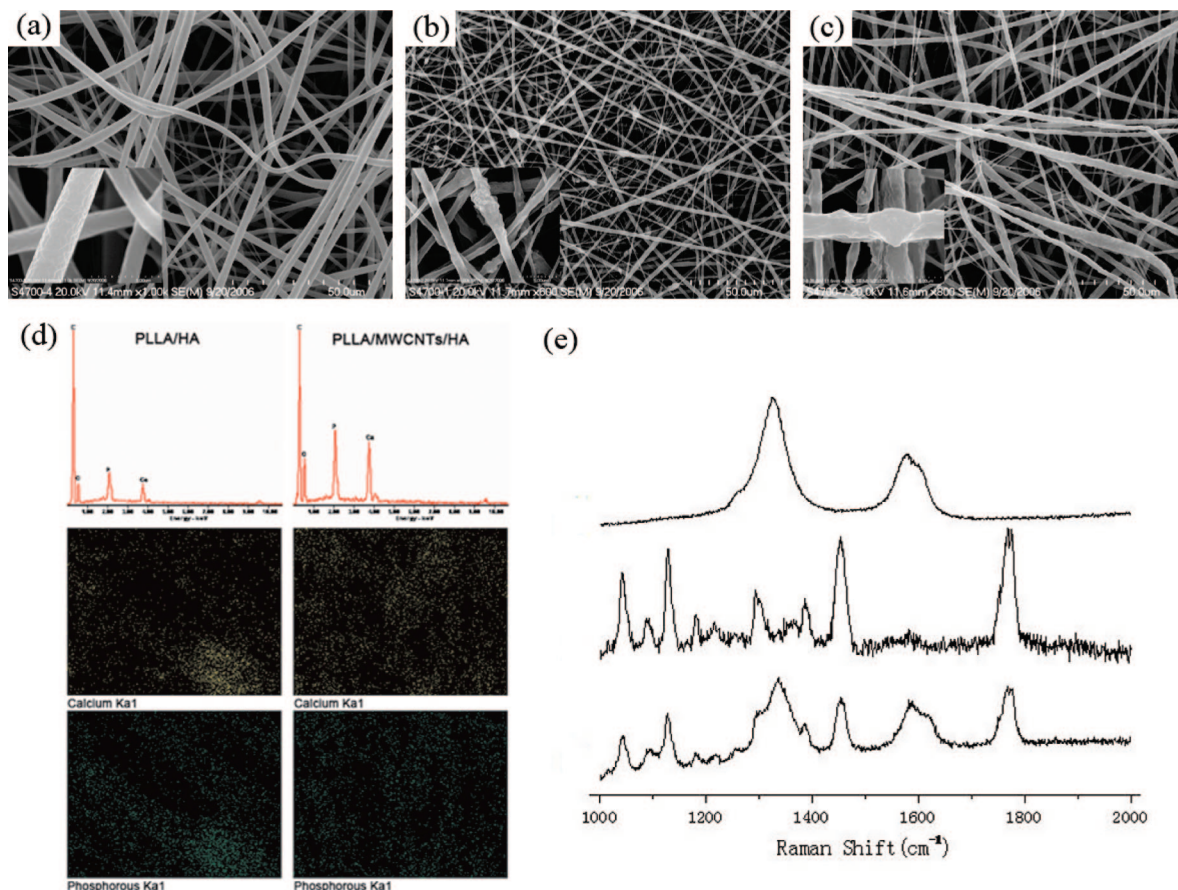


Figure 2. Characteristics of three kinds of membranes: a, b, and c represent typical SEM images of PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes, respectively; d represents the EDX mapping of PLLA/HA (left) and PLLA/MWNTs/HA (right) membranes for Ca (middle) and P (lower) elements; e represents Raman spectra of MWNTs (upper), PLLA/HA membrane (middle), and PLLA/MWNTs/HA membranes (lower).

trypsin/0.02% EDTA for 10 min and repeatedly pipetted to prepare a single-cell suspension. The cell pellets were collected and resuspended in α -MEM containing 10% fetal bovine serum and antibiotics supplemented with 10 ng mL^{-1} of epithelial growth factor (EGF; Sigma Chemical, MO). After reaching 80% confluence, the cells were passaged with 0.25% trypsin/0.02% EDTA. The cells between the third and the fifth passages were used in the following studies. Human GECs were harvested with 0.25% trypsin/0.02% EDTA and transfected by the recombinant adenovirus containing green fluorescent protein at a ratio of 1:20 for 2 h. GECs were seeded on three kinds of membranes and TCPS (control group) at a density of 5000 cells/well and cultured in α -MEM containing 10% fetal bovine serum and antibiotics supplemented with 10 ng mL^{-1} of EGF. After 1, 3, 5, and 7 days of culturing, cell counting and a MTT assay were carried out as previously described for PDLCs. All data were compared using a one-way ANOVA analysis with statistical significance at $P < 0.05$.

In Vivo Implantation of PLLA/MWNTs/HA Membranes with PDLCs. A total of 10 immunodeficient mice that were 8 weeks old were used in this study. Animal selection and management, surgical protocol, and preparation followed the routines approved by the Department of Laboratory Animal Science, Health Science Center, Peking University, China. PDLCs were cultured in an osteogenic differentiation medium for 7 days and then seeded into PLLA/MWNTs/HA membranes and cultured in an osteogenic differentiation medium for 48 h. Under general anesthesia, cell/membrane composites were implanted into one side of leg muscle pouches. After 4 weeks, all mice were sacrificed. Block sections, including the experimental sites, were removed at sacrifice. The sections were rinsed in sterile saline and fixed in 10% buffered formalin for 7 days and prepared for paraffin sections of $5 \mu\text{m}$ to conduct histologic examinations, including hematoxylin/

eosin staining, alizarin red staining for calcium deposits, and immunohistochemical staining for osteocalcin (OC). Goat antihuman antibody against OC (Santa Cruz, CA) was used as the primary antibody.

3. Results and Discussion

HA and MWNTs/HA nanoparticles were synthesized by the wet method.³² HA nanoparticles were well-combined on the surfaces of anodic oxidized MWNTs. The needlelike HA particles were about 40–70 nm in length and 15 nm in diameter, which were values very similar to the apatite in living bone (40–60 nm in length and 20 nm in diameter).

PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes were fabricated successfully. The SEM images of PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes are shown in Figure 2a–c. The diameters of three kinds of electrospun fibers were about $1 \mu\text{m}$, and three-dimensional porous structures were obtained. It is notable that the incorporation of HA or MWNTs/HA nanoparticles resulted in fibers more irregular in diameter and more beaded in morphology than pure PLLA fibers. EDX analysis shows the presence of P and Ca elements in membranes, in Figure 2d. P and Ca elements were detected and distributed uniformly in PLLA/HA and PLLA/MWNTs/HA membranes. The Raman spectra in Figure 2e also confirmed the presence of MWNTs in the PLLA/MWNTs/HA membrane. A crystalline carbon apex and an amorphous carbon apex, which were the two unique apices of MWNTs, were found in the PLLA/MWNTs/HA membrane.

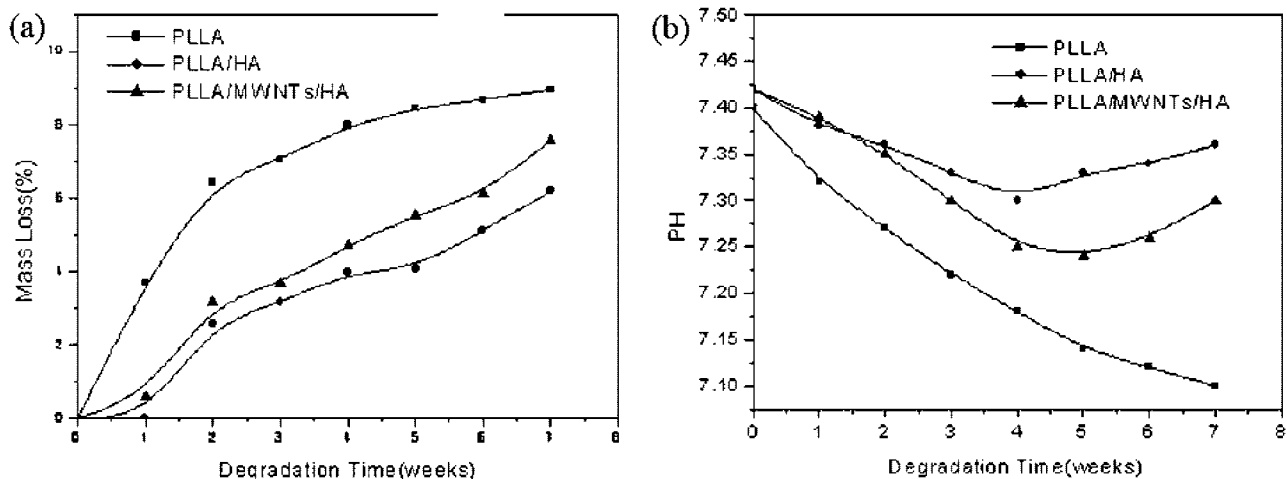


Figure 3. Characteristics of three kinds of membranes during in vitro degradation: a and b represent the changes of mass loss and pH, respectively.

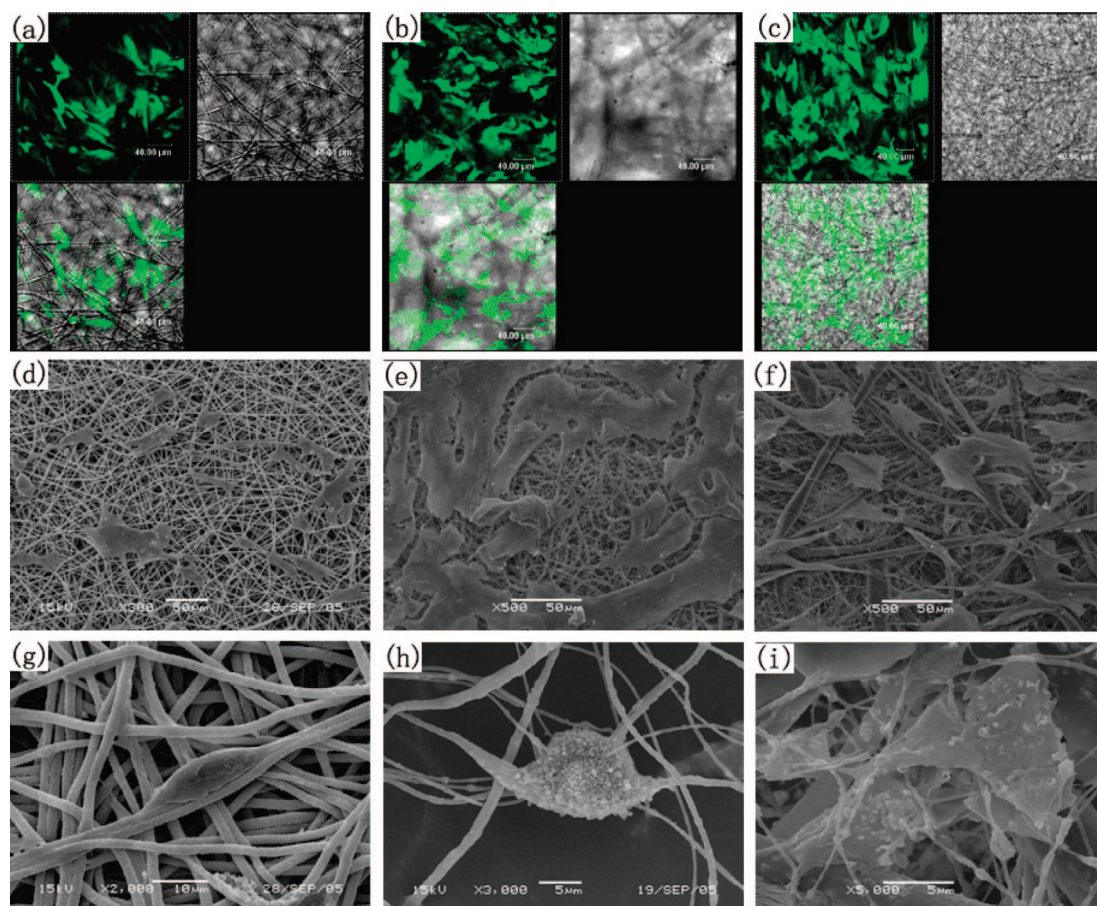


Figure 4. Morphology observation of PDLCs cultured on three kinds of membranes: a, b, and c represent confocal laser microscope images of PDLCs on PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes, respectively; d, e, and f represent SEM images of PDLCs on PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes, respectively, at low magnification, and g, h, and i represent them at high magnification.

The results of the in vitro degradation tests are shown in Figure 3, including mass loss and pH change. The weights of all membranes were continuously decreased due to the degradation of fibers. However, the incorporation of HA or MWNTs/HA slowed down the rate of mass loss, especially showing the lag phase in the early stage. PLLA is a hydrolytically biodegradable polymer and is easily degraded when immersed in a neutral aqueous medium such as PBS (pH 7.4).^{2,36} The ester bonds in the backbone chain of the polymer randomly decomposed and generated carboxyl groups, which autocatalytically

accelerated the degradation reaction and resulted in a fast decrease in the molecular weight. Therefore, the mass loss of the membrane increased as the degradation time increased.^{37,38} The incorporation of HA or MWNTs/HA nanoparticles has slowed down the degradation of PLLA because the dissolving alkaline HA particles act as a physical barrier, which can block off the entry of water. Figure 3b shows the pH change of solutions which were produced from the degradation of PLLA, PLLA/HA, and PLLA/MWNTs/HA membranes. For PLLA membranes, the pH of the solution decreased continuously

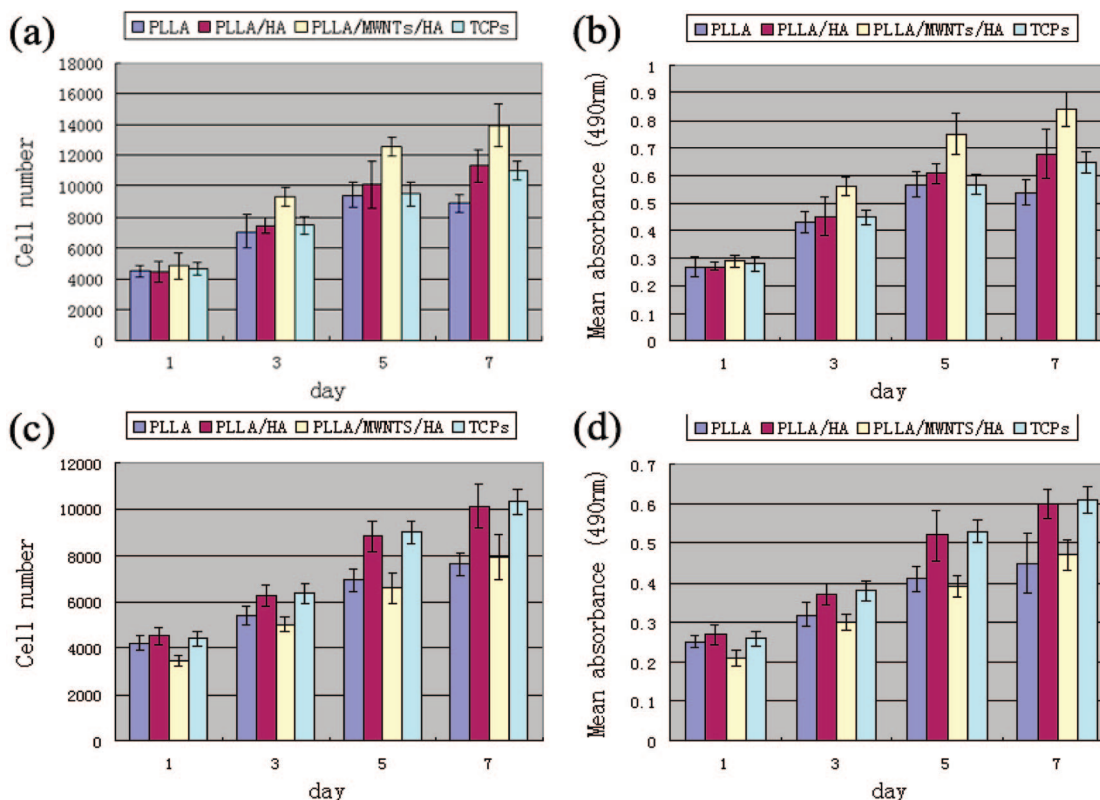


Figure 5. Effect of three kinds of membranes on the adhesion and proliferation of PDLCs and GECs: a and c represent the cell counting, and b and d represent the MTT assay of PDLCs and GECs, respectively. Standard deviations are shown as bars.

during the degradation time and reached 7.10 after 7 weeks. On the other hand, those for PLLA/HA and PLLA/MWNTs/HA membranes decreased from 7.42 to 7.30 and 7.25, respectively, at 4 weeks but slowly increased after 4 weeks. Other studies also reported similar results.^{39,40} This was due to the HA releasing OH^- after degradation, which neutralized the acid resulting from PLLA degradation.²² It was indicated that the incorporation of HA or MWNTs/HA could prevent PLLA membranes from rapid decomposition and a rapid decrease of pH in the surrounding environment during the degradation.

The response of human PDLCs seeded into three kinds of membranes was evaluated by morphology observation under a confocal laser scanning microscope and SEM and is shown in Figure 4. Cells were transfected with the recombinant adenovirus containing green fluorescent protein for direct observation under a fluorescence microscope. The transfection ratio was 92%, as determined by a flow cytometer. Figure 4a–c showed the confocal laser microscope images of transfected PDLCs, which were cultured on membranes for 7 days. It was observed that PDLCs attached to the three kinds of membranes with green fluorescence. SEM images (Figure 4d–i) show the attachment of PDLCs onto three membranes. The density of PDLCs on PLLA/MWNTs/HA membranes was the highest among those of the three membranes. The cells spread over the membrane fibers, linked with fibers by cytoplasmic extensions. PDLCs were more actively extended on the PLLA/MWNTs/HA membrane than on the PLLA and PLLA/HA membranes during the same culture time. More granulates, which was an implication of mineralization, were observed on the surface of attached cells on PLLA/HA and PLLA/MWNTs/HA membranes. Figure 4i shows that PDLCs had passed through the pores of fibers, entered into the inner space, and showed good status under the fibers. It is suggested that the PLLA/MWNTs/HA membrane is very suitable for human PDLC adhesion and proliferation.

The adhesion and proliferation of human PDLCs and GECs on membranes were examined by cell counting and a MTT assay, and the results are shown in Figure 5. The number of cells was directly counted under an inverted fluorescence microscope, while the number of viable cells was measured by a MTT assay. The principle mechanism of the MTT assay is that metabolically active cells react with a tetrazolium salt in the MTT agent to produce a soluble formazan dye which can be absorbed at a wavelength of 490 nm.^{34,35} The cell counting and MTT assay results showed a similar trend. The PDLC number was similar at 1 day for the three test and control groups, while the most active proliferation was observed on the PLLA/MWNTs/HA composite membrane, which was almost 3 times that of the initial seeding cells and 30% larger than that of the PLLA/HA membrane or control group for 7 days of culturing ($P < 0.05$). However, fewer human GECs were observed to attach on the membrane in the PLLA/MWNTs/HA group than in the other groups at 1 day ($P < 0.05$). During 7 days, the cell number in the PLLA/MWNTs/HA group was always lower than that in the PLLA/HA and control groups ($P < 0.05$). From the cellular response to membranes, we can conclude that the PLLA/MWNTs/HA membrane of the single structure may selectively promote the adhesion and proliferation of PDLCs and inhibit the adhesion and proliferation of GECs compared with the control group. Although there has been lots of research concerning the influence of CNT on the behavior of different cells,^{41,42} the exact mechanism is still unclear. According to the research of Webster's group,^{26,27} the higher surface energy and nanostructure of the material surface would increase the adhesion of osteoblast cells and decrease the adhesion of osteoblast competitive cell lines.

All animals survived without any local or general complications until the scheduled experimental time. Figure 6 shows representative microscopy photographs of paraffin sections that

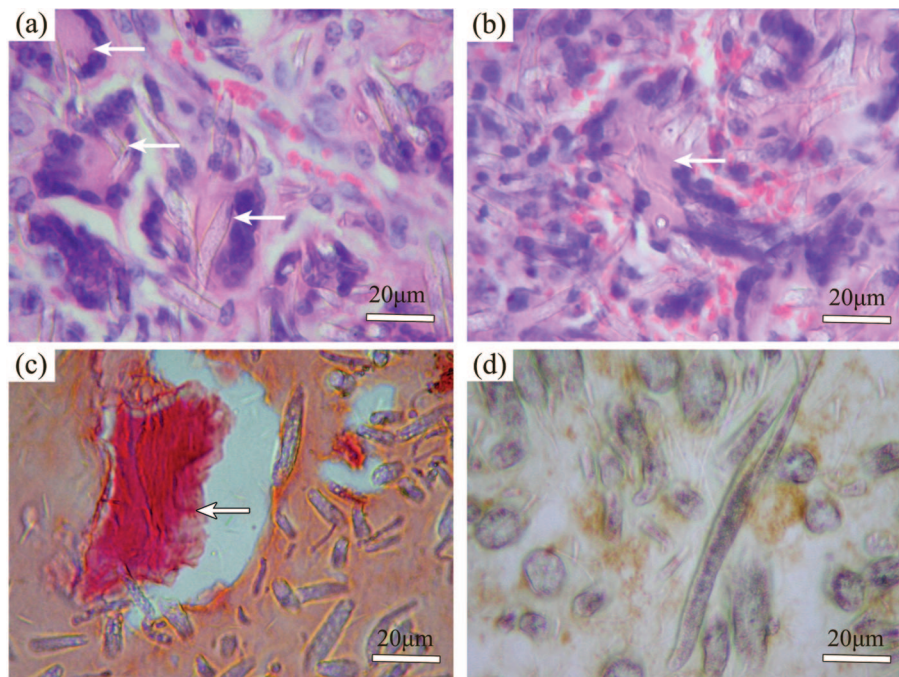


Figure 6. Histologic examination of cell/membrane composites implanted into immunodeficient mice: a–c show new-formed bonelike tissues in round or irregular shape (white arrow), and osteoblast-like cells were well arranged around bonelike tissues. Abundant blood vessels were found in the implanted area. In c, alizarin red staining confirmed calcium deposits in new-formed bonelike tissues. In d, osteocalcin, which was stained in brown, was detected in the cytoplasm and outside the cells.

underwent HE and immunohistochemical staining for osteocalcin. It is observed that residual electrospun fibers could be clearly identified; no obvious inflammation was found in the implant areas. Bonelike tissues were formed with a round or irregular shape and were stained into homogeneous pink by hematoxylin/eosin, and osteoblast-like cells were well-arranged around the bonelike tissues. Calcium deposits were confirmed in new-formed bonelike tissue by alizarin red staining. It is notable that abundant blood vessels were grown into the new-formed tissues. Osteocalcin, which was stained in brown, was detected in the cytoplasm and outside the cells. The results indicated that PLLA/MWNTs/HA membranes were of good biocompatibility *in vivo* during the 4-week period, and human PDLCs seemed to function well on the membrane. Three-dimensional porous structures of the implanted membranes may be helpful to guide the ingrowth of the blood vessels, which plays an important role in tissue regeneration.

Lundgren et al.¹ concluded that a bioresorbable GTR device designed to prevent epithelial downgrowth along the barrier surface has a higher potential to promote new attachment formation than a device which does not fulfill this purpose. Previous products or research usually utilized complex structures to achieve this function. Commercially available GTR membranes such as Bio-Gide (collagen type I and III; Geistlich Biomaterials, Wolhusen, Switzerland) and Guidor (PLLA; Guidor AB, Huddinge, Sweden) employed a two-layer design.^{3–6} Owen et al.⁷ reported a membrane design that had a surface topography that could inhibit epithelial cell migration and proliferation on one side and a topography that guided osteoblast migration to a desired area. A three-layer nanocarbonated hydroxyapatite/collagen/PLGA composite membrane was also fabricated for guided tissue regeneration and showed an enhanced protein content of osteoblastic cells cultured on the membrane.⁸ Kim et al.² utilized a functional biomedical membrane with a composition gradient between gelatin and HA, for use in guided hard/soft tissue regeneration.

Although more extensive work is required to investigate the exact mechanism, the unique biologic properties of the PLLA/MWNTs/HA membrane have shown great potential for application in tissue engineering. Compared with existing materials of similar function, this new type of material simplifies the manufacturing process, lowers the fabrication cost, and avoids possible mistakes in clinical application.

The GTR membranes were divided into resorbable and nonresorbable categories. Resorbable materials achieved wide attention because a second surgery procedure could be avoided. With respect to PLLA/MWNTs/HA membranes, PLLA is absorbable, while HA is a major inorganic component of bone and has shown good osteo-conductivity and bone-bonding ability, although it can not be degraded in the human body. Zanello et al.²⁸ proposed that CNTs can be used not only to stimulate bone regeneration but also to serve a permanent mechanical role. Therefore, PLLA/MWNTs/HA membranes need not be taken out after healing, which lightens the burden of patients.

Complex tissues, like periodontium, are composed of various types of cells; each cell plays a unique role when functioning as a whole or repairing after damage. The design of biomedical materials for a specific body area should take into account the detailed biological properties of composed cells or adjacent cells. The responses of various cells to the materials should be understood well before clinical application. In the current study, a new type of material was designed according to the previous report and was successfully fabricated. After further investigation of the physical, chemical, and biological characteristics, it was demonstrated to be a promising biomaterial for GTR. The most important result of the current study is the derivation of special consideration to design biomedical materials for the regeneration of complex tissues.

4. Conclusions

The PLLA/MWNTs/HA membrane could be successfully fabricated through an electrospinning technique. This new type of biomaterial was composed of a single layer but showed dual biological functions, promoting the adhesion and proliferation of human PDLs and inhibiting the adhesion and proliferation of GECs compared with the control group, which can satisfy the requirements of the GTR technique perfectly. In vivo experiments showed that human PDLs functioned well on the membrane. Compared with other membranes on sale or in research, it could simplify the manufacturing process, reduce the fabrication cost, and avoid possible mistakes in clinical application. Moreover, it need not be taken out after surgery. The PLLA/MWNTs/HA membrane has shown great potential for GTR.

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