Supporting Information

Bioinspired Triple-Layered Membrane for Periodontal Guided Bone Regeneration Application

Yang Yang[a,b], Deng-Cheng Yang[c], Xian-Yan Long[a], Xiang Liu[a], Jing-Wen Lu[a], Zhou-Jing Zhang[a], Qian-Qian Shi[a], Yong Zhou*[a,d], Duo-Hong Zou*[a,e]

[a]College & Hospital of Stomatology, Key Laboratory of Oral Diseases Research of Anhui Province, Anhui Medical University, Hefei, 230032, China.

[b]Department of Periodontology, College & Hospital of Stomatology, Anhui Medical University, Hefei, 230032, China.

[c]Department of Pathophysiology, School of Basic Medical Science, Anhui Medical University, Hefei, 230032, China.

[d]Department of Dental Implantology, College & Hospital of Stomatology, Anhui Medical University, Hefei, 230032, China.

[e]Department of Oral Surgery, Shanghai Key Laboratory of Stomatology, School of Medicine, National Clinical Research Center of Stomatology, Ninth People's Hospital, Shanghai Jiao Tong University, Shanghai, 200011, China.

Email: zouduohongyy@163.com (Duo-Hong Zou); zhouyong@ahmu.edu.cn (Yong Zhou)

This file contains descriptions of characterizations, 9 supplementary figures and 1 supplementary table.

Characterizations

SEM images and EDS mapping data were acquired with a GeminiSEM 300 field emission scanning electron microscope. AFM images were obtained from a Bruker JPK NanoWizard 4 XP BioScience atomic force microscope. Nanoindentation curves were measured with a Bruker Hysitron TI 980 nanoindenter. Each sample in Figure 2d was measured 5 times. Tensile tests were carried out on an Instron 3342 tensile tester. The samples were cut into stripes (30 mm × 3 mm) for the tests. The wet samples were prepared by soaking the dry samples into DIW for 30 minutes, and then removing the residual water on the sample with a filter paper. Each sample in Figure 2f was measured 5 times. FT-IR spectra were obtained with an INSA Optics FOLI20 Fourier transform infrared spectrometer. XRD data were acquired with a Haoyuan Instrument DX-2700BH X-ray diffractometer. Contact angle measurement was carried out with a Krüss DAS30S drop shape analyzer.

2D XRD graphs for orientation analysis were acquired with a Rigaku XtaLAB PRO 007HF X-ray single crystal diffractometer equipped with a Dectris Pilatus 200k pixel array detector. To prepare a specimen for the measurement, a membrane was cut into thin stripes (20 mm × 1 mm). These stripes were stacked together and pressed tightly, and then both ends were fixed with Kapton tapes. The incident X-ray was parallel to the surface of these stripes. To prepare a control group specimen, the as-prepared translucent Na-MMT solution was froze in liquid nitrogen and dried in a Yamato DC810 vacuum freeze dryer. The obtained powder was adhered to a Kapton tape for the following data acquisition. The sample-to-detector distance was kept the same for all samples.

The fatigue tests were carried out on an Instron ElectroPuls E3000 all-electric dynamic test instrument. The samples were cut into stripes (20 mm × 2 mm). The frequency for all tests was 0.25 Hz. Initially the load on each specimen was close to zero. Then the load was applied on both ends, resulting in the bend of the specimen until the load reached the peak value. Then the clamp returned to its initial position to start another

cycle. To keep a wet specimen wet during the test, a humidifier was used to generate fog around the specimen.

To carry out the degradation experiments, the membrane was cut into a square shape (30 mm × 30 mm), and the initial mass of each specimen was recorded. Then the specimen was immersed in 100 mL of phosphate buffered saline (PBS) solution (Thermo Fisher Scientific Inc.) in a 250 mL beaker. The beaker was fixed in a shaker incubator (Zhichu Instrument Co., Ltd., ZQZY-85CN) at 37 °C, and the oscillation speed was 10 rpm. After certain intervals, the specimen was taken out, washed gently with DIW, and dried in an oven at 45 °C for 6 hours, and then the remaining mass was measured. Three independent specimens were tested.

In vitro and in vivo experiments

The SD rats in this research were raised in the Experimental Animal Center of Anhui Medical University. This research was approved and supervised by the Experimental Animal Ethics Committee of Anhui Medical University (Approval: LLSC20221259). All experimental procedure were carried out according to the ethics to minimize the suffering of the rats.

To isolate BMSCs, the femur and tibia were taken from 4-week-old male SD rats. The bone marrow in the middle part of the bones were obtained via rinsing with PBS solution. The marrow was suspended in MEM α (Gibco minimum essential medium) containing fetal bovine serum (20 vol%), penicillin (100 U/mL) and streptomycin (100 U/mL), and then placed in an incubator (37 ºC, 5 vol% CO_2). Cell passage was carried out using trypsin-EDTA solution (0.25%, Thermo Fisher Scientific Inc.) when the cell confluency reached 70% to 80%. The third generation of BMSCs were used for the in vitro experiments. rGFs were obtained in a similar way, except that the initial tissue was acquired from the SD rats' gingiva instead of femur or tibia.

The cytotoxicity evaluation was based on the international standard ISO 10993-12. The membrane samples were first cut into rectangles (40 mm × 60 mm), immersed in 4 mL of MEM α medium containing fetal bovine serum (10 vol%), and incubated at 37 °C

for 72 hours. The supernatant was collected and stored at 4 °C before use. Then BMSCs (5×10^3 cells/mL) were inoculated in a 96-well plate (100 μL/well) and cultured for 24 hours (37 °C, 5 vol% CO_2). Then the culture medium was replaced by the stored supernatant, whereas the control group was replaced by complete medium. CCK-8 assay was performed after 1 day and 4 days of incubation (37 °C, 5 vol% CO_2). The cell viability was quantified by measuring the optical density (OD) value at 450 nm (OD450) with an Infinite 200Pro M nano microplate reader (Tecan Trading AG).

To observe the cell adhesion on the membrane surface, the membrane samples were trimmed into round shape (diameter was 14 mm) and placed at the bottom of a 24-well plate. BMSCs (2×10^4 cells/mL) were inoculated on the surface of the specimen in each well (1 mL/well) and cultured for 24 hours (37 °C, 5 vol% CO_2). Then the cells were fixed with paraformaldehyde (4%, Biosharp), and incubated in Triton X-100 permeabilization solution (0.5 vol%, Beyotime Biotechnology Inc.) for 15 minutes. After that, the cells were incubated in TRITC-Phalloidin (Solarbio Science & Technology Co., Ltd.) for 30 minutes to stain the actin filaments, and then in DAPI (Kingmorn Biotechnology Co., Ltd.) for another 5 minutes to stain the nuclei. Antifade mounting medium (Beyotime Biotechnology Inc.) was used to prevent fluorescence quenching. The stained specimens were observed with a fluorescence microscope (Olympus IX 51). For SEM observations, after being fixed with paraformaldehyde, the cells (along with the membrane specimens) were dehydrated gradually with a series of ethanol/DIW mixtures (ethanol volume ratios were 50%, 60%, 70%, 80%, 90%, 95% and 100% respectively; 10 minutes for each mixture). Then the specimens were supercritically dried with a K850 critical point dryer (Quorum Technologies Ltd.) before SEM observations.

To evaluate the anti-infiltration performance of the barrier membrane, the membrane samples were trimmed into round shape (diameter was 14 mm) and placed at the bottom of a 24-well plate (PLA side was upward). rGFs (2×10^4 cells/mL) were inoculated on the PLA side of the specimen in each well (1 mL/well) and cultured for 7 days (37 °C, 5 vol% CO_2). The cells (along with the membrane) were fixed with paraformaldehyde,

dehydrated gradually with a series of ethanol/DIW mixtures, and then dried in a critical point dryer. Both sides of the membrane were checked by SEM observations to determine if the rGFs could infiltrate the membrane.

To conduct the RT-qPCR assay, the membrane samples were trimmed into round shape (diameter was 35 mm) and placed at the bottom of a 6-well plate. BMSCs (2×10^4 cells/mL) were inoculated on the surface of the specimen in each well (2 mL/well) and cultured for 7 days (37 ºC, 5 vol% CO_2). TRI Reagent Solution (Thermo Fisher Scientific Inc.) was used for the extraction of total RNA, which was then quantified with a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc.). BCA Protein Assay Kit (Takara Bio Inc.) was used to transcribe 500 ng of total RNA into cDNA. The cDNA templates were amplified using the specific primers of the four osteogenesis markers (ALP, RUNX2, OPN, and OCN; Table S1). The relative expressions of the markers were assessed by using the GAPDH as the internal control.

To create animal models for the in vivo experiments, 8 SD rats were anesthetized by intraperitoneal injection with pentobarbital sodium solution (50 mg/kg). A crestal incision (4 mm) was made on the alveolar ridge of the maxillary first molar tooth of each rat, and the full-thickness mucoperiosteal flap was separated and reflected to expose the bone surface (Figure S8a). Then standard periodontal defects were made with a ball drill on the mesial surface of the two maxillary first molar teeth on both sides (Figure S8b). The square-shaped defects, which were 2 mm in length/width and 1.5 mm deep, were covered either with a bioinspired membrane or an unmineralized membrane (Figure S8c). The PLA side of the membranes was placed against the gingiva (outward). In the control group, no membrane was used. The wound was closed via stitching with a 5-0 suture. In the first 3 days after the surgery, each rat was given 8000 units of penicillin via intraperitoneal injection. The rats were sacrificed four weeks after the surgery, and the maxilla were collected and fixed with paraformaldehyde (4%) for micro-CT imaging analyses (AX-2000, Always Imaging Ltd.). The voltage for generating X ray was 90 kV with a current of 70 μA. The exposure time was 500 ms, and the resolution was 9 μm. The 3D images were reconstructed and analyzed with VG

Studio Max 3.4. The bone volume-to-total volume (BV/TV), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were calculated for the assessment of the new bone formation.

Supplementary Figures

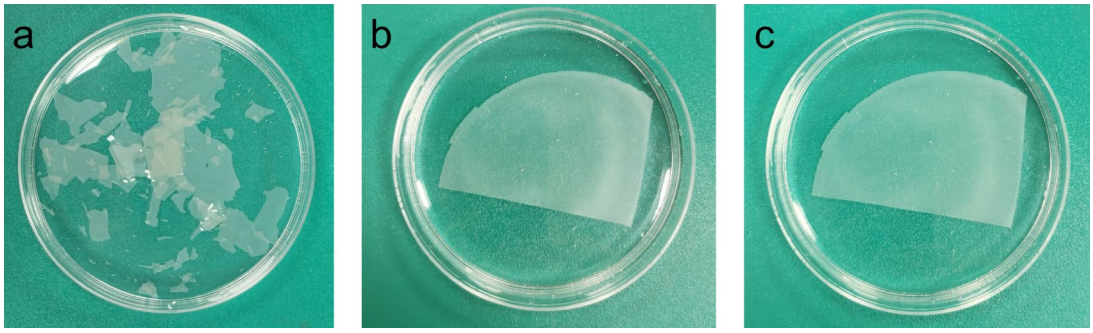

Figure S1. (a) Chitosan-clay membrane collapsed in 20 mL of DIW. In contrast, chitin-clay membrane was stable in DIW (b), even after adding 2 mL of acetic acid (c).

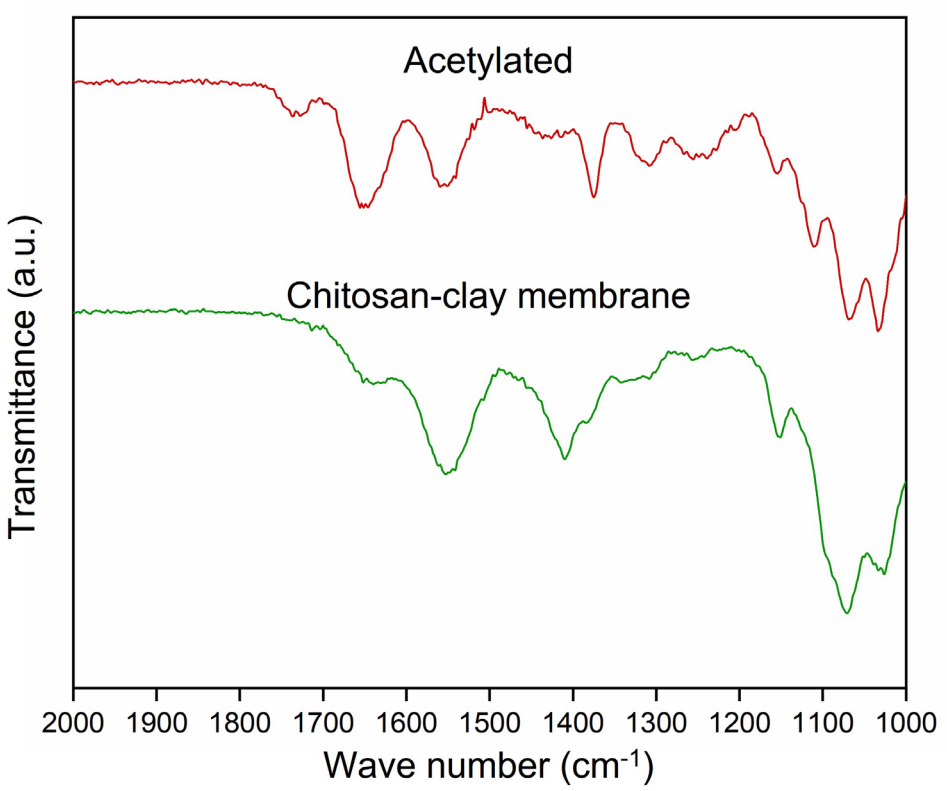

Figure S2. FT-IR spectrum of the chitosan-clay membrane and the membrane after acetylation.

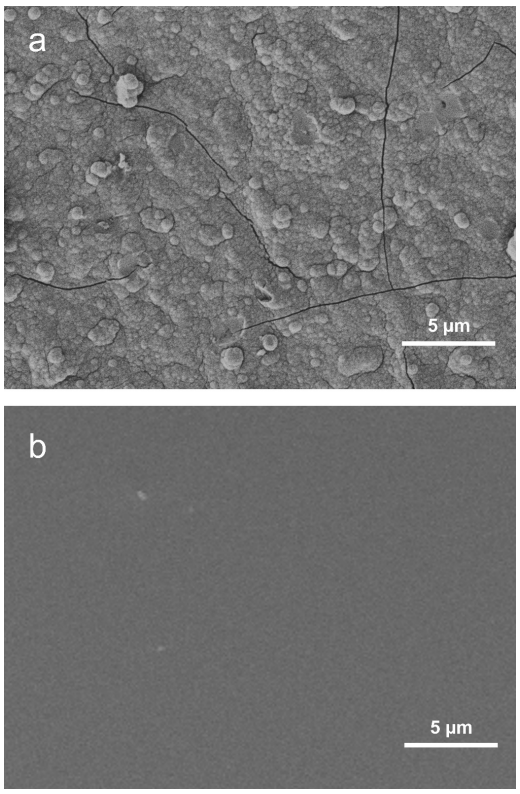

Figure S3. (a) Nanogranule structure of the mineral layer (a magnified view of Fig. 1e); (b) Magnified view of the PLA layer. The cracks were generated due to the high vacuum environment in the SEM sample chamber.

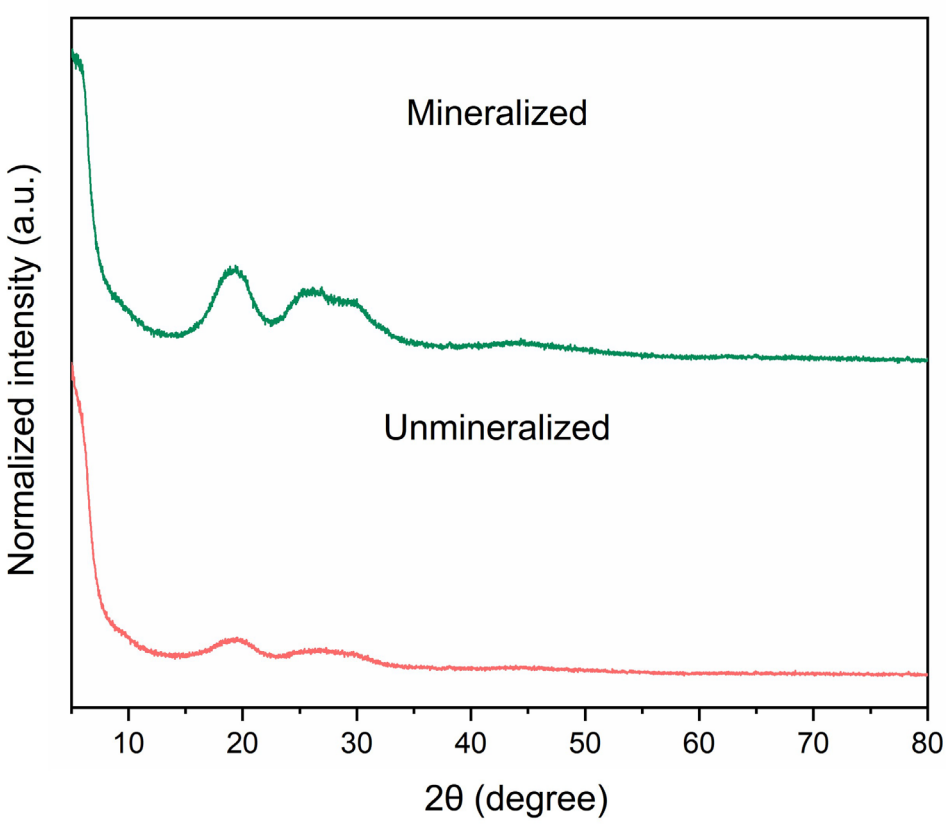

Figure S4. XRD patterns of the chitin-clay membrane before (red) and after (green) mineralization, showing the mineral phase was non-crystalline.

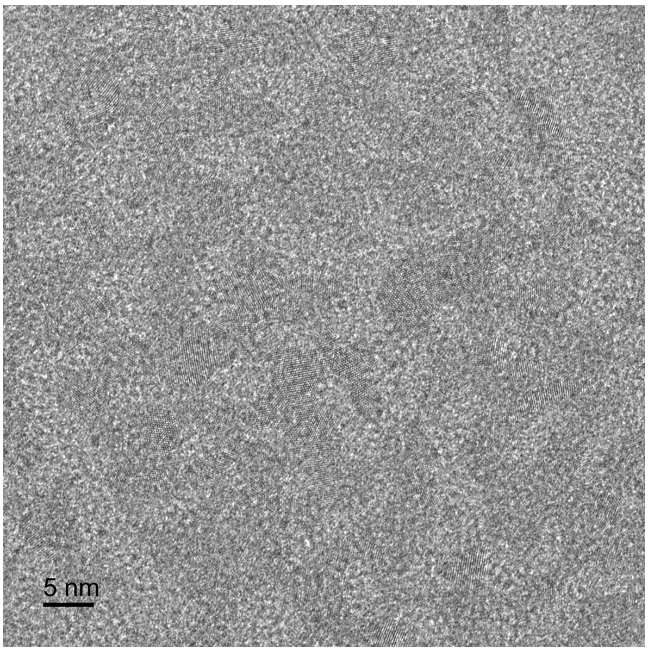

Figure S5. High-resolution transmission electron microscopy (HRTEM) image of the CaP mineral layer.

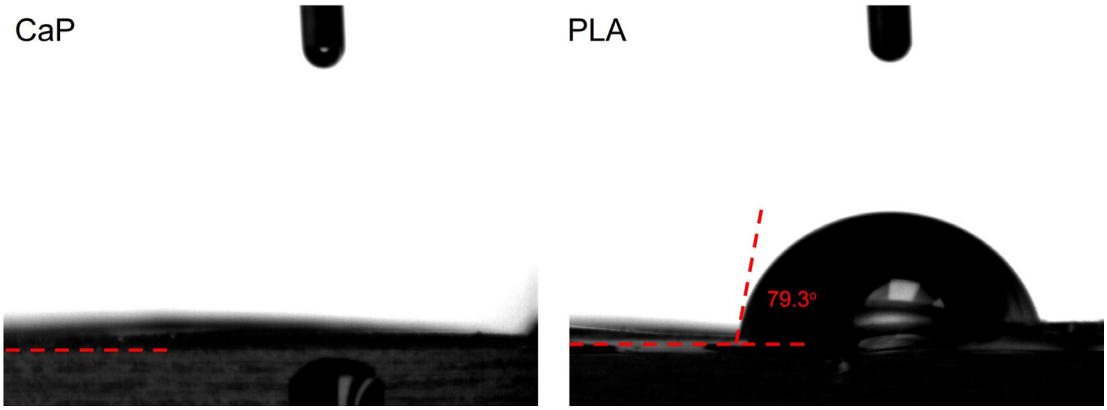

Figure S6. Water droplet completely spread on the mineral (CaP) surface, while maintained a relatively large contact angle (~ 80 degrees) on the PLA surface. The waviness in the left figure was due to the wavy membrane itself rather than the droplet.

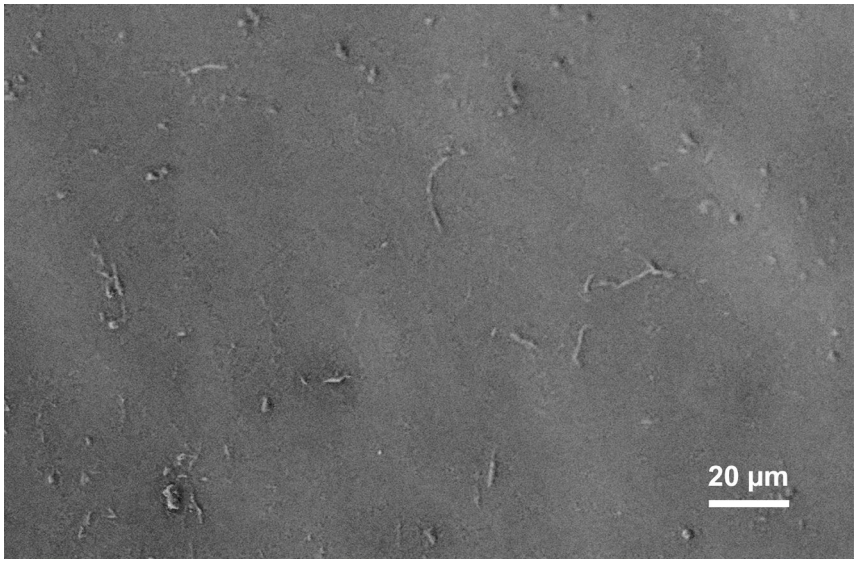

Figure S7. The PLA side of the bioinspired membrane after being soaked in the modified SBF (the same as that for the mineralization of the chitin-clay membrane) for 4 days. No mineralization was observed on the surface, suggesting the PLA surface can shield unwanted mineralization.

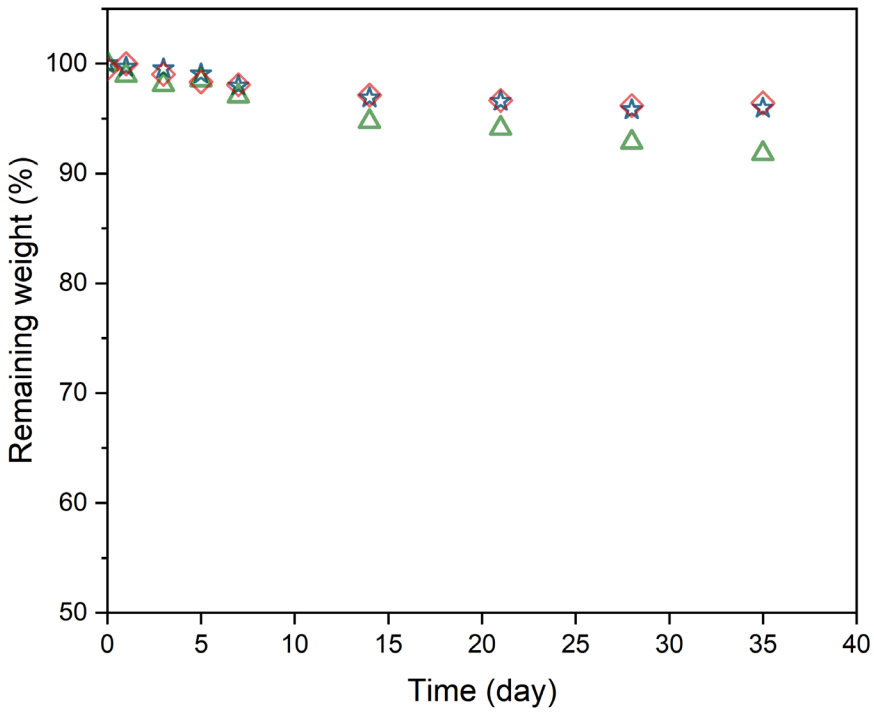

Figure S8. Degradation of the bioinspired membrane in PBS buffer solution.

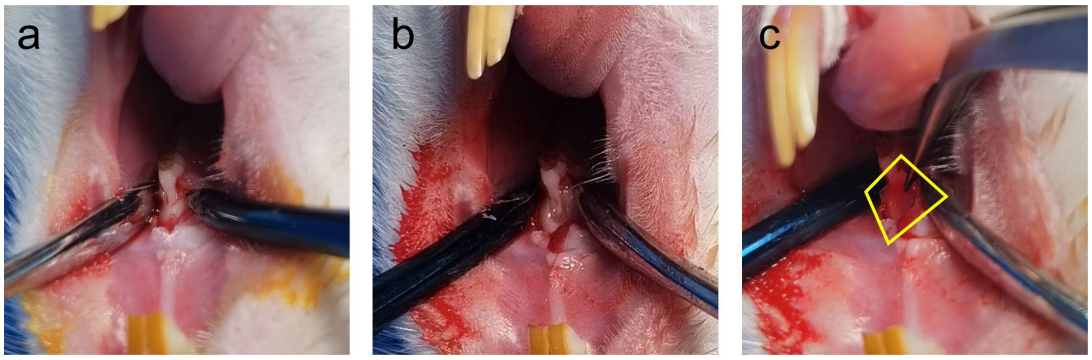

Figure S9. Surgery of the membrane implantation. (a) Flapping; (b) Drilling; (c) Membrane implantation. The yellow polygon in (c) indicates the implanted membrane.

Table S1. DNA sequences of the forward and the reverse primers of the four osteogenesis-related genes used for the RT-qPCR assay.

Genes	Forward primer sequence (5'->3')	Reverse primer sequence (5'->3')
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT
ALP	GCTGCTGCAGATGTACTACG	CTGCAGGCCTCTCATTCAAC
OCN	GCCCTGACTGCATTCTGCCTCT	TCACCACCTTACTGCCCTCCTG
OPN	GACGATGATGACGACGATGAC	GTGTGCTGGCAGTGAAGGACTC
RUNX2	TCCGCCACCACTCACTACCAC	GGAACTGATAGGACGCTGACGAAG