ELECTROSPUN PLLA/MWNT/HA MEMBRANE FOR GUIDED TISSUE REGENERATION

Xiaoping Yang[a,], Yunhua Yu[a] , Peng Li[a], Jigui Lu[b], Seungkon Ryu[b], Xuliang Deng[c], Qi Ma[c], Hong Yan[c]*

[a] The Key Lab. of Beijing City on Preparation and Processing of Novel Polymer, Beijing University of Chemistry and Technology, Beijing 100029, China

[b] Dept. of Chem. Eng., Chungnam National University, Daejeon 305-764, Korea

[c] School and Hospital of Stomatology, Peking University, Beijing 100081, China

Abstract

A new type of single layered poly (L-lactic acid)/multi-walled carbon nanotubes/hydroxyapatite (PLLA/MWNTs/HA) membrane was prepared by electrospinning method to use in guided tissue regeneration (GTR). MWNTs/HA nanoparticles were uniformly dispersed in the membranes. PLLA/MWNTs/HA membrane significantly enhanced the adhesion and proliferation of periodontal ligament cells (PDLCs) and inhibited the adhesion and proliferation of gingival epithelial cells (GECs). This new type membrane shows excellent dual biological functions and satisfied the requirement of GTR technique successfully, in spite of single layered structure in comparison with conventional double or triple layered membranes.

Keywords: Multi-walled carbon nanotubes (MWNT); Electrospinning; Periodontal ligament cells; Gingival epithelial cells; Guided tissue regeneration

1. Introduction

GTR membranes have recently attracted extensive interest because a considerable number of teeth could be preserved from extraction in periodontal diseases through GTR therapy [1-4]. 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 PDLCs to proliferate on the root surface. Hence, the ideal GTR membrane should have selectivity for adhesion and proliferation of different cells. Considering this selectivity, previous GTR membranes were designed as a two-layer or three-layer structure [2-4]. The layer 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 manufacture procedures, which means the increase of costs, time and errors and 2) bringing the possible mistakes of clinicians to confuse the surfaces of the membrane. So a new type of monolayer GTR membranes with dual biological functions should be developed.

The electrospinning technique could produce numerous nanofiber membranes, which have been introduced to tissue engineering and showed good biocompatibility [5-7]. PLLA has been widely investigated in tissue engineering because of its good biocompatibility [8-10]. However, local acidic environment during hydrolysis was toxic for cells, and the poor mechanical properties of highly porous PLLA scaffolds limit their use in bone-tissue regeneration [11]. Recently, carbon nanotubes (CNTs) are well known to have a high potential in biological applications due in part to their unique mechanical, physical, and chemical properties [12]. In vivo studies have confirmed good biocompatibilities of MWNTs with various cells, especially for osteoblast cells [13,14]. Researchers have studied that nano-scale hydorxyapatite(HA) crystallites could be well combined on MWNTs surface to improve the biological properties of PLLA, enhance the adhesion of osteoblast cells and inhibit the adhesion of osteoblast competitive cells [15,16],

In this paper, MWNTs/HA nanoparticles were incorporated into PLLA to fabricate the PLLA/ MWNTs/HA monolayer nanofiber membranes by using electrospinning technique to satisfy the specific requirements of GTR. Characteristics of the new type membranes were investigated using SEM, TEM, EDX, Raman spectra, and in vitro degradation test. The adhesion and proliferation of PDLCs and GECs on this membrane were examined by morphology observation, cell counting and MTT assay.

2. Experimental

2.1 Fabrication of PLLA/MWNTs/HA membranes

PLLA/MWNTs/HA membranes were fabricated by electrospinning technique under the conditions: voltage = 10 kV, injection rate = 1.0 mL h^{-1}, distance = 100 mm, inner diameter of spinneret = 0.7 mm. MWNTs were first modified by anodic oxidation, then MWNTs/HA nanoparticles (3 wt% MWNTs) were in situ synthesized by wet method with $Ca(NO_3)_2 \cdot 4H_2O$ and $(NH_4)_2HPO_4$ (Ca/P=1.67) by using ultrasonic vibrator. The resultant nanoparticles were washed with 1, 4-dioxane repeatedly to remove the water and then dispersed again in 1, 4-dioxane to form a suspension. Dichloromethane and PLLA particles were added to the suspension till the weight ratio of MWNTs/HA to PLLA approached to 1:9. Then the mixed solution was electrospun to fabricate the PLLA/MWNTs/HA membranes.

2.2 Characterization of electrospun membranes

The surface morphologies of the electrospun nanofiber membranes were observed by SEM (Cambridge 250MK3). Energy Dispersion X-ray (EDX) analysis (Oxford INCA) was carried out to confirm the existence of phosphorus (P) and calcium (Ca) elements in the membranes. Raman spectrum analysis (RM2000, Renishaw UK, 632.8 nm, He-Ne Laser machines) was conducted to confirm the existence of MWNTs in the PLLA/MWNTs/HA hybrid membrane.

2.3 Human PDLCs culture on electrospun membranes

PDLCs were obtained from the healthy teeth extracted for orthodontic reasons. Informed consents were obtained from the patients before the extractions. The midmost of the periodontal ligament attached to the root surface was removed with a curette, cut into small pieces and cultured in tissue culture medium. Alpha-modification of Eagle's medium (α-MEM, GIBCO BRL, Grand Island, NY, USA) containing 10% fetal bovine serum and antibiotics were used to culture the cells. After reaching 80% confluence, the cells were passaged with 0.25% trypsin/0.02% ethylene diaminotetraacetic acid (EDTA). The cells between the 3rd passage and the 5th passage were used in the following studies. PDLCs were harvested with 0.25% trypsin/0.02% EDTA and transferred to osteogenic differentiation medium, α-MEM containing 10% fetal bovine serum and antibiotics supplemented with 100 nM dexamethasone, 10 mM of β-glycero-phosphate and 50 mg mL^{-1} of ascorbic acid. After 7 days culture, PDLCs were transfected by the recombinant adenovirus containing green fluorescent protein at the ratio of 1 : 20 for 2 hours. Flow cytometer was used to determine the ratio of transfection. Membranes were cut into round shape, 10 mm in diameter, to fully cover the bottom of wells of tissue culture plates, and then fixed. PDLCs were seeded into 3 kinds of membranes and tissue culture polystyrene (TCPS, control group) at a density of 5,000 cells/well and cultured in osteogenic differentiation medium. The PDLCs were detached with 0.25% trypsin/0.02% EDTA after 1, 3, 5 and 7 days culture, centrifuged, and resuspended. The number of suspended cells were counted by hemocytometer under inverted fluorescence microscope. Five random fields were counted per substrate. The number of viable cells were also determined by 3-(4,5-dimethylithiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay, which has previously been described.[17,18]. Data were compared using one-way ANOVA analysis

with statistical significance at $p < 0.05$. After PDLCs were cultured on membranes for 7 days, the specimens were observed by confocal laser microscope and SEM.

2.4 Human GECs culture on membranes

Cells were obtained from gingival tissue of systemic healthy individuals removed during periodontal surgery. Informed consents were obtained from the patients before surgeries. The explants were treated with 6 mg mL^{-1} of dispase (Sigma Chemical, MO, USA) 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% 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} epithelial growth factor (EGF, Sigma Chemical, MO, USA). After reaching 80% confluence, the cells were passaged with 0.25% trypsin/0.02% EDTA. The cells between the 3rd passage and the 5th passage 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 the ratio of 1 : 20 for 2 hours. GECs were seeded on 3 kinds of nanofibrous membranes and tissue culture polystyrene (TCPS, control group) at a density of 5,000 cells/well and cultured in α-MEM containing 10% fetal bovine serum and antibiotics supplemented with 10 ng mL^{-1} EGF. After 1, 3, 5 and 7 days culture, cells counting and MTT assay were determined as previously described for PDLCs. All data were compared using one-way ANOVA analysis with statistical significance at $p < 0.05$.

3. Results and Discussion

The SEM images of PLLA, PLLA/HA and PLLA/MWNTs/HA membranes are shown in Figure 1(a)-(f). The diameters of three kinds of electrospun fibers were about 1 μm and three-dimensional porous structures were obtained. It is notable that incorporation of HA or MWNTs/HA nanoparticles into PLLA resulted in irregularity in diameter and more beaded morphology. EDX result in Figure1(g) showed the phosphorous(P) and calcium(Ca) elements distributed uniformly in the PLLA/MWNTs/HA membranes. Raman spectra in Figure 1(h) were also confirmed the presence of MWNTs in PLLA/MWNTs/HA membrane. Crystalline carbon apex and amorphous carbon apex, which were the two unique apices of MWNTs, were found in the PLLA/MWNTs/HA membrane.

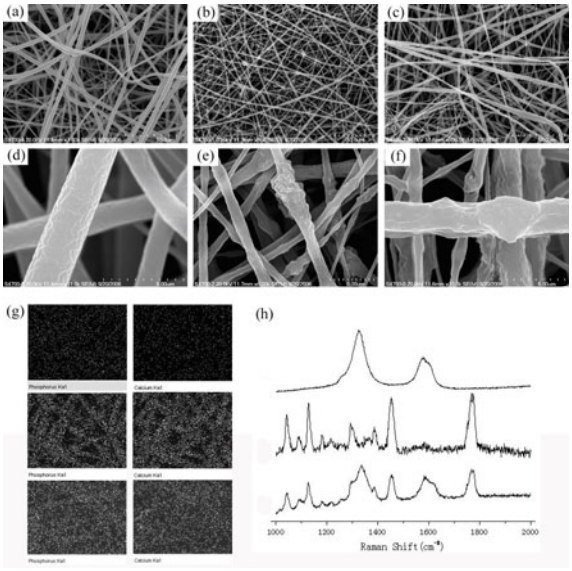

Figure 1. Characteristics of three kind of membranes: (a), (b), and (c) represent typical SEM images of PLLA, PLLA/HA, and PLLA/MWNTs/HA membrane respectively in low magnification, (d), (e), and (f) in high magnification; (g) represents EDX mapping of PLLA (upper), PLLA/HA (middle), and PLLA/MWNTs/HA (lower) membrane for P (left) and Ca (right) element; (h) represents Raman spectra of MWCNTs (upper), PLLA/HA membrane(middle), and PLLA/MWCNTs/HA membrane (lower).

Morphology observation of PDLCs cultured on PLLA, PLLA/HA and PLLA/MWCNTs/HA membrane for 7 days under confocal laser scanning microscope and SEM are showed in Figure 2. Cells were transfected with the recombinant adenovirus, containing green fluorescent protein for direct observation under fluorescence microscope. The transfection ratio was 92 % determined by flow cytometer. Figure 2 (a)-(c) showed the confocal laser microscope images of transfected PDLCs, which were cultured on membranes It was observed that PDLCs attached on the three kinds of membranes with green fluorescence. SEM images (Fig. 5(d)-(i)) have shown the attachment of PDLCs onto three membranes. PDLCs density on PLLA/MWNTs/HA membranes was the highest among the three membranes. The cells had spreaded over the membrane fibers, linked with fibers by cytoplasmic extensions. PDLCs were more actively extended on the PLLA/MWNTs/HA membrane than PLLA and PLLA/HA membranes during the same culture time, suggesting that the PLLA/MWNTs/HA membrane is very promisable for human PDLCs adhesion and proliferation.

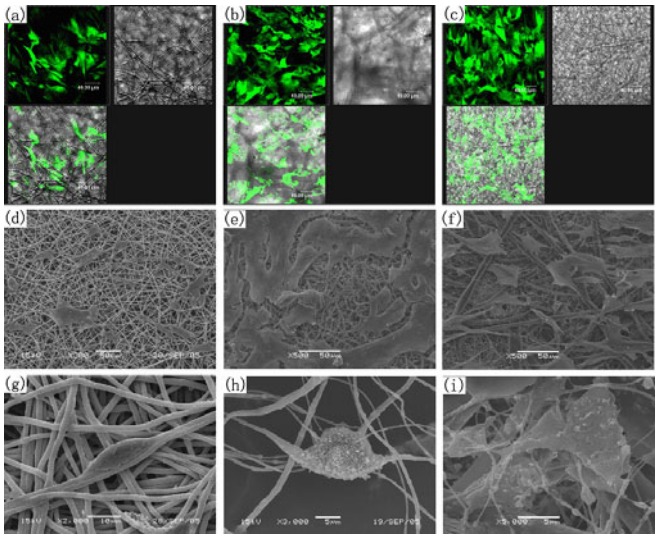

Figure 2. Morphology observation of PDLCs cultured on three kind of membranes: (a), (b), and (c) represent confocal laser microscope images of PDLCs on PLLA, PLLA/HA, and PLLA/MWNTs/HA membrane, respectively, (d), (e), and (f) represent SEM images of PDLCs on PLLA, PLLA/HA, and PLLA/MWNTs/HA membrane, respectively at low magnification: (g), (h), and (i) at high magnification.

Adhesion and proliferation of human PDLCs and GECs on membranes were examined by cell counting and MTT assay [17,18], and the results are shown in Figure 3. Cell counting and MTT assay results showed a similar trend. PDLCs number was similar at 1 day for the three tests and control groups, while the most active proliferation was observed on the PLLA/MWNTs/HA hybrid membrane, which was almost three times of initial seeding cells and 30 % larger than that of PLLA/HA membrane

or control group for 7 days culture ($p < 0.05$). However, less human GECs were observed to attach on the membrane in PLLA/MWNTs/HA group than other groups at 1 day ($p < 0.05$). In 7 days, the cell number in PLLA/MWNTs/HA group was always lower than PLLA/HA and control group ($p < 0.05$). From cellular response to membranes, we can conclude that PLLA/MWNTs/HA membrane of single structure may selectively promote the adhesion and proliferation of PDLCs and inhibit the adhesion and proliferation of GECs.

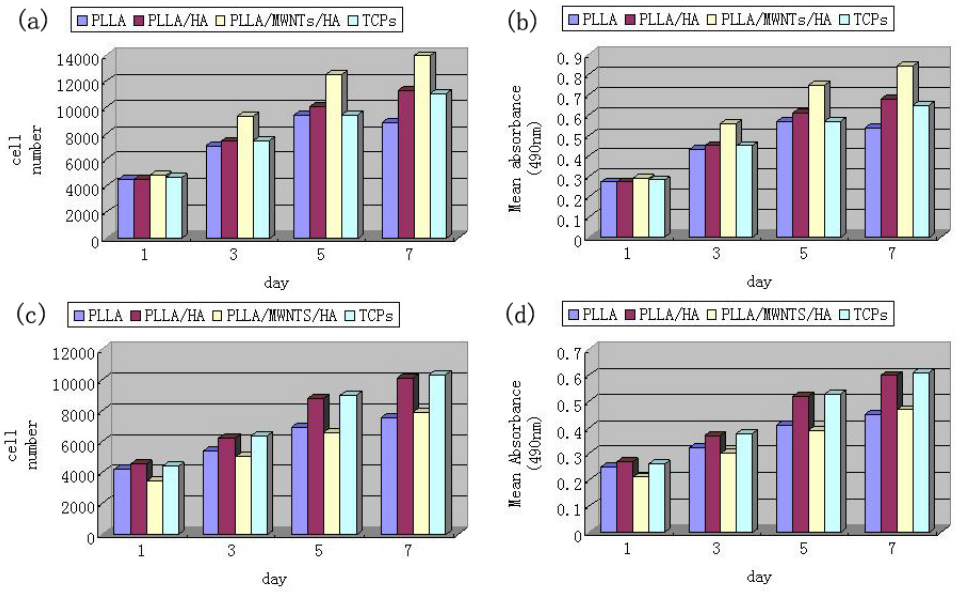

Figure 3. The effect of three kind of membranes on the attachment and proliferation of PDLCs and GECs: (a) and (c) represent the cell counting, (b) and (d) represent the MTT assay of PDLCs and GECs, respectively.

4. Conclusion

PLLA/MWNTs/HA hybrid membrane with uniformly distributed MWNTs/HA nanoparticles could be successfully fabricated through electrospinning technique. Cytological results indicated PLLA/MWNTs/HA membrane promoted the adhesion and proliferation of human PDLCs and inhibited the adhesion and proliferation of GECs. This new type biomaterial was composed of single layered but showed dual biological functions, which can satisfy the requirements of GTR technique.

Reference

[1] G. Hillmann, A. Steinkamp-Zucht, W. Geurtsen, G. Gross, A. Hoffmann, *Biomaterials* **2002**, *23*, 1461.

[2] H. W. Kim, J. H. Song, H. E. Kim, *Adv. Funct. Mater.* **2005**, *15*, 1988.

[3] G. R. Owen, J. Jackson, B. Chehroudia, H. Burt, D. M. Brunette, *Biomaterials* **2005**, *26*, 7447.

[4] S. Liao, W. Wang, M. Uo, S. Ohkawa, T. Akasaka, K. Tamura, F. Cui, F. Watari, *Biomaterials* **2005**, *26*, 7564.

[5] W. J. Li, C. T. Laurencin, E. J. Caterson, R. S. Tuan, F. K. Ko, *J. Biomed. Mater. Res.* **2002**, *60*, 613.

[6] E. R. Kenawy, J. M. Layman, J. R. Watkins, G. L. Bowlin, J. A. Matthews, D. G. Simpson, G. E. Wnek, *Biomaterials* **2003**, *24*, 907.

[7] F. Yang, R. Murugan, S. Ramakrishna, X. Wang, Y. X. Ma, S. Wang, *Biomaterials* **2004**, *25*, 1891.

[8] M. O. Montjovent, L. Mathieu, B. Hinz, L. L. Applegate, P. E. Bourban, P. Y. Zambelli, J. A. Manson, D. P. Pioletti, *Tissue. Eng.* **2005**, *11*, 1640.

[9] C. G. Simon, N. Eidelman, S. B. Kennedy, A. Sehgal, C. A. Khatri, N. R. Washburn, *Biomaterials* **2005**, *26*, 6906.

[10] H. Tsuji, M. Ogiwara, S. K. Saha, T. Sakaki, *Biomacromolecules* **2006**, *7*, 380.

[11] R. Zhang, P. X. Ma, *J. Biomed. Mater. Res.* **1999**, *44*, 446.

[12] M. F. Yu, B. S. Files, S. Arepalli, R. S. Ruoff, *Phys. Rev. Lett.* **2000**, *84*, 5552.

[13] R. L. Price, M. C. Waid, K. M. Haberstroh, T. J. Webster, *Biomaterials* **2003**, *24*, 1877.

[14] L. P. Zanello, B. Zhao, H. Hu, R. C. Haddon, *Nano. lett.* **2006**, *6*, 562.

[15] T. Akasaka, F. Watari, Y. Sato, K. Tohji, *Mater. Sci. Eng.* **2006**, *26*, 675.

[16] S. Aryal, K. C. R. Bahadur, N. Dharmaraj, K. W. Kim, H. Y. Kim, *Scripta. Materialia.* **2006**, *54*, 131.

[17] H. J. Gong, X. P. Yang, G. Q. Chen, *Acta Polymerica. Sinca.* **2005**, *2*, 297.

[18] J. C. Wataha, R. G. Craig, C. T. Hanks, *Dent. Mater.* **1992**, *8*, 65.