Supplementary information

Fabrication and evaluation of electrospun PCL-gelatin micro-/nanofibers membrane for anti-infective GTR implant

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Materials and methods

S1 Materials

Poly (ε-caprolactone) (PCL: M_n =70-90 kDa) and 3-(4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) (MTT) were obtained from Sigma-Aldrich and used as received. Gelatin was purchased from Rousselot (France). Metronidazole (MNA) was purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Dulbecco's modified eagle medium (DMEM), Fetal bovine serum, 0.05% Trypsin EDTA and phosphate buffer saline (PBS) were purchased from Gibco (USA). Methanol with HPLC reagent was purchased from Alfa-Aesar Chemical Inc. (USA). WST-8 was purchased from Beyotime Institute of Biotechnology. Other chemical reagents such as dichloromethane (DCM), N-N'dimethylformamide (DMF) and trifluoroethanol (TFE) were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. (China) and used as received without further purification. L929 fibroblast cell lines were kindly donated by Jishuitan Hospital.

S2 Molecular dynamics simulation approach and the interaction of PCL and MNA

In this force-field approach, the total energy E_T of the system is represented by the sum of bonding and nonbinding interactions given as $E_T=E_b+E_0+E_\psi+E_{loop}+E_{pe}+E_{vdw}+E_q$. (1)

The first five terms represent the bonding interactions, which correspond to energies associated with the bond E_b, bond angle bending E_0, torsion angle rotation E_ψ, out of loop E_{loop}, and potential energy E_{pe}. The last two terms represent the nonbonding interactions, which consist of the van der Waals term E_{vdw} and electrostatic force E_q. This force-field approach describes the intramolecular and intermolecular interactions in the electrospinning system of MNA and PCL. The COMPASS force field has been widely used to optimize and predict the structural, conformational, and thermophysical condensed phase properties of molecules including polymers. Initial velocities were set by using the Maxwell −Boltzmann profiles at 298 K. The Verlet velocity time integration method was used with the time step of 1 fs.

Amorphous cells were constructed containing blends of four chains of 30 repeat units of PCL and MNA with different content. First, the PCL polymer chains and MNA were built in periodic boundary cell (Figure S1). Then the cell was taken by energy minimization to get the cell with the lowest energy. After that, the systems were equilibrated in the isothermal-isobaric (NPT) ensemble at 298 K. This equilibration was usually done for 5 ps with the dynamics that was followed by the data accumulation running for at least 100 ps with the configurations saved every 5 ps. At last, the proper cell can be used to analyse hydrogen bonds.

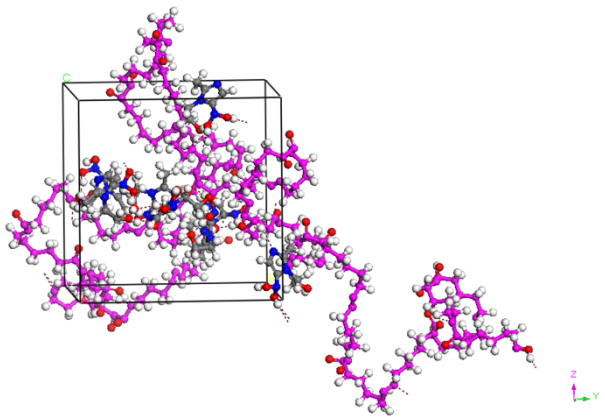

Fig. S1. Simulation for hydrogen bonding through molecule modeling using Materials Studio Software (in the unit cell: red, O; white, H; gray, C; blue, N; purple, back bonds of PCL matrix).

S3 FTIR, DSC, XRD and mechanical analysis

FTIR analysis was performed on a Bruker Tensor 27 FTIR spectrometer. The scan range was 4000 cm^{-1} to 600 cm^{-1} with a resolution of 2 cm^{-1}. The thermal properties of the membranes were investigated by the DSC measurements (Mettler-Toledo International Inc., Switzerland). Samples with a weight of approximately 5 mg were loaded in an aluminum crucible under dry condition. The samples were cooled from 30 °C to -100 °C and stayed for 5 minutes, then heated to 220 °C under nitrogen atmosphere at a heating rate of 10 °C /min.

X-ray diffraction (XRD) analysis was carried out on a Rigaku D/max-Ultima III X-ray diffractometer equipped with Cu-K α source and operating at 40 kV and 40 mA. The diffraction patterns were obtained at a scan rate of 5 °/min.

The mechanical properties of the membranes both at dry state and wet state were evaluated by using a BOSE ElectroForce 3200 test instrument with a 50-N load cell at a crosshead speed of 5 mm/min at the ambient temperature of 25 °C. All samples were cut into rectangles with dimensions of 25 mm × 4 mm. Five samples at dry state were tested for each membrane. Another five samples were soaked in deionized water for 1 h for wet state tensile property measurements. The thicknesses of the samples were measured with a micrometer accurate to 1 μm.

S4 Cytotoxicity evaluation

MTT assay was used to test the cytotoxicity of the membranes with different content of MNA. The 20 mm × 30 mm samples were sterilized by washing with a 75% (v/v) ethanol solution in sterilized water, exposed to Co^{60} for 15 min, and then incubated in DMEM at a proportion of 3 cm^2/mL for 24 h at 37 °C to get the extract solution of the samples. The extract solution was then filtered (0.22 μm pore size) to eliminate the possible presence of solid particles of the sample. L929 cells were resuspended in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) at a density of 1.0 × 10^4 cells/mL and 100 μL cell resuspension solution was pipetted into 96-well micrometer plates. After incubated at 37 ℃ under 5% CO_2 atmosphere for 24 h, the medium was replaced by the previously prepared extracted dilutions (50%, volume proportion), the culture medium as blank control and DMSO as negative control. After 24 h, 48 h and 72 h of incubation, morphology of the cells in plate were observed using inverted phase contrast microscope (Olympus

IX50-S8F2), then the cells were treated with 20 μL/well MTT (5 mg/mL in PBS solution) and incubated for another 4 h at 37 °C in a humidified atmosphere of 5 % of CO2. At this stage the MTT and culture medium were removed and 200 μL/well of DMSO was added to dissolve the forming formazan crystals. After shaking the plate for 15 minutes, the optical density (O.D.) was read on a multi-well microplate reader at 630 nm. All materials' extracts were tested for six averages.

Cytotoxicity of PG30 was also tested with human periodontal ligament fibroblasts (hPDLFs) and ROS cells using a Presto Blue assay (Life Technologies). Prestoblue™ is a blue non-fluorescent, cell permeable compound (resazurin-based solution), that is reduced by living cells into a fluorescent compound (resorufin). Commercially available hPDLFs were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/mL penicillin and 50 U/mL streptomycin. ROS cells were cultured in α-MEM supplemented with 10% (v/v) FBS. P30 was cut into 20 mm × 30 mm rectangle. The samples were disinfected in 100% isopropanol for 20 minutes under shaking followed by three rinses in PBS under sterilization environment. After that, 8 mL cells culture medium (DMEM+10 % FCS for hPDLFs and α-MEM+10 % FBS for ROS cells) were separately added into the sample in a sterilization tube to get the extract substrates at 37 °C for 72 hours. Cells were resuspended accordingly in culture medium at a density of 5×10^5 cells/mL and 100 μL cell resuspension solution was pipetted into 24-well plate after 1 mL culture medium was added in the plate. After incubation for 24 hours, medium were replaced with 900 μL extract substrates per well. The viability of the cells cultured in the extract solution was assessed using the PrestoBlue assay. With another 24 hours incubation, 100 μL PrestoBlue™ was pipetted per well. At 1 hour incubation, 200 μL dye-containing medium of each well was placed into 96-well plate in duplicate, and fluorescence intensity was measured using a fluorescence reader (TECAN Spectrophometer) at an excitation wavelength of 570 nm and emission wavelength of 600 nm. Every sample experimented for 3 averages.

S5 Proliferation of cells on the membranes

The membrane was cut into circle 2.5 cm in diameter, sterilized, fixed on a 24-well plate Cell-Crown (Sigma, USA), and then put into the well of 24-well plate. L929 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 μg/mL penicillin and 100 mg/mL streptomycin) at a density of 4.0×10^4 cells/mL. Cell culture medium(800μL) and cell suspension (100μL) were plated onto the sample carefully. Cell suspensions added into wells with no materials were regarded as control. Plates were incubated at 37 °C under 5% CO_2 atmosphere. The medium was changed every two days. After incubating for 4 h, the sample was washed with PBS for three times to remove the cells didn't attach on the membrane and 100 μL WST-8 (final dilution: 1:10) which can react with dehydrogenase in mitochondria getting a water soluble formazan was added onto the sample to test the alive cells. After incubating for another 4 h, 100 μL of supernatants was transferred into a 96-well plate to read the optical density (O.D.) at 450 nm. At days 1, 3, 5 and 7 of culture, cell proliferation and viability were also assessed using CCK-8 test as above. The cells-cultured membrane was afterwards washed with PBS for three times, fixed with 3% glutaraldehyde at 4 °C for 2 h, soaked in 0.18 M sucrose solutions at 4 °C for 2 h and then was dehydrated through a series of graded ethanol solutions and lyophilized overnight. The morphology of cells on the membranes were observed by using SEM.

The PG30 membrane was cut into circles 2.5 cm in diameter. PG30 was first disinfected in 100% isopropanol for 20 minutes and then rinsed in PBS three times. The circular membranes were fixed

on Cell-Crown (Sigma, USA) and placed inside the wells of a 24-well plate. After the addition of 800 µL of culture medium on the membrane per well, hPDLFs and ROS cells separately suspended in 100 µL of culture medium at a density of 5×10^5 cells/mL were added gently on each membrane. The viability of the cells on the membrane was measured by using a fluorescence reader after 2 days of post-seeding.

S6 Barrier function to fibroblast cells

The schematic diagram of characterization of barrier function of membranes is shown in Figure S2. The membrane was cut into a circle 2.5 cm in diameter, sterilized, fixed on a CellCrown™, and put into a 24-well plate without touching the bottom of the well. L929 cells were resuspended in DMEM supplemented with 10% (v/v) FBS at a density of 4.0×10^4 cells/ mL. Then 1 mL of culture medium without cells was added to the well from the outside of the CellCrown™, and 900 µl of culture medium plus 100 µl of cell resuspension was added onto the sample. After incubated at 37 °C under a 5% CO_2 atmosphere for 1 day, 3 days, 5 days, and 7 days, the CellCrown™ was taken out, and the bottom of the 24-well plate was observed under an inverted phase contrast microscope (Olympus IX50-S8F2) to determine whether the cells got through the membrane to the bottom of the well or the culture medium. The other side of the membrane, which had no contact with the cells, was observed under a scanning electron microscope to find out whether the cells penetrated the membrane. The barrier function of the membranes after 1 month of degradation in PBS was also evaluated as above.

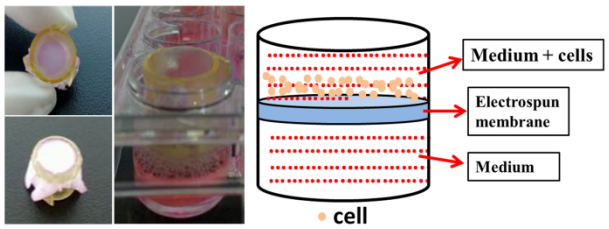

Fig. S2. Schematic diagram of barrier function characterization of electrospun membrane.

Results and discussion

S7 Interaction between PCL and MNA at different MNA content analyzed by molecular dynamics simulation.

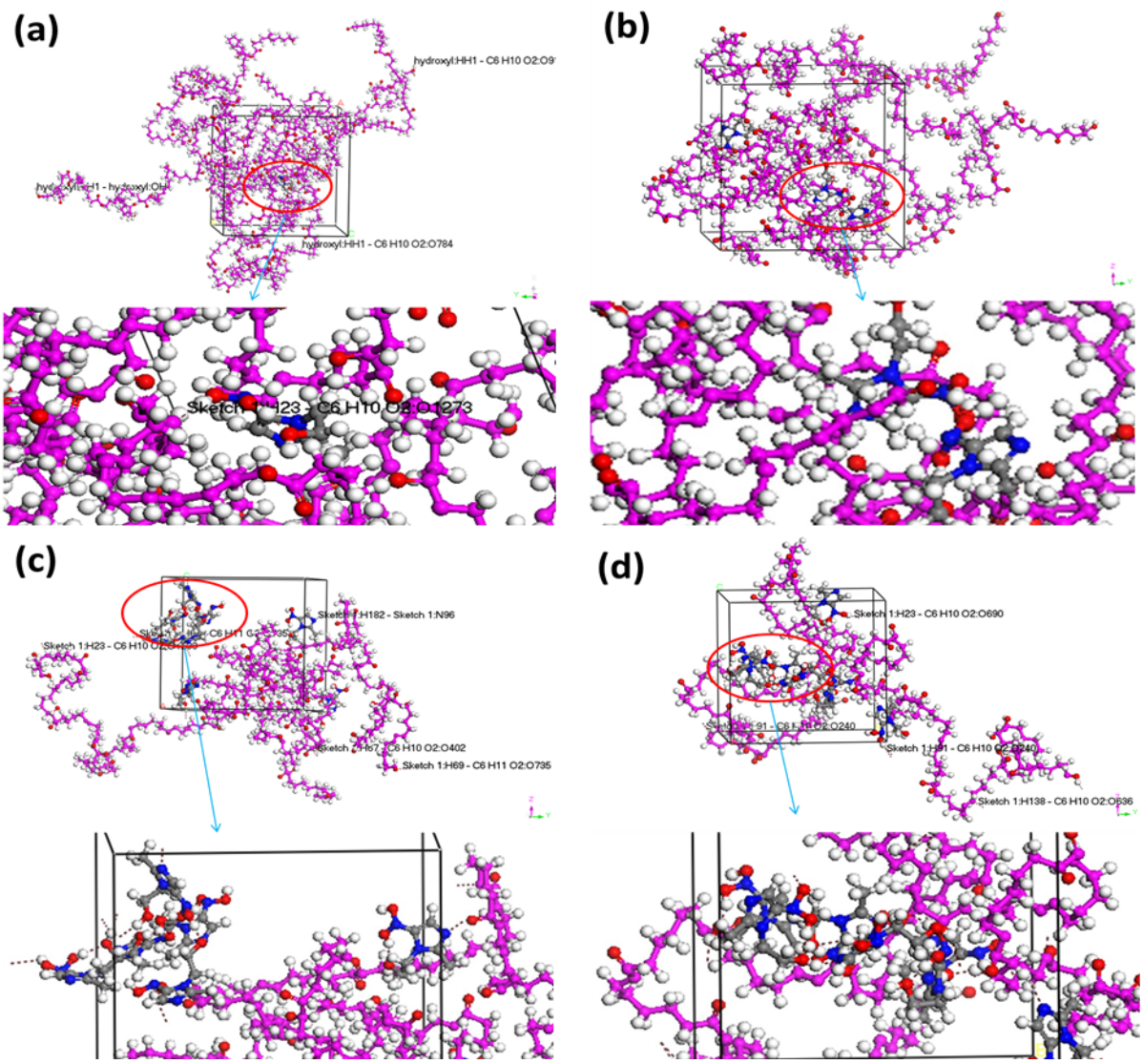

Fig. S3. Formation of HBs between MNA and PCL with different MNA content of PCL: (a) 1 wt.%; (b) 5 wt.%; (c) 20 wt.%; (d) 40 wt.% simulated by molecular modeling using Material Studio Software. (In the unit cell: red, O; white, H; gray, C; blue, N; purple, back bonds of PCL matrix).

S8 Sink condition studies of the drug release experiment

Sink condition is the fundamental requirement of drug release studies. Metronidazole's solubility in buffers at pH 7.0 was reported as 11.6 mg/mL at 37 ^{o}C [S2, S3]. The weight of a membrane with circle 2 cm in diameter was no more than 10 mg. At the highest drug content of 40 wt.%, with the encapsulation efficiency of 84.1%, MNA loaded in the sample was no more than 3.4 mg. Even if all MNA in the sample released into 5mL PBS, the concentration of MNA would be 0.68 mg/mL. The

saturation solubility of metronidazole in buffers is 11.6 mg/mL. The highest drug concentration during the drug release experiment was lower than 0.68 mg/mL. Thus the saturation solubility of MNA in the dissolution medium is ten times more than the drug concentration, reflecting that the sink condition was maintained.

Dissolution study of MNA pure powder was also performed as the same method that the drug release profile of MNA-loaded membrane was tested. MNA with a weight of 10 mg, which is 3 times more than the drug weight in the electrospun membrane, was incubated in 5 mL of PBS at 37 ^{o}C with mild shaking. At predetermined timed intervals (5, 10, 15, 20, 30 minutes until MNA dissolved completely in PBS), 1 mL of the dissolution medium was withdrawn and filtered for HPLC detection to determine the amount of drug dissolved. The remaining medium was removed and replaced with another 5 mL of fresh PBS. The dissolution curve is shown in Fig. S4, after 10 minutes, almost 90% drug dissolved in PBS.

Besides, the dissolve test of MNA at a concentration of 2 mg/mL, which is 3 times more than the highest drug concentration during drug release profile test of the electrospun membrane, also showed that the MNA dissolved completely in PBS within 20 minutes. The highest drug concentration released from the membranes only can reach to 0.34 mg/mL in 1 day.

It can be concluded that the dissolution rate of the not-incorporated drug is much faster than the entrapped drug in the membranes, reflecting that the sink condition was maintained. Thus, the release rate of MNA from electrospun membrane is not a result of the drug solubility but a "matrix-effect". Volume of the release study was acceptable.

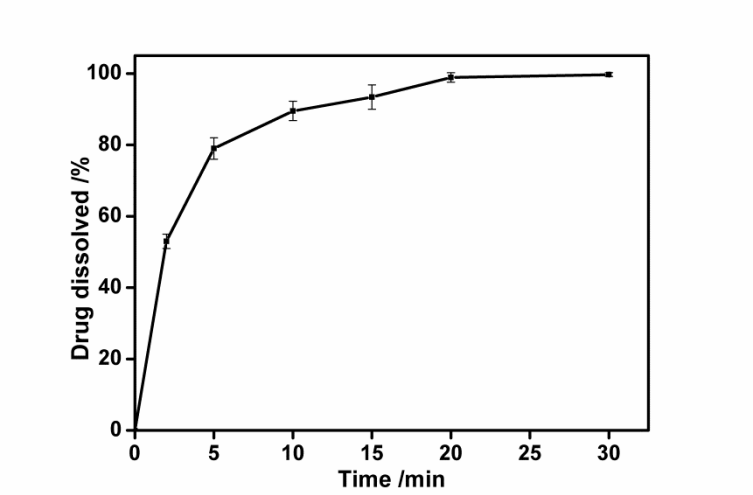

Fig. S4. The dissolution profiles of metronidazole powder released from PBS.

S9 Cytotoxicity of the electrospun membranes

The relative growth rate (RGR) reflects the viability of L929 cells growing in the material extract. From Fig. S5(a), the RGR of the cells is all higher than 80%; that is, all membranes show no cytotoxicity. As indicated in Fig. S5(b), the morphologies of L929 cells incubated for 24 h in the extract substrates of the electrospun membranes all show spindle, triangular, and quadrangular shapes with good growing conditions.

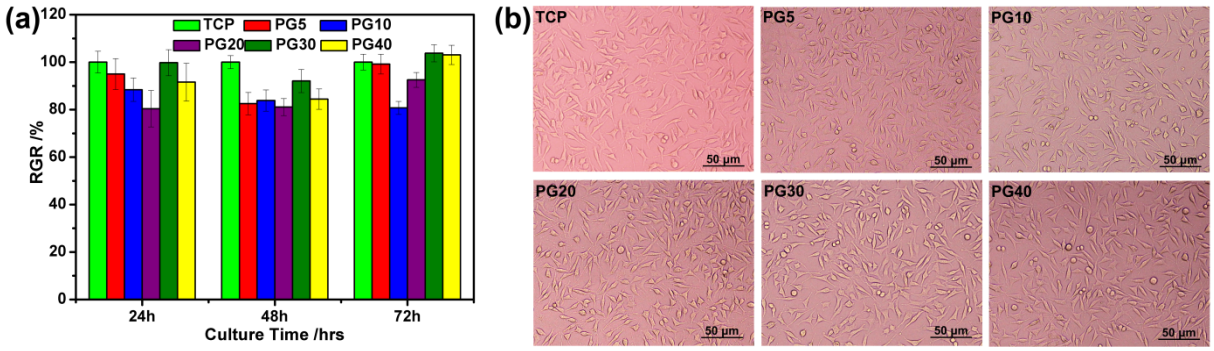

Fig. S5. Results of the in vitro cytotoxicity: (a) RGR of L929 cells cultured in extract substrates of membranes and (b) microscope images (10 x magnifications) of L929 cells cultured for 24 h in extract substrates of membranes.

The cytotoxicity of PG30 with hPDLFs and ROS cells was tested. The fluorescence intensities of hPDLFs and ROS cells cultured for 24 hours in the PG30 extract substrate are higher than those in the blank control as shown in Fig. S6, indicating that the membrane shows no cytotoxicity.

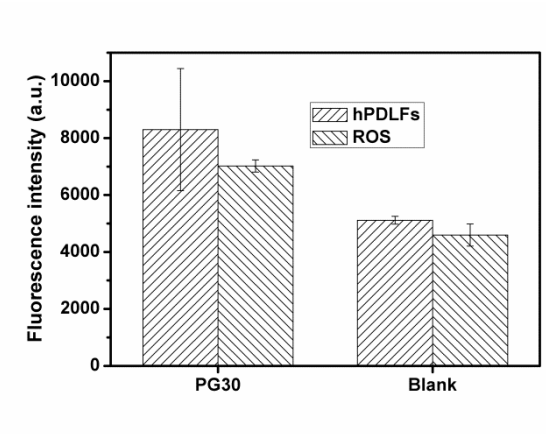

Fig. S6. Fluorescence intensities of hPDLFs and ROS cells cultured for 24 hours in extract substrate of PG30.

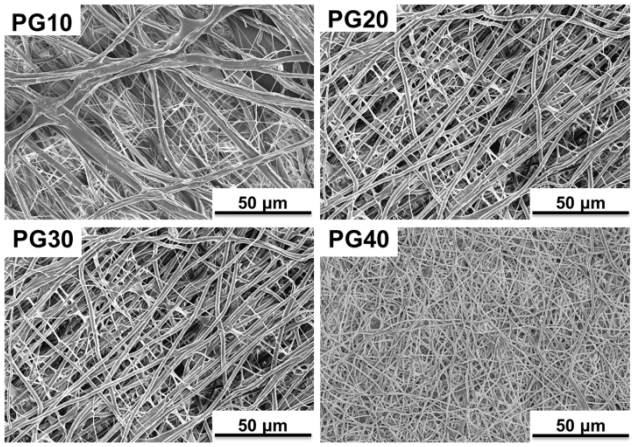

Fig. S7. SEM micrographs of opposite side of cells-seeded membranes after seeding for 3 days.

Reference:

S1. R. Wang, J. Ma, X. Zhou, Z. Wang, H. Kang, L. Zhang, K.C. Hua, J. Kulig, Macromolecules, 2012, 45, 6830-6839.

S2. H. Ogata, N. Aoyagi, N. Kaniwa, T. Shibazaki, E. Ejima, Y. Takagishi, T. Ogura, K. Tomita, S. Inoue and M. Zaizen, Int. J. Pharm., 1985, 23, 1618-627.

S3. M. O. Koeppe, R. Cristofoletti, E. F. Fernandes, S. Storpirtis, H.s E. Junginger, S. Kopp, K. K. Midha, V.P. Shah, S. Stavchansky, J. B. Dressman and D. M. Barends, J. Pharm. Sci., 2011, 100,1618-1627.