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RESEARCH ARTICLE

Prodrug inspired bi-layered electrospun membrane with properties of enhanced tissue integration for guided tissue regeneration

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Abstract

Guided tissue regeneration (GTR) membranes play a vital role in periodontal surgery. Recently a series of composite electrospun membranes have been fabricated to improve the unexpected biodegradation of collagen-based GTR membranes. However, their tissue integrity needs to be studied in depth. In this study, a bi-layered electrospun membrane (BEM) inspired by "prodrug" was fabricated, which contained a dense-layer (BEM-DL) and a potential loose-layer (BEM-LL). The nanofibers of BEM-DL were composed of poly(L-lactic-co-glycolic acid) and tilapia skin collagen (TSC). Whereas the BEM-LL consisted of two types of nanofibers, one was the same as BEM-DL and the other was made from TSC. The morphology, degradation in vitro, cytocompatibility and biocompatibility in rats were investigated with a poly(lactic-co-glycolic acid) electrospun membrane (PLGA) as the negative control. The pore size of BEM-LL soaked for 7 days became larger than the original sample (164.8 ± 90.9 and 52.5 ± 21.0 μm^2, respectively), which was significantly higher ($p < .05$) than that of BEM-DL and PLGA. The BEM-LL displayed a larger weight loss rate of 82.3 ± 3.6% than the BEM-DL of 46.0 ± 2.8% at day 7 because of the rapid degradation of TSC fibers. The cytocompatibility test demonstrated that L929 cells were only spread on the surface of the BEM-DL while MC3T3-E1 cells grew into the BEM-LL layer. The subcutaneous implantation test further proved that BEM-DL performed as a cellular barrier, whereas BEM-LL was conducive to cell infiltration as deep as 200 μm with reduced fibrous encapsulation. Herein, the BEM inspired by "prodrug" is a promising GTR membrane with a property of enhanced tissue integration.

KEYWORDS
bi-layered electrospun membrane, guided tissue regeneration, potential loose-layer, tilapia skin collagen, tissue integration

1 | INTRODUCTION

Guided tissue regeneration (GTR) is a necessary surgical technology in the treatment of defects of periodontal tissue, which could regenerate some lost periodontal tissue including periodontal ligament, bone and cementum. GTR membrane, an essential biomaterial used in this procedure, could provide a barrier for selective cells to grow into the defect area.[1,2] Currently, a variety of GTR membranes could be generally divided into resorbable and non-resorbable membranes according to their degradation characteristics. The non-resorbable membranes need a second surgery for membrane removal and increase the risk of bacterial infection.[3] Conversely, resorbable membranes could be degraded in vivo to avoid the second surgery and attract increasing attention,[2,3] which can be fabricated with collagen, chitosan, polylactic acid and so on.[4–6] Because of its excellent biocompatibility, collagen membrane is widely used in the clinic, among which Bio-Gide® (Geistlich, Swiss) is the outstanding representative. However, collagen membranes still have some disadvantages: high cost, risk of disease transmission, poor mechanical strength and unexpected degradation in vivo.[1,3]

There is an increasing concern about medical-grade fish collagen to reduce the cost and the risk of disease transmission.[7–14] Numerous studies have proved that the biocompatibility of tilapia collagen was excellent, including the cytocompatibility, sensitization, chromosomal aberrations, intracutaneous reactions, acute systemic toxicity, pyrogenic reactions, and immunogenicity.[7–11] Furthermore, tilapia collagen could be processed into different medical products such as micro/nanofiber and hydrogel.[12–14] These studies indicated that tilapia collagen could be used as a potential raw material for GTR membranes. However, membranes prepared by pure collagen lacked mechanical strength and degraded quickly in vivo.[15,16]

Synthetic biodegradable polymers, including poly(lactic-co-glycolic acid) and poly(caprolactone), have been extensively studied for GTR applications due to their good biocompatibility, adjustable biodegradability and United States Food and Drug Administration (FDA) approved compliance.[17] However, GTR membranes based on some synthetic biodegradable polymers had disadvantages including moderate cytotoxic reactions and reduced cellular adhesion.[3] Additionally, GTR membranes based on synthetic polymers were often encapsulated by fibrous tissue after implantation owing to its poor tissue integration.[18] To overcome the disadvantages of synthetic polymers, a series of hybrid membranes have been fabricated recently based on electrospinning.[18,19] As an attractive and cost-effective technique for composite biomaterials, electrospinning could regulate the properties of materials by changing the composition of electrospinning solutions.[20–23]

Most researches focused on improving the effectiveness of regeneration or antimicrobial activity for GTR membranes.[18,19,24] However, the properties of cellular infiltration and host tissue integration in vivo should have gotten more attention. GTR membranes not only served as a barrier to prevent connective tissue from growing into the defect area but also provided the microenvironment to allow the reconstruction of periodontal tissue. The chemical composition and topological structure of GTR membranes played a vital role in periodontal tissue regeneration.[1,3] Just as the manufacturer claimed, Bio-Gide® was designed as a bi-layered structure in which the "dense layer" is a barrier and the "loose layer" is suitable for inducing cell penetration. But it is a challenge to prepare the "loose layer" by the traditional electrospinning technology because the pore sizes of electrospun membranes are usually too small to allow cell infiltration.

As a classical concept of pharmacokinetics, the "prodrug" usually refers to a series of derivatives for a certain drug, which can be transformed into a drug with higher biological activity once it is absorbed in vivo. Based on the inspiration of the "prodrug", we fabricated a bi-layered membrane by a two-step electrospinning process (Figure 1). Firstly, a potential loose layer of BEM (BEM-LL) was fabricated using the conjugated electrospinning with two different solutions (A and B), and then the dense layer of BEM (BEM-DL) was fabricated onto BEM-LL. Thus BEM-LL consisted of two types of fibers, while BEM-DL contained only one type of fiber prepared from solution A. We hypothesized that a type of fiber in BEM-LL could degrade quickly to create space for cell growth and tissue integration. Whereas the fibers of BEM-DL degraded slowly enough to achieve cellular barrier function. In this study, we intended to fabricate a bi-layered electrospun membrane (BEM) using the composite material of collagen and poly(lactic-co-glycolic acid) and investigate its properties of tissue integration for GTR with a single-layered poly(lactic-co-glycolic acid) electrospun membrane as the negative control.

2 | MATERIALS AND METHODS

2.1 | Materials

Purified tilapia skin collagen (TSC, Figure S1[A]) was provided by Shandong International Biotechnology Park Development Co., Ltd (Yantai, China). The poly(L-lactic-co-glycolic acid) (82:18) with a composition of 82 mol.% L-lactide and an intrinsic viscosity of 1.9 dl/g was supplied by Jinan Daigang Biomaterial Co., Ltd. (Jinan, China). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was purchased from Shanghai Fine Chemicals Co., Ltd. (Shanghai, China). Mouse fibroblasts (L929 cells) and preosteoblast (MC3T3-E1 cells) were supplied by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All culture media and reagents were purchased from Gibco Life Technologies Co., (USA) unless specified. All of the other chemicals were of analytical grade and were used without further purification.

2.2 | Fabrication of electrospun membrane

2.2.1 | Fabrication of bi-layered electrospun membrane

The preparation process of BEM could be divided into two steps (Figure 1), which were implemented using the electrospinning machine (SS-3556H, Ucalery, China). Briefly, BEM-LL was firstly prepared by electrospinning solutions A and B, and then BEM-DL was fabricated by solution A onto the BEM-LL layer. For the preparation of solution A (w/v [%] = 10), a mixture of poly(L-lactic-co-glycolic acid) and TSC (mass ratio = 60:40) was added to HFIP and stirred at room temperature until complete dissolution. Whereas solution B was obtained by dissolving TSC in HFIP (w/v [%] = 10). Two syringes that loaded solutions A and B were placed on the upper left and upper right of the drum collector respectively (Figure S1[B]). The distance between syringe needles and the rotating drum was set at 15 cm. Two oppositely charged voltages of 15 kV were applied to the two needles, and the flow rate of pumps was set at 1.0 ml/h. Thus two kinds of nanofibers were collected together on the drum collector and referred to as BEM-LL. For the preparation of BEM-DL, solution A was withdrawn in a syringe and electrospun at a rate of 1 ml/h with a voltage of 15 kV. Finally, BEM-DL was prepared with BEM-LL as the receiver and the BEM was obtained.

2.2.2 | Fabrication of control samples

As the control samples, the single-layer membrane of BEM-LL and BEM-DL were also fabricated according to the above method. In addition, poly(L-lactic-co-glycolic acid) or tilapia skin collagen was dissolved in HFIP at the concentration of 10% (w/v) and referred to as solution PLGA and solution TCOL, respectively. Then solution PLGA or solution TCOL was withdrawn in a syringe and electrospun at a

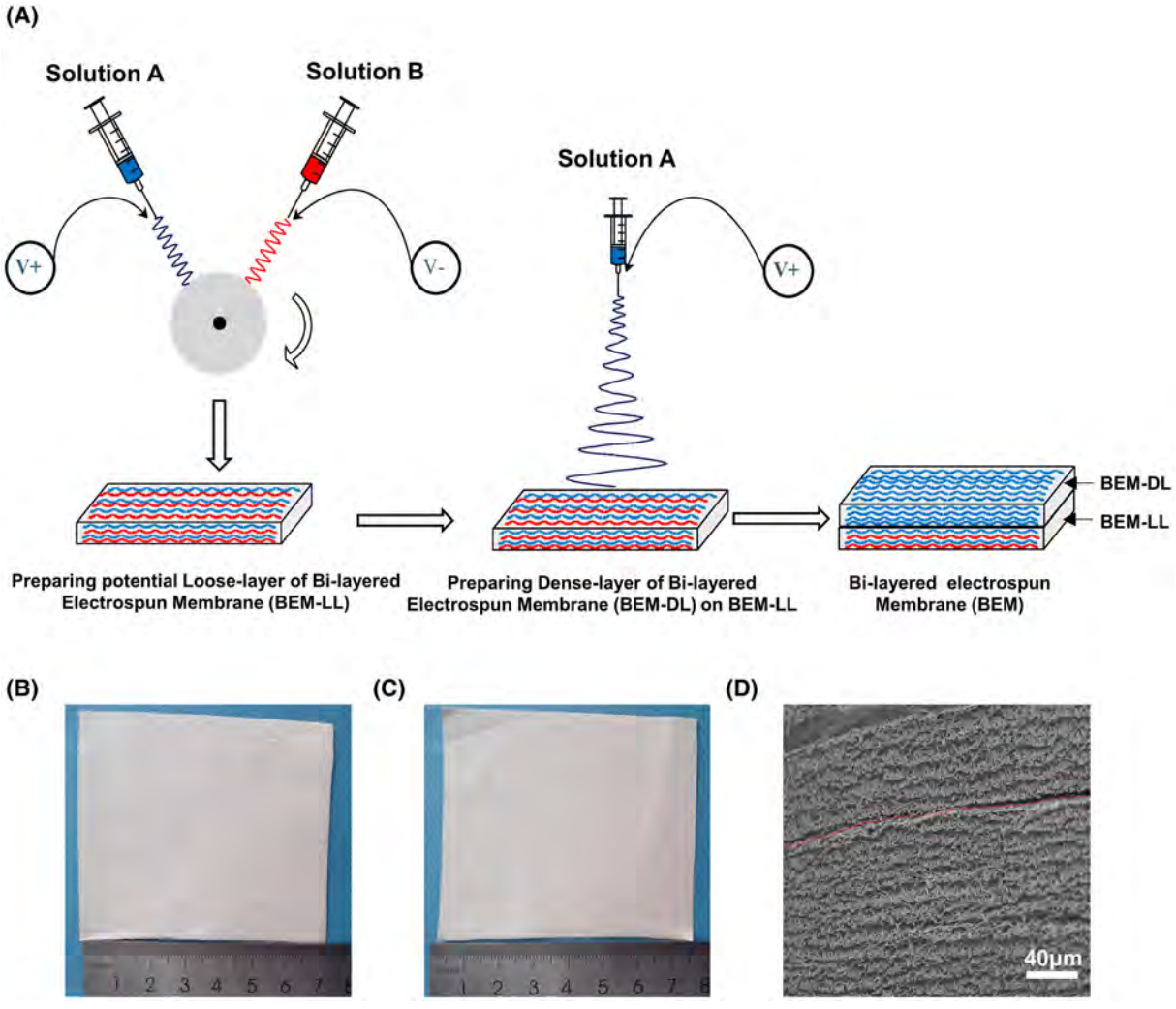

FIGURE 1 The schematic diagram of the bi-layered electrospun membrane (BEM) production process (A), photos of the bi-layered electrospun membrane-loose-layer (BEM-LL) (B) and bi-layered electrospun membrane-dense-layer (BEM-DL) (C) and the scanning electron microscopy images of the cross-sections for BEM (D). The red dotted line represents the interface between BEM-DL and BEM-LL. Scale bars are 40 μm

rate of 1 ml/h with a voltage of 15 kV. Thus the poly(L-lactic-co-glycolic acid) electrospun membrane (refer as PLGA) and the tilapia skin collagen electrospun membrane (refer as TCOL) were obtained.

2.2.3 | Post-treatment of electrospun membrane

All electrospun membranes were dried under vacuum for 48 h to facilitate volatilization of the residual solvent, heated under vacuum at 50°C for 16 h as a thermo-crosslinking process, and finally were sterilized by 15 kGy Co-60 γ-ray irradiation for following experimental study.

2.3 | Characterization of electrospun membrane

In this section, the characteristics of morphology and degradation in vitro for the BEM-LL, BEM-DL, PLGA and TCOL were tested to verify the hypothesis of this study: the TSC fibers in BEM-LL could be rapidly degraded and then the pore size of BEM-LL become larger. While the fibers in BEM-DL degraded slowly and could maintain a dense structure. Moreover, the hydrophilicity and mechanical properties for BEM were further studied with the sample PLGA as control.

2.3.1 | Morphology

The samples of BEM-LL, BEM-DL, PLGA and TCOL were firstly cut into square specimens with the size of 3 × 4 cm, immersed in phosphate buffer saline (PBS, pH 7.4) at 37°C for 7 days, and lyophilized for 48 h to make the hydrated samples. Then the morphology of the hydrated and original samples was observed via scanning electron microscopy (EVO LS15, Zeiss, Germany). In brief, samples taken from the different electrospun membranes were previously prepared employing gold sputter coating and carbon conductive tape to guarantee proper adhesion and electronic conductivity. For each sample, 100 pores were randomly chosen and the area was semi-quantitatively measured using the image analysis software (Image J, National Institutes of Health, USA, Figure S2). Results were expressed as the mean pore size of different pores.

2.3.2 | In vitro degradation

The samples of BEM-LL, BEM-DL, PLGA, TCOL together with BEM were cut into square specimens with a size of 1 × 1 cm and weighed (W_0). Then the pieces ($n = 3$) were soaked in a sealed tube containing 5 ml of sterile PBS at 37°C.[17] The PBS solution was refreshed every 7 days. After incubating at 37°C for 1, 3, 7, 14, 28 and 56 days, the degraded samples were carefully removed from the tubes and rinsed several times with distilled water, freeze-dried and weighed (W_t). The weight loss rate (ΔW %) at each time interval was calculated according to the following equation: $\Delta W\ \% = (W_0 - W_t)/W_0 \times 100\%$.

2.3.3 | Hydrophilicity

The hydrophilicity of samples was estimated from the contact angles. Twenty microliter deionized water was dropped onto the surface of each sample ($n = 3$) and the water contact angles at the fifth second were measured by a Contact Angle Analyzer (OCA40, Dataphysics, Germany).

2.3.4 | Mechanical properties

Samples were cut into a uniform size of 50 × 10 mm ($n = 3$), soaked in normal saline for 10 min, and the mechanical property was measured by a universal material testing machine (CMT8502, MTS systems, China) at 25°C with a stretching speed of 25 mm/min.

2.4 | Cytocompatibility test

2.4.1 | Cells incubation and seeding

L929 cells and MC3T3-E1 cells were used to study cytocompatibility in this section. The membranes of BEM (group BEM) and PLGA (group PLGA) were firstly cut into discs with a diameter of 15 mm. On one hand, the BEM discs were placed into 24-well plates with the BEM-DL layer facing up and soaked in the Dulbecco's modified Eagle medium (DMEM) overnight. L929 cells were seeded on membranes individually at a density of 2.5×10^4 cells/well and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). On the other hand, MC3T3-E1 cells cultured in Minimum Essential Medium α (α-MEM) were seeded on the BEM discs with the BEM-LL layer facing up. In addition, L929 cells or MC3T3-E1 cells were seeded on PLGA discs as the control group. The culturing medium was refreshed every 2 days as well. Then the cell morphology, proliferation and infiltration property were evaluated according to the following methods.

2.4.2 | Cell morphology

The morphology of cells culturing for 1, 3, 7 days was observed via scanning electron microscopy. Briefly, The samples were rinsed twice with PBS after removing the culture media, fixed with 4% paraformaldehyde for 2 h at 4°C, and were rinsed with PBS twice and then dehydrated in gradient concentrations of ethanol (30%, 50%, 60%, 70%, 80%, 90%, 95%, and 100%). After being dried by carbon dioxide supercritical fluid, samples were gold-coated in a sputter coater and observed at an acceleration voltage of 5 kV by scanning electron microscopy (Phenom XL, Phenom-World BV, Nederland).

2.4.3 | Cell proliferation

Cell proliferation on day 1, 3 and 7 was measured by methythiazol tetrazolium (MTT) assay. In this section, DMEM and α-MEM were used

for culturing with L929 cells and MC3T3-E1 cells respectively. Briefly, MTT solution was prepared by dissolving MTT in a culture medium at a concentration of 5 mg/ml. Samples culturing in 24-well plate were rinsed twice with PBS after removing the culture media. Fifty microliter MTT (5 mg/ml) was added into wells together with 200 μl of culture medium, and the samples with cells were further incubated for 4 h at 37°C, 5% CO_2. Then MTT solution of each well was removed, 200 μl dimethyl sulfoxide (DMSO) was added to each well. After swaying this plate for 10 min, 50 μl solution in each well was transferred to another 96-well plate, and the optical density at 492 nm of each sample was tested using a microplate absorbance reader.

2.4.4 | Cell infiltration

To qualitatively observe whether the cells grew into the membranes, the cell- membrane constructs culturing for 14 days were embedded in optimal cutting temperature compound (OCT), frozen at −80°C for 12 h, and then their cross sections in 8 μm thickness were obtained using a Cryotome E cryostat (CM1950, Leica Biosystems, Germany). The slices were permeabilized (0.1% Triton X-100 /PBS), stained with 4′, 6-diamidino-2-phenylindole (DAPI) for 10 min, and then examined using an inverted fluorescence microscope (Revolve FL, Echo Laboratories, USA).[25] Furthermore, the membrane constructs cultured for 14 days were stained with Hoechst 33342/PI Double Stain Kit (Solarbio Ltd., Beijing, China) following the manufacturer's protocol, and then visualized using a confocal microscope (LSM800, Zeiss, Germany).[17] Cell infiltration was further analyzed with the 3D imaging software that provided a visual image of the stained cells growing into the membranes.

2.5 | Subcutaneous implantation

The biocompatibility and biodegradability of the membranes in vivo were evaluated by the subcutaneous implantation test in Sprague–Dawley (SD) rats.[17] The protocol for animal experiments was approved by the Committee on the Ethics of Animal Experiments of Yantai University. BEM and PLGA membranes were cut into a piece of 1 × 1 cm before implantation. Twelve female SD rats (6–8 weeks, female, 200–250 g) were acclimatized for 7 days, anesthetized with sodium pentobarbital and two 1.5 cm long incisions were made on the back of each rat. Then four pockets were made by bluntly separating the deep fascia, two of which were implanted with the BEM (group BEM), and the other two pockets were implanted with the PLGA (group PLGA). Four animals were sacrificed at 2, 4 and 8 weeks after implantation. The implanted materials together with the around tissue were harvested, embedded with paraffin and stained using hematoxylin–eosin (H&E). The specimens were observed using optical microscopy (DM1000, Leica Biosystems, Germany) to qualitatively evaluate the degree of cell growth and fibrous encapsulation at the interface between the membrane and surrounding tissue.

2.6 | Statistical analysis

The data were analyzed using SPSS Statistics 26 (IBM, USA). Results were expressed as mean ± SD. Comparisons between two groups were performed using one-way Analysis of Variance (ANOVA). A value of $p < .05$ was considered significant.

3 | RESULT AND DISCUSSION

3.1 | Fabrication of electrospun membrane

The BEM was successfully prepared by the two-step electrospinning process (Figure 1A), and photos of layer BEM-LL and BEM-DL are shown in Figure 1B–D. Currently, biomaterials are becoming increasingly complicated by adding various expensive drugs or growth factors. While the simplicity of the production process and its compliance with medical regulations have been ignored, such as the terminal sterilization processes recognized by the medical industry. In the present study, we used two common biomaterials, fish skin collagen and poly(L-lactic-co-glycolic acid) as raw materials, and fabricated a bi-layered membrane based on traditional electrospinning technology. This process complied with terminal sterilization regulations for medical devices. Moreover, fish skin collagen was easily available due to fish skins being produced in large quantities.

In a preliminary experiment, we examined several polymers and found that poly(L-lactic-co-glycolic acid) (82:18) was more suitable for preparing electrospun membranes due to its higher stability in the wet state. This property is vital for implantable medical devices. Compared with membranes made from poly(L-lactic-co-glycolic acid) (82:18), the ones from poly(DL-lactic-co-glycolic acid) or poly(DL-lactic acid) shrank sharply when put in water, and the ones from poly(L-lactide-co-caprolactone) appeared curled. Furtherly, the influence of different ratios between poly(L-lactic-co-glycolic acid) (82:18) and TSC on deformation rate was investigated. The result showed that no significant difference was observed in the deformation rate when the proportion of TSC was <40%. The methods and results of preliminary experiments were present in Supporting Information and Figure S3. Considering the better cytocompatibility of TSC, a mixture of poly(L-lactic-co-glycolic acid) (82:18) and TSC (mass ratio = 60:40) was chosen for BEM-DL. Additionally, we found that the TCOL membrane without thermal crosslinking could be dissolved instantly in water, whereas the thermally cross-linked TCOL membrane had not completely dissolved after soaking for 3 days. Therefore, the post-treatment process of thermal crosslinking was adopted in this research to further reduce the shrinkage effect of the electrospun membrane.

3.2 | Characterization of electrospun membrane

3.2.1 | Morphology and degradation in vitro

The SEM images showed that some flat fibers were found in BEM-LL (Figure 2A [II]), but not found in BEM-DL (Figure 2A [I]). Because

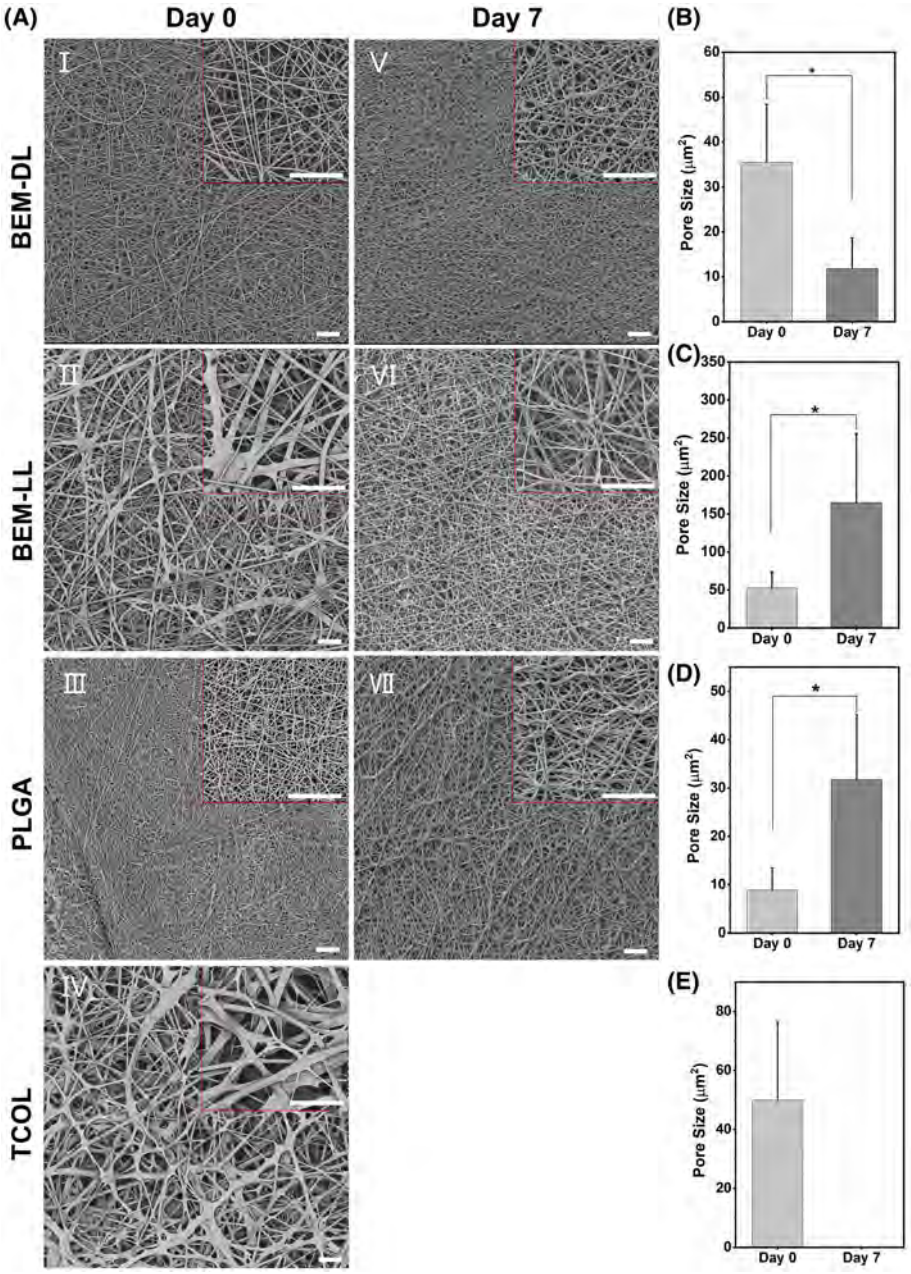

FIGURE 2 Morphological characterizations of the original electrospun membranes and the ones that soaking in the phosphate buffer solution for 7 days. (A) Representative 1000× and 5000× images of scanning electron microscopy for bi-layered electrospun membrane-dense-layer (BEM-DL), bi-layered electrospun membrane-loose-layer (BEM-LL), poly(lactic-co-glycolic acid) (PLGA) and TCOL soaking in the phosphate buffer solution for 0 days (original samples) and 7 days, image of TCOL for 7 days was not available, because TCOL was completely dissolved after 7 days. All scale bars are 20 μm. (B–E) Comparison of the pore size of BEM-DL (or BEM-LL, PLGA and TCOL) samples after soaking in the phosphate buffer solution for 0 days and 7 days ($n = 100$). *$p < .05$

BEM-LL contained fish collagen fibers that were prepared with solution B, we speculated that the flat fibers in BEM-LL were fish collagen fibers. The TCOL prepared from fish collagen fibers also contained flat fibers (Figure 2A [IV]), which just confirmed that the fish collagen fibers were flat in this study. Figure 2A (V–VII) is the morphology of the membranes soaking in PBS for 7 days, and Figure 2B–E represented the change in pore size of BEM-DL (or BEM-LL, PLGA and TCOL) between the original sample and one soaking in PBS for 7 days. The results showed that compared with the original samples (0 days), the pore size of BEM-DL became significantly smaller after 7 days of soaking (Figure 2B). Whereas the BEM-LL soaking for 7 days displayed a significantly larger ($p < .05$) pore size than the original samples (164.8 ± 1.28% and 62 ± 1.13 respectively, Figure 2C), which should be related to the disappearance of flat fibers in BEM-LL (Figure 2A [VI]). This speculation was consistent with the observation results of the TCOL membrane, which completely dissolved when soaked in PBS for 7 days (Figure 2E).

Additionally, we further compared the pore size of the original membranes (Figure 3A) and the ones soaked 7 days (Figure 3B). Whether it was the original sample or soaked for 7 days, the pore size of BEM-LL was significantly larger ($p < .05$) than that of BEM-DL. It was clear that the fish collagen fibers of BEM-LL lead to its larger pore size. In addition, the weight loss curves (Figure 3C) showed that all membranes degraded gradually when incubating in PBS as evidenced by increasing weight loss rate over time, and the degradation rate was BEM-LL > BEM > BEM-DL in the order of 1–56 days of degradation in vitro. The TCOL and BEM-LL groups displayed a larger weight loss rate of 99.0 ± 1.3% and 82.3 ± 3.6% respectively at day 7 than the

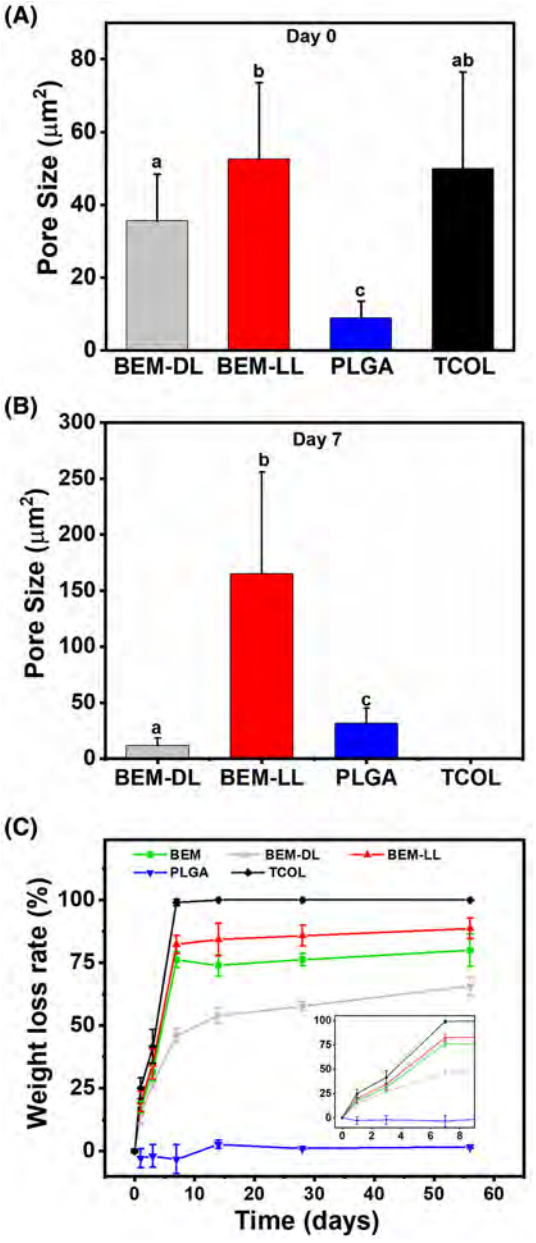

FIGURE 3 Pore sizes of bi-layered electrospun membrane-dense-layer (BEM-DL), bi-layered electrospun membrane-loose-layer (BEM-LL), poly(lactic-co-glycolic acid) (PLGA) and TCOL samples soaking in the phosphate buffer solution for (A) 0 days or (B) 7 days. The mean ± SD that are indicated with different small letters are significantly different from each other ($p < .05$). (C) Degradation profile in vitro for BEM, BEM-DL, BEM-LL, PLGA and TCOL samples

BEM-DL of 46.0 ± 2.8%. It should be mentioned that the PLGA sample degraded <10% from beginning to end. As we expected, fish collagen fibers were easily degraded and poly(L-lactic-co-glycolic acid) fibers degraded slowly. In short, the results of SEM and degradation in vitro verified the hypothesis of this study: the fish collagen fibers in BEM-LL could be degraded rapidly and the pore size of BEM-LL became larger, while the fibers in BEM-DL degraded slowly and it could maintain a dense structure.

3.2.2 | Hydrophilicity and mechanical properties

As shown in Figure 4A, the water contact angles of PLGA, BEM-DL layer and BEM-LL layer of BEM were 123.7 ± 3.1°, 105.9 ± 0.8° and 55.4 ± 0.3°, respectively. In addition, the water dropped onto TCOL membrane was absorbed instantaneously, which indicated that the TCOL membrane was super hydrophilic. The BEM-DL layer prepared by poly(L-lactic-co-glycolic acid) and fish collagen was more hydrophilic ($p < .05$) than the PLGA membrane prepared from pure PLGA, which indicated that adding fish collagen to poly(L-lactic-co-glycolic acid) could increase the hydrophilicity of the electrospun membrane. Moreover, the contact angle of the BEM-LL layer was significantly smaller ($p < .05$) than that of the BEM-DL layer. This might be because the BEM-LL contained fish collagen fibers, which improved the hydrophilicity of the electrospun membrane. Membranes with a suitable hydrophilic-hydrophobic balance allow easy cell attachment.[26,27] Therefore, the addition of fish collagen might improve the cell compatibility for the PLGA membrane. However, fish collagen might reduce the mechanical properties of the electrospun membrane. In a preliminary experiment, we have found that the TCOL membrane made from pure fish collagen was fragile. Figure 4B–D illustrated the stress–strain curves, tensile strength and elongation at break of BEM and PLGA samples. The tensile strength of BEM (3.05 ± 0.14 MPa) was significantly lower than the PLGA (8.85 ± 0.64 MPa), and the elongation at break of BEM (65.07 ± 5.96%) was also obviously lower than the PLGA (96.02 ± 6.82%).

3.3 | Cytocompatibility test

In periodontal surgery, the dense layer of GTR membranes generally plays as a barrier to block connective tissue from growing into the defect area, while the loose layer acts as a scaffold to promote bone regeneration. L929 cells and MC3T3-E1 cells are the representative cells of connective tissue and bone tissue, respectively. Thus the two types of cells were inoculated on the BEM-DL and BEM-LL respectively to evaluate the cytocompatibility of BEM.

3.3.1 | Cell morphology

The morphology of L929 cells on the BEM-DL layer and PLGA membrane culturing for 1, 3 and 7 days were shown in Figure 5A. The cells were already spread on the surface of the BEM-DL layer at day 1 (Figure 5A[I]), whereas only adhered to the PLGA membrane in a spherical shape (Figure 5A[IV]). L929 cells spread fully on the surface of the BEM-DL layer at day 3 and 7 (Figure 5A[II, III]), and adhere to the PLGA membrane in a spindle shape (Figure 5A[V, VI]), suggesting that the BEM-DL layer prepared by the compound of poly(L-lactic-co-glycolic acid) and fish collagen was more conducive to the adhesion and spreading for L929 cells than the PLGA membrane prepared by poly(L-lactic-co-glycolic acid).

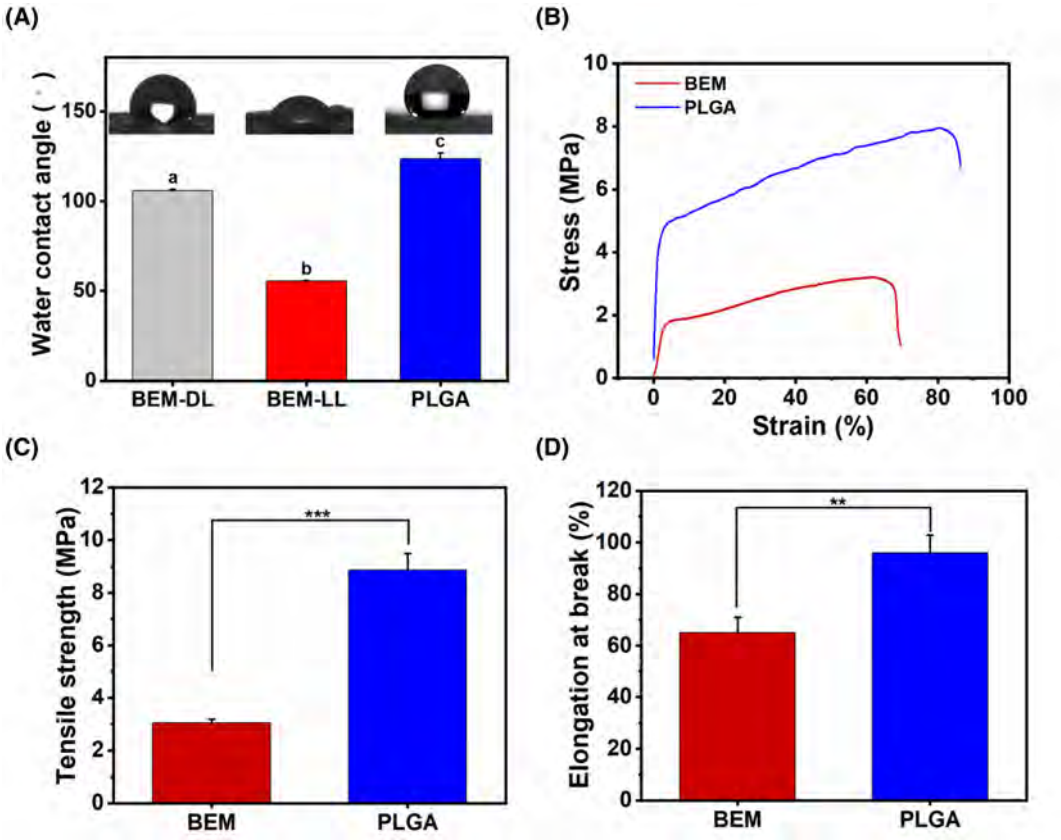

FIGURE 4 Hydrophilicity and mechanical characterization of bi-layered electrospun membrane (BEM) and poly(lactic-co-glycolic acid) (PLGA) samples. (A) The contact angle of bi-layered electrospun membrane-dense-layer (BEM-DL), bi-layered electrospun membrane-loose-layer (BEM-LL) and PLGA samples. The mean ± SDs that are indicated with different small letters are significantly different from each other ($p < .05$). (B) Stress–strain curves, (C) tensile strength and (D) elongation at break of BEM and PLGA samples. $^{**}p < .01$, $^{***}p < .001$

Moreover, the morphology of MC3T3-E1 cells on the BEM-LL layer and PLGA membrane culturing for 1, 3 and 7 days were shown in Figure 5B. Few cells were growing on the two membranes on day 1 (Figure 5B[I, IV]). Over a culture time from day 3 to day 7, increasing numbers of MC3T3-E1 cells grew into the BEM-LL layer (Figure 5B[II, III]), while only a few cells adhered to the surface of the PLGA membrane (Figure 5B[V, VI]). As a potential loose layer, the BEM-LL was verified to be conducive to the growth of cells into the membrane.

3.3.2 | Cell proliferation

The MTT assay is a common method to detect the degree of cell proliferation. The results showed that the OD_{492nm} of L929 cells for group BEM and PLGA increased over time (Figure 5C), but no statistically significant difference was evident between the two groups ($p > .05$). For the MTT assay of MC3T3-E1 cells (Figure 5D), the OD_{492nm} of the group BEM was significantly higher than that of the group PLGA from day 3 to day 7. As the SEM images shown (Figure 5B), MC3T3-E1 cells grew into the BEM-LL layer instead of the PLGA. So it was the unique three-dimensional structure of the BEM-LL layer that made it more conducive to cell proliferation.

3.3.3 | Cell infiltration

L929 and MC3T3-E1 cells cultured on the membrane for 14 days were evaluated by immunofluorescence staining, including the DAPI staining and 3D reconstruction of Hoechst 33342/PI Double Staining. It is well known that the nucleus stained by DAPI can emit blue fluorescence, while 33,342/PI Double Staining can color the chromatin and show blue or red fluorescence depending on the cell state. The images of DAPI staining showed that L929 cells were spread on the surface of the BEM-DL layer (Figure 6A), while MC3T3-E1 cells grew into the BEM-LL layer (Figure 6C). Additionally, L929 cells and MC3T3-E1 cells only adhered to the surface of the PLGA membrane (Figure 6B,D). The 3D reconstructed images of Hoechst 33342/PI Double Staining confirmed that MC3T3-E1 cells grew into the BEM-LL layer to a certain depth (Figure 6G) and only grew on the surface of membrane for the other groups (Figure 6E,F,H). Therefore, the above results verified our hypothesis that the BEM-DL layer could act as the cellular barrier and BEM-LL as the scaffold for cell infiltration (Figure 6I). The cellular barrier is the basic function of GTR membranes, and the property of cell infiltration is more conducive to the regeneration of blood vessels as a scaffold, thereby promoting the regeneration of peripheral tissues.

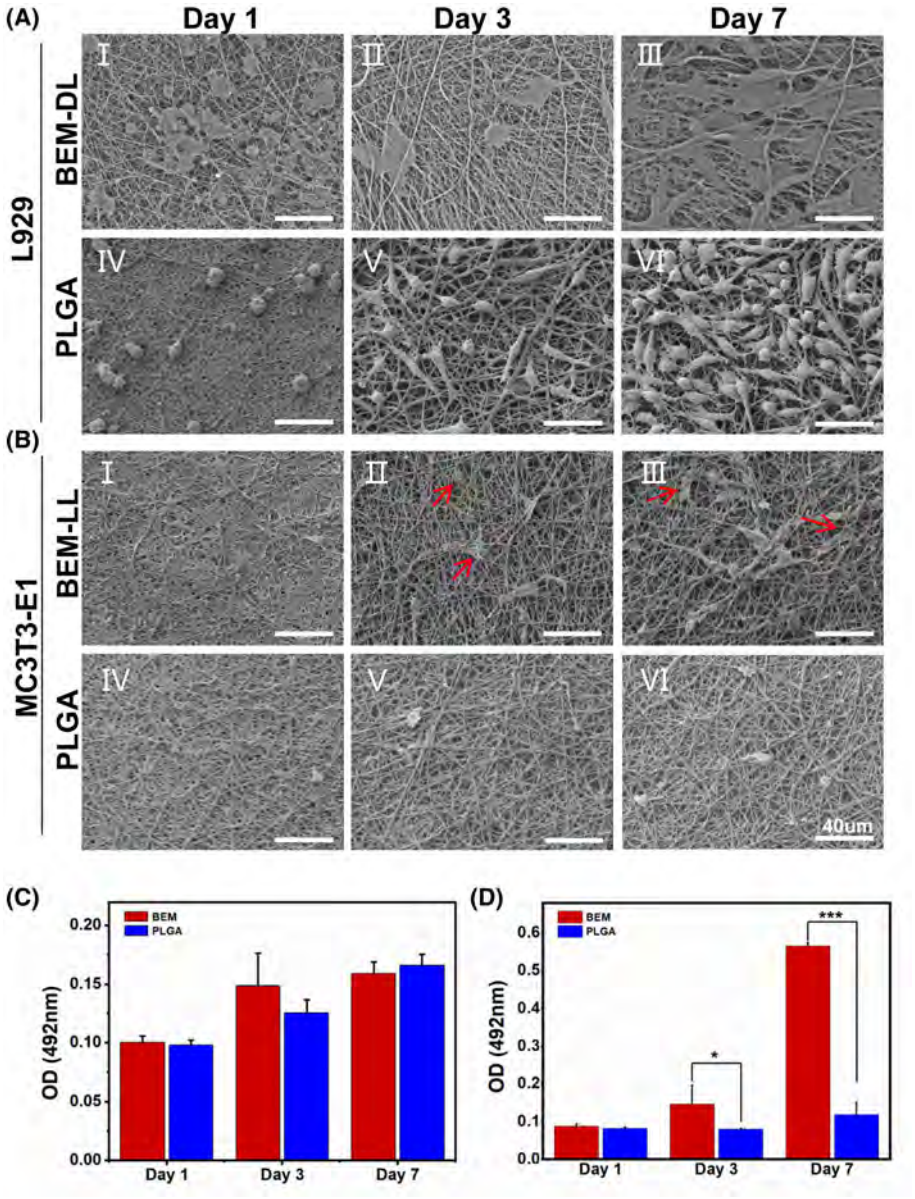

FIGURE 5 Cell proliferation for bi-layered electrospun membrane (BEM) and poly(lactic-co-glycolic acid) (PLGA) samples via scanning electron microscopy (SEM) and methythiazol tetrazolium (MTT) assay. (A) SEM images of bi-layered electrospun membrane-dense-layer (BEM-DL) layer and PLGA seeded L929 cells for 1, 3 and 7 days; (B) SEM images of bi-layered electrospun membrane-loose-layer (BEM-LL) layer and PLGA seeded MC3T3-E1 cells for 1, 3 and 7 days. Red arrows represent the cells that grew into the fibrous membrane. Scale bars are 40 μm. (C) MTT results of BEM and PLGA samples culturing L929 cells for 1, 3, and 7 days and (D) culturing MC3T3-E1 cells for 1, 3, and 7 days. $^{*}p < .05$, $^{***}p < .001$

3.4 | Subcutaneous implantation

In the clinical application, one side of the GTR membrane is usually in contact with the gingival tissue and the other side is with the alveolar bone tissue. Take Bio-Gide® as an example, its denser layer faces gingival tissue to prevent connective tissue from growing into the bone defect area, and its loose layer faces the bone tissue to guide the growth of cells and avoid foreign body reactions. Referring to the usage of Bio-Gide®, the BEM was implanted subcutaneously with BEM-DL facing upward in the present study, and the host response of BEM was investigated by subcutaneous implantation in rats. H&E staining images (Figure 7) could reflect the cell growth and fibrous capsule of the GTR membrane at different periods after implantation. Obviously, the PLGA membrane has consistently resisted cell growth (Figure 7B,D,F). Meanwhile, a small number of cells mainly appeared in a thin layer around the BEM-DL, which proved that the BEM-DL could perform as a cellular barrier for 6–8 weeks in vivo and met the requirements of GTR membrane as reported in the literature.[1] While, many cells had deeply infiltrated into the BEM-LL from 2 to 8 weeks after implantation, with a migration distance of almost 200 μm (Figure 7A,C,E). The reason might be the rapid degradation of fish collagen fibers in the BEM-LL. These results further confirmed that the BEM-LL inspired by "prodrug" was technically feasible, and this finding did support the previous research,[28] in which B. M. Baker et al. argued that removal of soluble sacrificial fibers could improve cell infiltration of electrospun scaffolds. It was just that fish collagen fibers in the BEM-LL played the role of sacrificial fibers, and they degraded in vivo rather than be removed artificially during the preparation process. Bottino et al. criticized that selective removal of soluble sacrificial fibers could jeopardize structural integrity.[1] But the macroscopic layer-delamination did not appear in BEM (Figure 7A,C,E). This is the advantage of the design concept of "prodrug" over "sacrificial fibers".

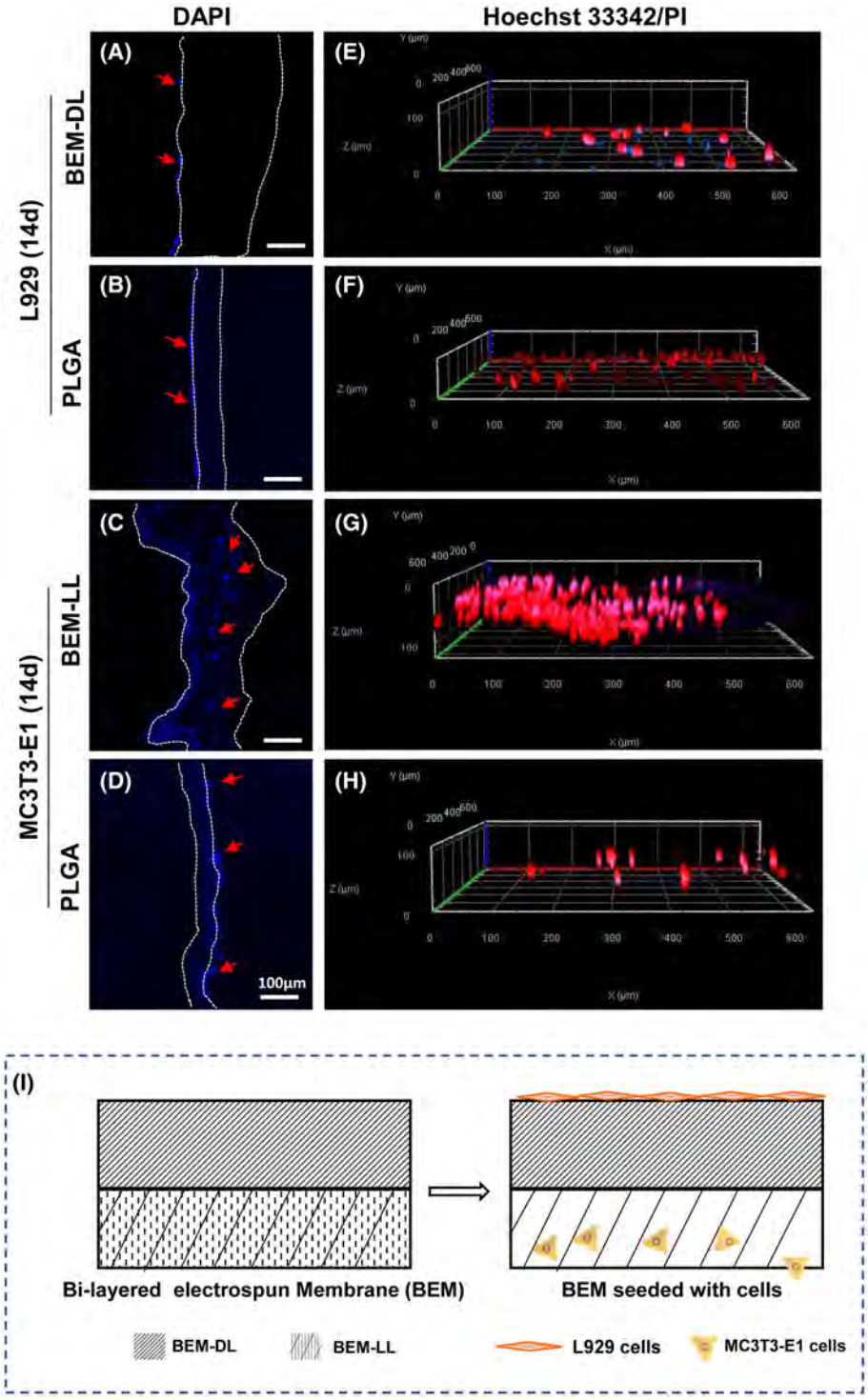

FIGURE 6 Cell tests for bi-layered electrospun membrane (BEM) and poly(lactic-co-glycolic acid) (PLGA) samples via fluorescence imaging. (A–D) DAPI staining images of the cross-sections of bi-layered electrospun membrane-dense-layer (BEM-DL) and PLGA samples seeded with L929 or MC3T3-E1 cells for 14 days, respectively. The white dotted lines indicate the outline of the fiber membranes, and red arrows represent cells growing on the surface or inside the membranes. Scale bars are 80 μm. (E–H) Confocal microscopy reconstructed stacks of three-dimensional fluorescence images of BEM-DL and PLGA samples seeded with L929 or MC3T3-E1 cells for 14 days, respectively. (I) Schematic diagram of the BEM-DL blocking L929 cells or the BEM-LL guiding MC3T3-E1 cells infiltration

Additionally, the tissue integration performance of biomaterials could be evaluated by examining the thickness of the fibrous capsule around the implanted materials.[29,30] The results showed that the PLGA membrane was wrapped by a thick fibrous capsule (Figure 7B, D,F), while the BEM was covered with a relatively thin fibrotic tissue, especially at the interface of BEM-LL (Figure 7A,C,E). In other words, fibrous capsules around BEM were thinner than those around the PLGA membrane. Compared with the PLGA membrane, fish collagen had significantly improved the tissue integration performance of BEM-LL as a GTR membrane.

It should be mentioned that a certain number of inflammatory cells could be observed inside BEM-LL and BEM-DL (Figure 7A,C,E), especially obvious lymphocyte and multinucleated giant cells (Figure S4). In another study, we found that there was no obvious inflammation for fish collagen implanted subcutaneously from 2 to 8 weeks (Figure S5), and the electrospun nanofiber materials based fish skin collagen had

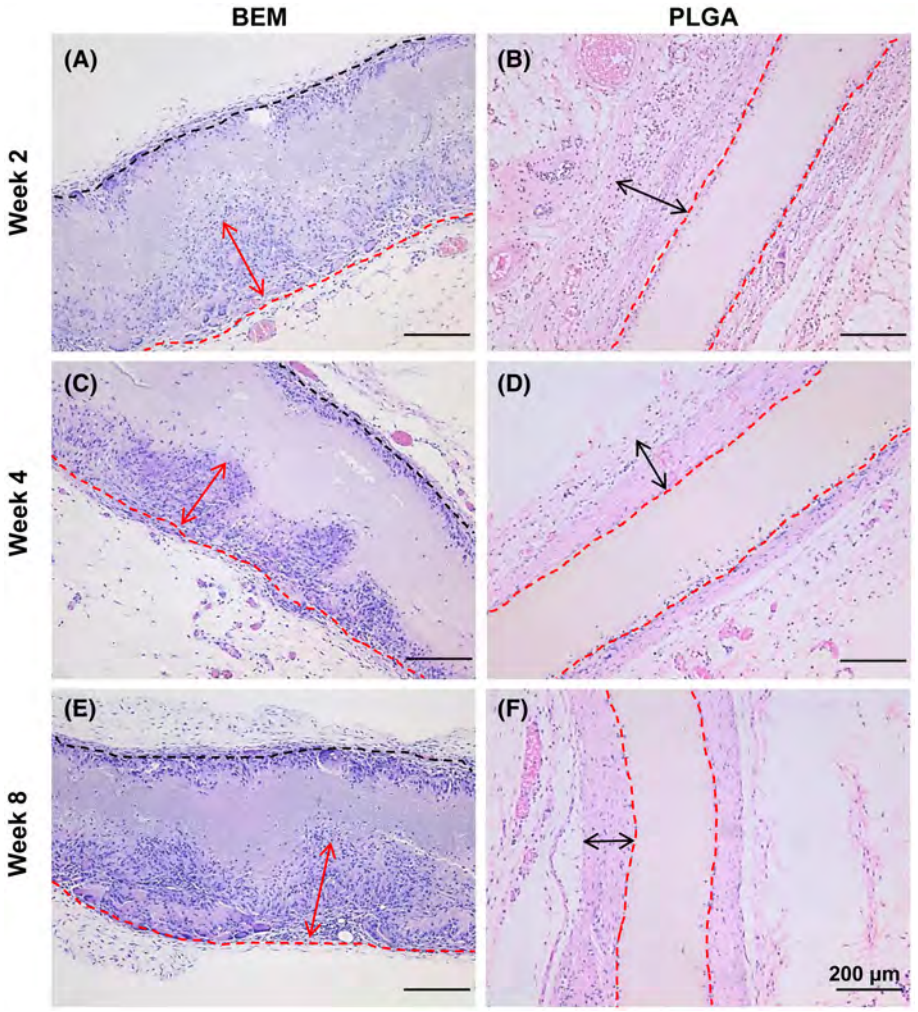

FIGURE 7 H&E staining images of bi-layered electrospun membrane (BEM) and poly(lactic-co-glycolic acid) (PLGA) samples subcutaneous implanted into SD rats after 2, 4 and 8 weeks. The membranes are highlighted with dotted lines to indicate their location. The red dotted line and black dotted line in images (A, C, E) respectively represent the interface between bi-layered electrospun membrane-loose-layer (BEM-LL) and surrounding tissues, or the interface between bi-layered electrospun membrane-dense-layer (BEM-DL) and surrounding tissues. The red arrows in images (A, C, E) and black arrows in images (B, D, F) represent cell layers grown into BEM-LL and fibrous encapsulation around PLGA, respectively. Scale bars are 200 μm

good biocompatibility.[7] In view of the literature reported that the degradation of poly(L-lactic-co-glycolic acid) could cause non-infectious inflammation in vivo,[3] we speculated that the inflammatory cells inside BEM might be caused by the acidic hydrolysates of poly(L-lactic-co-glycolic acid). There have been a large number of researches on poly (L-lactic-co-glycolic acid) based electrospun membranes in recent years, but negative reviews about their biocompatibility were relatively rare. The unexpected biocompatibility of BEM prepared with poly(L-lactic-co-glycolic acid) in this study should be widely known and valued by researchers in the field of periodontal tissue regeneration materials. Furthermore, whether polyester-related polymers are suitable for guiding tissue regeneration should be reassessed, especially the production process of biomaterials should be carried out following the standards of the medical industry. For instance, the samples for characterization and biological tests could be sterilized by γ-ray irradiation instead of soaking with the 70% ethanol solution, the latter of which is still used in many studies.

A previous study has reported that the foreign body reaction could be affected by the biodegradation of the GTR membrane,[31] and the biodegradation of electrospun membrane was related to its chemical components.[17] Therefore, the biodegradation of BEM could be regulated by adjusting the proportion of collagen and poly(L-lactic-co-glycolic acid)]. This view has been initially confirmed by the degradation experiment in vitro. Compared with pure poly(L-lactic acid-glycolic acid), adding fish collagen to poly(L-lactic acid-glycolic acid) could accelerate the degradation of electrospun membrane (Figure 3C). Considering that some papers reported that the immune response of GTR membrane might play a positive role in GTR,[32,33] the influence of inflammation for BEM should be further verified by other animal experiments. In addition, it should be meaningful to further optimize the chemical composition of BEM to regulate properly the number of inflammatory cells growing into the membrane.

4 | CONCLUSION

In this study, we used fish skin collagen and poly(L-lactic-co-glycolic acid) as two raw materials to fabricate a BEM inspired by "prodrug", which consisted of a dense-layer (BEM-DL) and a potential loose-layer (BEM-LL). The pore size of BEM-LL became larger as it degraded to facilitate cell growth and tissue integration, which had been confirmed in this study through the experiments of morphology, degradation in vitro, cytocompatibility and subcutaneous implantation in rats. These results indicated that BEM-LL could provide space to allow

tissue regeneration, and BEM-DL could serve as a barrier to cells. In brief, BEM could be a promising candidate for GTR membrane by further optimizing the composition to regulate its biodegradation, and the concept of a potential loose-layer could be used to design the antifibrotic interface for synthetic polymer implant. This article also raised some issues that were easily overlooked in the field of periodontal regeneration, including the terminal sterilization of biomaterials and the acidic inflammatory response of polyester-related polymers. These issues need to be taken seriously and systematically researched.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Performed experiments and wrote the paper: Dongsheng Li. *Performed animal experiments*: Tong Wang and Yonglin Gao. *Directed the experiments in vitro*: Jinglei Wu. *Performed the cell experiments*: Juanjuan Zhao. *Designed the research and directed the writing of the paper*: Chuanglong He, Meifang Zhu, Shumin Zhang and Xiumei Mo. *Directed the writing of the paper*: Mohamed EL-Newehy, Hany EL-Hamshary and Yosry Morsi.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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