In vitro evaluation of electrospun PLGA/PLLA/PDLLA blend fibers loaded with naringin for guided bone regeneration

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The present study was to evaluate fiber mesh loaded with naringin via electrospinning to guide bone regeneration in vitro. The naringin-loaded fiber mesh was prepared via electrospinning of PLGA, PLLA, PDLLA blending solution with naringin. SEM showed the naringin decreased the fiber’s diameter according to the concentration of naringin. After 20 days’ degradation in PBS, the drug-loaded fiber meshes still kept their stability with about 10% decrease in tensile strength. In vitro release experiments showed a sustained and steady naringin releasing profile with little initial burst releasing. Compared to the mats without naringin, the fiber mats loaded with naringin showed the most pronounced enhancement of cell growth when MC3T3-E1 cells were cultured on the fiber mats. The blend fiber loaded with naringin has optimized physical properties and sustained release profile in vitro. The study presents a promising fibrous mesh material for guided bone regeneration therapy.

Keywords: Electrospinning, Naringin, PLGA/PLLA/PDLLA, Guided bone regeneration

INTRODUCTION

In dentistry, guided bone regeneration (GBR) applying membrane directs the growth of new bone1-9. The most important function of the membrane materials is the ability to regenerate bone and to prevent connective tissue ingrowth. Used in conjunction with sound surgical technique, GBR has become a reliable and validated procedure for local periodontitis defect treatment and increasing the width and height of the alveolar ridge before implant installation and in the treatment of peri-implant bone defects. In order to improve the effects of this procedure, drugs and bioactive factors have been administered along with the GBR membrane4,5. Naringin is a double-hydrogen flavonoid compound extracted from Rhizomadrynariae. It has many kinds of biological activity and pharmacological action including anti-oxidation, anti-tumor and anti-radiation effects, bacteriostasis, and inhibiting tumor necrosis factor-a releasing6-12. Naringin has been shown to inhibit HMG-CoA reductase, activate the BMP-2 promoter11 and induce the expression of osteogenic markers of osteoblasts12. Naringin can also dose-dependently suppress the number of osteoclasts formed by the treatment with interleukin-1 (IL-1)9. Moreover, several recent in vitro studies also revealed that naringin improved the performance of osteoblasts and promoted bone restoration6,13. When naringin is incorporated into the GBR membrane, which served as a controlled-release GBR scaffold, the drug-loaded mats can deliver drugs locally after implantation, which is suitable for bone tissue reconstruction and regeneration.

The materials that are used as a membrane for GBR procedures should meet several prerequisites. As the membrane is supposed to be implanted in the body, it must be biocompatible, non-immunogenic and non-toxic. To avoid the removal of the membrane after healing, it would be better to be composed of biodegradable materials. The degradation time should be long enough to achieve bone regeneration before membrane disintegration. Other properties such as tissue integration, cell occlusivity, nutrient transfer, space making ability and ease of use in the clinic are also of interest. Due to their high surface-area-to-volume ratio, drug-loaded electrospun polymer fibers show pronounced drug encapsulation efficiency and better stability than other drug carriers. Electrospinning is a widely used method for fiber fabrication in many fields, especially the biomedical application. The electrospun nanofiber also affords the promising prospect of preparing useful polymer GBR membrane with controlled drug release property6,14-18. The materials selected for drug loading include poly lactic acid (PLA), poly(lactide-co-glycolide) (PLGA), poly(D, L-lactic acid) (PDLLA), polycaprolactone (PCL), and other biocompatible and biodegradable polymers6,16,19,20. Among different classes of biodegradable polymers, the thermoplastic aliphatic polyesters, such as PLA, PLGA and so on, have generated much interest due to their favorable properties5,19-23.
Considering the GBR application, the drug loading membrane should have balanced properties between the biodegradability and mechanical strength. However, these two contradictory properties are both influenced by the molecular weight, the copolymer ratio and the fabrication conditions simultaneously. That means when one property improved the other must be deteriorated. Single polymer formula can hardly meet all the GBR membrane requirements at the same time. The blending degradable polymer formula should be a solution to this problem. One polymer designed for a proper degradation speed while the other polymer maintains the strength.

In the present study, naringin loaded PLGA/PLLA/PDLLA blend fiber meshes were fabricated using electrospinning. The PLLA/PDLLA in the blend was designed to increase the strength and keep the basic frame for a relative long time during GBR therapy, while PLGA was designed to increase the degradation of the blend with a desire to control the drug release. This mat releases essential amounts of its ingredients within a period of time suitable for GBR treatment. Properties of mechanical strength, release profile and biocompatibility in vitro were characterized systematically.

**MATERIALS AND METHODS**

**Materials**

PLGA (M<sub>n</sub>=48,000, PLA/PGA=75/25), PDLLA (M<sub>n</sub>=50,000), and PLLA (M<sub>n</sub>=100,000) were purchased from Jinan Daigang Biological Technology (Jinan, China). Methylene dichloride (DCM) was purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Hexafluoroisopropanol (HFIP) was purchased from Alfa Aesar (Shanghai, China). Naringin was purchased from Sigma-Aldrich (Shanghai, China). All chemicals and solvents were used without further purification.

**Methods**

1. **Blend mesh preparation**

The preparation processing was similar to the work we published<sup>24</sup>. Electrospinning was performed using a high-voltage power supply (GF-2, Dongwen High voltage power, Tianjin, China) at 15 kV, an syringe pump (KDS-100, KD Scientific, Holliston, MA, USA) at a constant flow rate (2.0 mL/h), and a syringe equipped with a stainless steel blunt-ended needle (inner diameter 0.57 mm) with an gap of 10 cm between the collector and the needle tip. The DCM and HFIP mixed solvent with volume ratio of 2:3 were made before the experiment. The compositions of samples A, B and C are listed in Table 1.

2. **Morphological characterization**

The blend fiber meshes were coated with gold and examined using a scanning electron microscopy (SEM, XL-30, PHILIPS, Amsterdam, The Netherlands) at an accelerating voltage of 5 kV.

3. **Differential scanning calorimetry (DSC)**

DSC was used as a rapid test method to investigate the glass transition temperatures (T<sub>g</sub>) and other phase transitions of the polymer fibers. These experiments were performed on dry fiber mats in a Pyris1 apparatus (Perkin Elmer, Shanghai, China). The samples were analyzed in perforated and covered aluminum pans under a nitrogen purge. The samples were subjected to a heating scan from 25°C up to 200°C at 10°C/min. After cooling down to 25°C at a rate of 10°C/min, another heating run was performed from that temperature up to 200°C at 10°C/min. The first scans are those analyzed here.

4. **Degradation in vitro**

Naringin-loaded electrospun blend fiber meshes were soaked in a vial filled with 50 mL of PBS buffer (pH=7.4). The vials were incubated for 20 days at 37°C in a thermostat-controlled shaker with a shaking speed of 90 rpm. The meshes were removed, air-dried, and then cut into rectangular shape with 30×6 mm for mechanical testing. Mechanical characterization was performed using testing loads to the specimens at a speed of 0.1 mm/s, and five samples were evaluated for each composition (AG-1, Shimadzu, Kyoto, Japan). The surface morphology of the samples after degradation in vitro was coated with gold for SEM observation.

5. **Drug release in vitro**

To study drug release behavior, a naringin-loaded electrospun blend fiber mesh (200 mg) was soaked in a vial filled with 50 mL of PBS buffer (pH=7.4). The vials were incubated at 37°C in a thermostat-controlled shaker at a shaking speed of 90 rpm. In the case of control test, the blended fiber without drugs was added. At the predetermined time intervals (1, 4, 16, 24 h and 2–7 days), 5 mL of the original buffer solution was transferred for release testing, and then another 5 mL

| Sample | Composition | Naringin  |
|--------|-------------|-----------|
| A      | PLGA, PLLA, and PDLLA (m₁/m₂/m₃=2:1:1) | 0         |
| B      | PLGA, PLLA, and PDLLA (m₁/m₂/m₃=2:1:1) | 0.7 wt%   |
| C      | PLGA, PLLA, and PDLLA (m₁/m₂/m₃=2:1:1) | 7.0 wt%   |

Solvent: HFIP/DCM=3/2(v/v)
fresh buffer solution was added. The naringin released from the original buffer solution was centrifuged at 10,000 rpm and the supernatant was collected and monitored by a UV-visible spectrophotometer at the wavelength of 278 nm using a ultra-violet spectrometer (UV-2550, Shimadzu). Then, the accumulative weight and relative amount of the naringin released were calculated as a function of incubation time. Three samples were evaluated for each composition.

6. Cell culture
The blend fiber meshes with a diameter of 10 mm were disinfected by Co\(^{60}\) γ-ray at a dosage of 5 kGy. Preosteoblasts (MC3T3-E1) were used. The fiber samples were put at the bottom of a 24 wells plate in three groups with a cell density of 1×10\(^4\) cells/well in 800 μL DMEM with 10% of fetal calf serum and 0.1% of AAS (antibiotic antimycotic solution) in a 37°C, 5% CO\(_2\) incubator. The tissue culture plates were used as control. To investigate the effects of the naringin in the fiber mats on the viability of MC3T3-E1, the MTT test was used according to the instruction of MTT assay kit (Beyotime Biotech, Shanghai, China). The MTT assay was as follows: 20 μL of MTT solution (5 mg/mL) in PBS (pH=7.4) was added to each well. The incubation was continued for another 4 h, and then the solution was carefully aspirated from each well. Thereafter, the MTT derivative was completely dissolved with 100 μL DMSO, and the optical density (OD) of the solution was measured with a microplate reader (Multiskan MK3, Thermo Fisher Scientific, Waltham, MA, USA) at 492 nm. The OD values in one sub-group were averaged. Relative cell viability was calculated by dividing the OD value of the test subgroup by that of the test cell subgroup in the same plate. All experiments were performed in triplicate.

To prepare the cell-cultured samples for SEM observation, the scaffolds were first gently rinsed twice with PBS followed by fixation with 4% formaldehyde for 30 min. The samples were again rinsed twice with distilled water and then dehydrated in graded concentrations of ethanol (30, 50, 70, 90, and 100% at 10 min each). Finally, they were air-dried overnight under a fume hood. Dry cellular constructs were gold sputtered and observed under the SEM at an accelerating voltage of 15 kV.

**Statistical analysis**
All the quantitative data were recorded as mean±standard deviation. One-way analysis of variance (ANOVA) was used to determine statistical significance between groups. A \(p\)-value less than 0.05 was considered as statistically significant.

**RESULTS**

**SEM observation**
The microstructures of the meshes are as shown in Fig. 1. As shown in Fig. 1, the fibers all were successfully fabricated by electrospinning whether naringin was loaded or not. All the fibers’ surfaces were smooth. The blank blend fiber meshes are in size with a fiber diameter of 2–3 μm, and no obvious bead occurs under SEM observation. The naringin-loaded blend fiber also shows no bead. However the drug-loaded fibers with a diameter of about 300±50 nm for sample B and 1,100±500 nm for sample C respectively are smaller than the blank fibers. Few particles were observed on the surfaces of the electrospun fibers in the sample C (loaded 7.0 wt% naringin). These particles were granular naringin. This is because naringin crystallizes readily when solvent evaporates after electrospinning. However, no obvious particles on the blend fiber loading 0.7 wt% naringin are observed in Fig. 1B.

**DSC**
Figure 2 shows the DSC analysis of pure materials and their blends containing 7.0 wt% naringin. A single glass-transition step as shown for the material firstly occurs in each sample, except naringin. Figure 2 also shows that the naringin’s inoculation into the fiber mat led to a lower Tg (47.33°C at the cure of \(e\) in Fig. 2). By adding drug into the electrospinning polymer fibers, the small molecule drug acted on the molecular chains and made the molecular chains move easily, leading to a lower Tg\(^{23}\). The exothermic peaks in the range of 100–140°C are attributed to crystallization of PLLA, the PLLA-blend and the PLLA-blend with naringin respectively.

![Fig. 1](image-url) SEM images of blend fiber mesh: (A) blank blend (sample A); (B) 0.7 wt% naringin-loaded blended mesh (sample B); (C) 7.0 wt% naringin-loaded blend fiber mesh (sample C).
Both exothermic peaks of the PLLA-blend and the PLLA-blend with naringin are obviously weaker than that of pure PLLA. It is suggested that the crystallization of PLLA in the blends and blends with naringin would decrease. By the way, the obvious shift of the exothermic peak of the blend crystallization occurs, which is relative to the existence of naringin. A third phase transition was detected in the range of the melting point of naringin of 159–160°C, also indicating the presence of crystallized naringin, which is consistent with SEM results in Fig. 1. The endothermic peak shifted with the addition of naringin, implied that an interaction would exist between naringin and the blend.

**Drug release curve**

Figure 3 shows that the cumulative release profiles of naringin from the blend fiber meshes (samples B and C) within 7 days. The release of naringin was sustained
and steady and less pronounced than the initial burst release. The black and red curves show the slow release of 0.7 wt% and 7.0 wt% naringin-loaded ternary blend fiber mesh, respectively. The Figure shows the cumulative release of 0.7 wt% blend fiber mesh to be more pronounced than that of the 7.0 wt% naringin-loaded blend. The cumulative release of 0.7 wt% naringin-loaded blend at the end of the experiment was 82%, and the cumulative release of 7.0 wt% naringin-loaded blend was only 11% on the 21st day. At the initial 4 h duration, the release of 0.7 wt% naringin-loaded blend was obviously stronger than that of 7.0 wt% naringin-loaded, although the naringin granular precipitated from the 7.0 wt% naringin-loaded blend fiber mesh like the SEM morphology of sample C (Fig. 1).

Degradation test
Most of the fibers appeared more swollen than they had in their original formation, as shown in Fig. 4. This was because of a chain relaxation that took place in the matrix polymer after 20 days of incubation in the medium. The web mats retained their fibrous structures after 20 days of degradation. No obvious fragments or break down were observed. It is suggested that the mesh may retain its basic frame even after 20 days of constant rinsing. This may facilitate tissue reconstruction in vivo. The mechanical properties of decreased visbly were as shown in Table 2. The tensile strength of the blend loaded with 7.0 wt% naringin decreased more than that of the 0.7 wt% mesh. At the same time, the representative stress-strain curve (Fig. 5) showed that the mechanical behavior had changed from the elastic-plastic behavior of the undegraded samples to the brittle behavior of degraded samples. The change may be attributable to the degradation of PLGA in the blend, because the flexible chains degraded quickly and the ratio of ductile chains of PLGA comparatively increased after 20 days degradation.

Biocompatibility
In this study, the cytotoxicity of the fiber mats to MC3T3-E1 cells was examined by MTT, as shown in Fig. 6. Cell viability increased with incubation time and naringin content. The blend loaded with 7.0 wt% naringin showed more improvement in cell viability than that loaded with 0.7 wt% naringin. The cytotoxicity tests demonstrated that naringin had no cytotoxic effects but rather fostered cell viability. The morphology of the cells on the surface of the membrane is shown in Fig. 7. All cells attached to the membranes very well, and kept increasing in number over time. The results confirmed that naringin could improve the proliferation of bone-like cells in vitro and the relatively high concentration of naringin increased cell viability.

Table 2 Tensile strength of the degraded blended fiber meshes after 20 days

| Sample            | A       | B       | C       |
|-------------------|---------|---------|---------|
| Undegraded (MPa)  | 2.043 (0.875) | 2.856 (0.851) | 3.546 (0.877) |
| Degraded (MPa)    | 1.910 (0.433) | 2.605 (0.675) | 3.095 (0.719) |
| Decreasing ratio (%) | 6.50     | 8.79     | 12.72   |

Fig. 5 Representative stress-strain curves of undegraded and degraded blended fiber meshes.

Fig. 6 Cell viability of MC3T3-E1 on blended fiber mesh as indicated by MTT assay (*”, represents a p<0.05).
From these results, it can be concluded that the biocompatibility of the loaded naringin fiber mats is closely related to the release behavior of the drugs. The fiber mats loaded with 7.0 wt% naringin showed the most pronounced enhancement of cell proliferation.

**DISCUSSION**

A variety of membrane materials has been developed for GBR. The thermoplastic aliphatic polyesters has been proven as a suitable materials for GBR. For three main reasons PLGA/PLLA/PDLLA blend was used as a matrix material: First, all of them are bioreabsorbable materials based on polyesters; second, their features are suitable mechanical stability; third, they all are easy to fabricate via electrospinning. Usually, a typical GBR-membrane is supposed to stay intact for several months. It would take about 3 months for the PLGA scaffolds to degrade to half of the original mass. The form stability of the mats as well as their constituent fibers (Fig. 4) in a liquid medium such as PBS over a test period of 20 days is an advantage that supports their clinical usability in the bone regeneration. A too-quick degradation of the blend fibers to lactic acid could lead to an acidosis and interfere with wound healing. The mechanical stability of the fiber mats is also essential to resist the mechanical stress caused by the high production of sulcus fluid. By the way, the mechanical properties of the composite fiber membranes with naringin are better than that without naringin. This may be due to strengthening by naringin,
which acted as a crystal particle to enhance the polymer composite matrix.

Many resorbable drug delivery systems were developed during recent decades, such as drug loaded hydroxypropyl cellulose films, which were first described by Noguchi et al., or drug carrying gels such as Elyzol (Dumex, Bad Vilbel, Germany) dental gel, based on melted glycerol mono-oleate. However, also for these systems, the periodontal milieu often poses the major problem that the required period of drug exposure (7 days) cannot be achieved, let alone bone formation which would require several months. Usually, a slower but longer release would be beneficial to the bone formation. Unfortunately, most drugs loaded in carriers, not matter whether the loading materials are, show obvious burst release. A rapid burst release is the typical problem of drug-release in its carrier. One of the purposes of our study is to solve the problem. The values obtained from the UV spectrometer measurements for the fibers loaded with 7.0 and 0.7 wt% naringin are very close to a sustained and steady drug-liberation in the duration of 21 days, as seen in Fig. 2. Like other polyester biopolymers, PLLA and PDLLA are quite hydrophobic. Hydrophilic naringin could not be encapsulated effectively even amphiphilic PDLLA has been added in the blends system. Fine Naringin precipitated on the fibers loaded with 7.0 wt% naringin, as seen in Fig. 1C. Due to the low solubility of naringin in water at room temperature and the interaction between the blend and naringin (as shown in DSC curves in Fig. 2), an initial burst of naringin did not occur. Naringin was embedded into the PLGA/PLLA/PDLLA fibers, therefore a steady and slow release of naringin from the system was obtained. The polymer fibers gradually degraded, rather than directly diffused into the medium. For the degradation of polyester biopolymers, the stage I (the initial 20 days) would have a rapid decrease in the molecular weights with little weight loss, therefore a duration of “21 days” was selected as the period of drug-release in vitro.

The major impact of the fiber diameters on the initial drug release was demonstrated in the study of Reise’s report. The thinner the fiber diameter was, the larger the drug release was. Therefore, compared with that of 7.0 wt% blend fiber, the cumulative release of 0.7 wt% blend fiber with a thinner fiber diameter was more pronounced.

Naringin inhibits retinoic acid-induced osteoporosis in rats, and in addition, may enhance bone morphogenetic protein levels in osteoblasts. Naringin enhanced the proliferation and osteogenic differentiation of human bone marrow stromal cells. Naringin in a collagen matrix increases local bone formation. In this study, the naringin-loaded fiber mats also enhance the cell proliferation of MC3T3-E1. It is suggested that electrospun naringin-loaded fiber can be served as a controlled-release GBR scaffold.

Looking on the intended clinical use of the naringin-loaded PLGA/PLLA/PDLLA fiber mats, the main advantage of such a local drug delivery systems is the relatively steady and sustained amount of drug that is necessary to achieve lasting concentrations in the GBR treatment. Moreover, excellent stability and good cell adhesion might be another advantage of the fiber mats enabling them to retain within the repairing site despite high sulfus-fluid rates.

CONCLUSION

The PLGA/PLLA/PDLLA blend fibers loaded with naringin were prepared via electrostatic spinning. The drug-loaded blend fibers were smooth and uniform in size. The addition of naringin decreased the fiber diameter obviously. As naringin increased, the mechanical properties of the blended fiber membranes improved. A sustained and steady release of naringin from the drug-loaded blend fibers was obtained, altogether with less pronounced than the initial burst release. The in vitro degradation results showed that the web mats retained their fiber structure and that their mechanical properties worsened after 20 days of incubation in the medium. An in vitro cytotoxicity assay showed that the electrospun blend fiber mats loaded with naringin showed enhancement on MC3T3-E1cells. The present results have implications for the development of new implantable polymeric devices for GBR therapy.

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