Supplementary Information

For

Sequential electroporation and biomimetic mineralization on E. coli biotemplate: Green synthesis of functional porous hollow calcium phosphate capsules

Wen Jiang,[†a] Fengwu Zhang,[†b, c] Mengyao Bian,[a] Jingxuan Han,[a] Changyu Shao*[a]

[a]Stomatology Hospital, School of Stomatology, Zhejiang University School of Medicine, Clinical Research Center for Oral Diseases of Zhejiang Province, Key Laboratory of Oral Biomedical Research of Zhejiang Province, Cancer Center of Zhejiang University, Hangzhou, 310016, China

[b]Cancer Institute, The Second Affiliated Hospital Zhejiang University School of Medicine, Hangzhou, 310009, China

[c]Institute of Translational MedicineZhejiang University School of Medicine, Hangzhou, 310029, China

Table of Contents

Experimental Methods

2.1 Bacterial activation

Retrieve the *E. coli* glycerol stock from -80^{o}C storage and briefly thaw it on ice for approximately 5 min. Using sterile technique, aseptically streak the thawed culture onto an appropriate solid LB agar medium and incubate the inverted plate at 37°C for 16-18 h to obtain well-isolated single colonies. Subsequently, select a morphologically uniform colony with a sterile pipette tip or inoculation loop, inoculate it into 3-5 mL of sterile LB medium, and disperse the biomass thoroughly by vortexing. Incubate the culture at 37^{o}C with vigorous shaking (220 rpm) for 12-16 h to achieve the late-logarithmic growth phase until the optical density at 600 nm (OD_{600}) reaches 0.8-1.2, yielding an activated bacterial suspension ready for electroporation and mineralization.

2.2 Mineralization of *E.coli*

The *E. coli* was obtained by centrifuging the bacterial suspension at 6000 rpm for 15 min (Centrifuge 5804 R, Eppendorf, Germany) and subsequently washed twice with deionized water. The *E. coli* (10 mg/mL based on wet weight) were distributed in the mineralization medium comprising 4.5 mM $CaCl_2$, 2.1 mM K_2HPO_4, 50 mM HEPES, 150 mM NaCl, and 100 μg/mL polyaspartic acid (pAsp) at pH 7.4. The mineralization persists for 1, 2, and 3 days at room temperature (RT) with stirring at 400 rpm. After mineralization, the *E.coli* product underwent centrifugation, was rinsed with deionized water and ethanol, and was vacuum-dried overnight. These mineralized *E.coli* were utilized for subsequent characterization. Furthermore, the calcination conducted was necessary to eliminate the cells to obtain the PHCPCs. The samples were first heated to

450°C for 3 h, and then to 600°C for a further 2 h. They were then collected after cooling to RT.

2.3 Preparation of metal oxide-loaded PHCPCs

Culture the *E. coli* in 500 ml of fresh LB medium and incubate at 37°C with vigorous shaking until the OD_{600} reaches approximately 0.4. Centrifuge the cells at 5000 rpm for 15 min at 4°C to pellet them. Then discard the supernatant and wash the cells three times with ice-cold sterile distilled water, followed by two washes with ice-cold sterile 10% glycerol. To attain a high transformation efficiency, it is crucial to maintain the cells on ice from this point onwards. Ultimately, the cells were resuspended in 1 mL GYT medium containing 10% glycerol, 0.25% tryptone, and 0.125% yeast extract. Electroporation was employed to facilitate the uptake of nanoparticles by the cells. The solution of 10 μL of 20 mg/mL of either TiO_2 alone or a mixture of ZnO and TiO_2 was mixed with 90 μL of electrocompetent cells into an ice-cold electroporation cuvette (0.2-cm gap). Gently tap to mix and then incubate on ice for 10 min. Insert the cuvette into the electroporation apparatus (Gemini X2, BTX, USA), wiping the sides of the cuvette dry. Set the electroporation conditions on a Gemini X2 electroporator to 3.0 kV, 25 μF, 5 ms, and 200 Ω. After pulsing the cells, add them to the mineralization medium mentioned above and mineralize for 3 days at RT. After mineralization, the *E.coli* underwent centrifugation, was rinsed with deionized water and ethanol, and was vacuum-dried overnight. Furthermore, the calcination conducted was to eliminate the cells in order to obtain the metal oxide-loaded PHCPCs.

2.4 Calculation of mineralization rate of *E. coli*

E. coli were cultured at 37°C in LB medium until the OD_{600} reached 0.6. The medium was diluted to an OD_{600} of 0.3 with fresh medium containing 0.1 mM FITC-d-Lys (Xiamen Bioluminor Bio-Technology Co., Ltd). The diluted bacteria were further incubated at 37°C until an OD_{600} of 1.0-1.5. The bacteria were centrifuged, washed thrice with saline solution, and subsequently resuspended in mineralization medium. After mineralization, the bacteria were centrifuged, washed thrice with saline solution, and subsequently resuspended in 10 μM Calcein Deep Red™ (AAT Bioquest, USA) for 20 min at RT. Then, the cells were subjected to confocal laser scanning microscope (CLSM, Nikon A1, Nikon, Japan) analysis.

2.5 Scanning electron microscopy

The surface morphology (or microstructure) of the samples was characterized using a scanning electron microscope (SEM, GeminiSEM560, Zeiss, Germany). Before observation, the samples were sputter-coated with a thin layer of platinum to enhance conductivity. SEM images were acquired under an accelerating voltage of 4 kV, with a working distance of approximately 8 mm. Elemental composition was analyzed using an energy-dispersive X-ray spectroscopy (EDS) detector (X-Max 80, Oxford Instruments, UK) attached to the SEM system. The EDS analysis was performed at an accelerating voltage of 15 kV with a live time of 60 s. Spectra were processed using Aztec software for elemental analysis.

2.6 Transmission electron microscopy

The microstructure and morphology of the samples were characterized by transmission electron microscopy (TEM, JEM-1400Flash, JEOL, Japan) operated at an accelerating

voltage of 100 kV. The samples were dispersed in ethanol, ultrasonicated for 10 min, and a drop of the suspension was deposited onto a carbon-coated copper grid for observation. High-resolution TEM (HRTEM) and scanning transmission electron microscopy (STEM) images were acquired by a JEM-F200 (JEOL, Japan) operated at an accelerating voltage of 200 kV. Elemental mapping and compositional analysis were performed using an integrated EDS system (JED2300T, JEOL, Japan).

2.7 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS, Thermo Scientific K-Alpha, USA) was performed on three samples: hydroxyapatite (HAP, purchased from Macklin, China), pristine *E. coli*, and mineralized *E. coli*. XPS measurements were performed using a K-Alpha X-ray photoelectron spectrometer (Thermo Fisher Scientific, USA) equipped with a hemispherical electron analyzer and a monochromatic Al Kα (1486.6 eV) radiation. Data analysis was conducted with Thermo Avantage Software (v6.8.1), and all binding energies were calibrated by referencing the C1s peak to 284.8 eV.

2.8 Powder X-ray Diffraction

The crystalline phase of the samples was identified by powder X-ray diffraction (XRD, D8 Advance, Bruker, Germany) with Cu Kα radiation (λ = 1.5406 Å) operated at 40 kV and 40 mA. Data were collected in the 2θ range of 10^{o}-80^{o} with a step size of 0.02^{o} and a scan rate of 5 ^{o}/min. The samples were evenly spread on a sample holder for measurement. Furthermore, the average crystallite size was estimated using the Scherrer equation based on the FWHM of the (101) diffraction peak for TiO_2 and ZnO.

2.9 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was carried out using a thermoanalyzer [TG 209 F1, Netzsch, Germany]. Approximately 5 mg of sample was placed in an alumina crucible and heated from RT to 800^{o}C at a heating rate of 10 ^{o}C/min under a continuous flow of air atmosphere. The thermal decomposition behavior and weight loss were recorded as a function of temperature.

2.10 Brunauer-Emmett-Teller (BET) surface area and pore size measurement

The specific surface area (SSA) of PHCPCs was determined via N_2 adsorption-desorption isotherms at 77 K using the BET method and performed on an analyzer (Quantachrome Autosorb IQ3, Quantachrome Instruments, USA). In addition, the pore size distribution was determined from N_2 adsorption-desorption isotherms using the Barrett-Joyner-Halenda (BJH) model.

2.11 In vitro DOX drug loading

The HPMCs powder (15 mg) was dispersed in 5 mL of a 2 mg/mL DOX solution and incubated in the dark for 24 h. The mixture was then centrifuged at 8000 rpm for 15 min, and the pellet was washed once with deionized water to remove free DOX. After washing, the pellet was dried under vacuum to obtain DOX-loaded PHCPCs. To determine the DOX loading capacity (DLC) of PHCPCs, the supernatant from the initial centrifugation was collected. The absorbance of this supernatant was measured at 480 nm, and the concentration of free DOX (Fr.DOX) was calculated using a DOX standard curve. The DLC was then calculated as follows:

$$DLC = \frac{To.DOX - Fr.DOX}{mHPMC}$$

Where To.DOX represents the total mass of DOX added (mg), Fr.DOX represents the

mass of unloaded DOX in the supernatant (mg), and mHPCPC represents the mass of HPCPC powder used (mg).

2.12 Cell viability assay

Mouse embryonic fibroblasts (MEF) cells were isolated from E13.5 embryos of C57BL/6 mice according to the protocol described in our previous work[S1]. MEF cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS; Gibco, A5256701), 1% non-essential amino acids (Gibco, 11140050), and 1% (v/v) penicillin-streptomycin (Gibco, 15140163).

For the cell viability assay, the MEF cells were seeded into a 96-well plate at a density of 3000 cells per well and incubated for 12 h. Subsequently, the culture medium was replaced with fresh medium containing the HPCPCs (0, 25, 50, 100 μg/mL), and the MEF were further incubated for 12, 24, and 48 h, respectively. Then, 10 μL of Cell Counting Kit-8 (CCK-8; MCE, HY-K0301) solution was added to each well, and the absorbance at 450 nm was measured using a microplate reader (SpectraMax i3x, Molecular Devices, USA) after incubation at 37°C. Optical images of the MEF co-cultured with various concentrations of the HPCPCs for 48 h were captured using an Olympus DP80 microscope (Olympus, Japan). Subsequently, the MEF was observed using a confocal laser scanning microscopy (CLSM, Nikon A1, Nikon, Japan) after nuclear staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Beyotime Biotechnology).

2.13 In vitro anti-tumor efficacy test

The human lung cancer cell line A549 was obtained from the American Type Culture

Collection (ATCC, Manassas, VA, USA; RRID: CVCL_A549). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin.

To test the in vitro cytostatic activity on tumor cells, A549 cells were seeded into a 96-well plate at a density of 5000 cells per well and incubated for 24 h. Sterilized DOX-loaded HPCPCs powder was added to the cells at concentrations of 25, 50, and 100 μg/mL, after which the cells were co-cultured for 12, 24, 36, and 48 h. A CCK-8 test was then performed. The optical images of the A549 cells after co-culturing with various concentrations of DOX-loaded HPCPCs were obtained using an Olympus DP80 microscope. Subsequently, the A549 cells were observed using a CLSM after nuclear staining with DAPI.

2.14 Statistical Analysis

Data are expressed as means ± SD. A one-way ANOVA test was performed to determine statistically significant differences between groups, where appropriate. Statistical significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Supplementary Figure

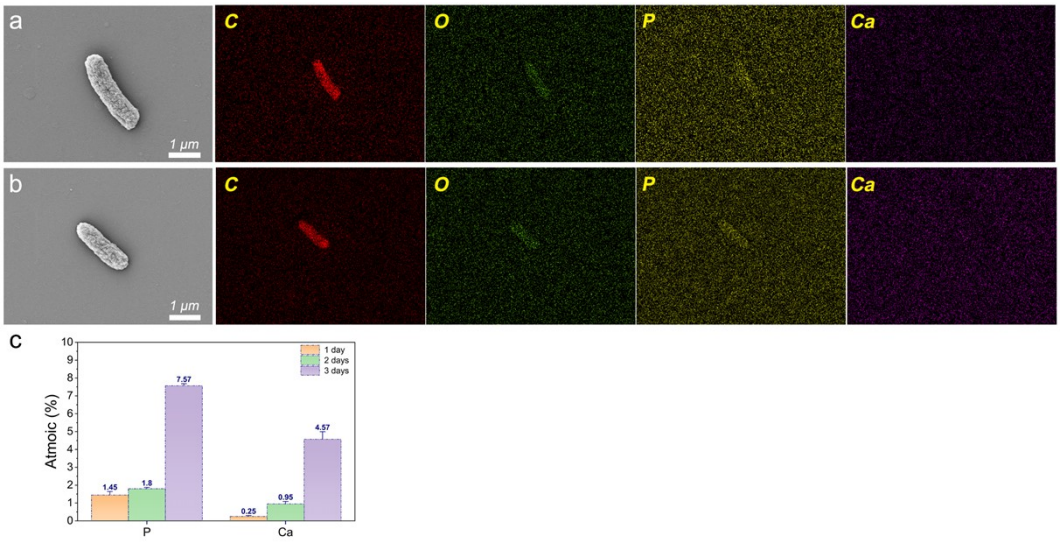

Figure S1. The elemental analysis of the mineralized E. coli. SEM images and corresponding elemental maps of the samples after 1 day (a) and 2 days (b) of mineralization are presented. (c) The atomic percentage (Atomic%) of calcium (Ca) and phosphorus (P) in the mineralized *E. coli* after mineralization of 1, 2, and 3 days. The atomic percentages (Atomic%) of Ca and P reflect the amount of calcium phosphate deposited on *E. coli.* As mineralization progressed, the calcium phosphate content increased continuously and reached a relatively high level of Ca and P by day 3. Furthermore, the elemental mapping showed that Ca and P were uniformly distributed across the surface, and a well-defined outline of the mineralized structure was clearly observed in the SEM and STEM images. Thus, a 3-day mineralization period represents an appropriate duration.

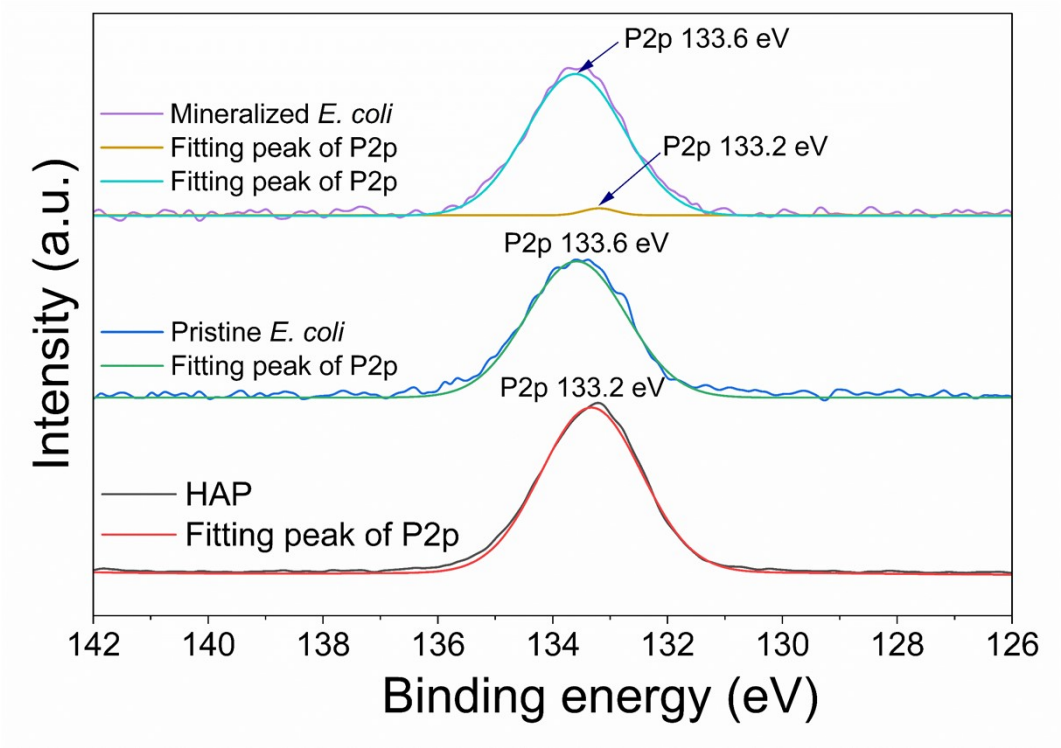

Figure S2. XPS spectra of HAP, pristine and mineralized *E. coli*. The binding energies of P2p were detacted: HAP, 133.2 eV, pristine *E. coli* 133.6 eV, and mineralized *E. coli*, 133.2 and 133.6 eV. The P2p peak at 133.6 eV, observed for both pristine and mineralized *E. coli*, is primarily attributed to phospholipids. The P2p peak at 133.2 eV corresponds to HAP formation in mineralized *E. coli*.

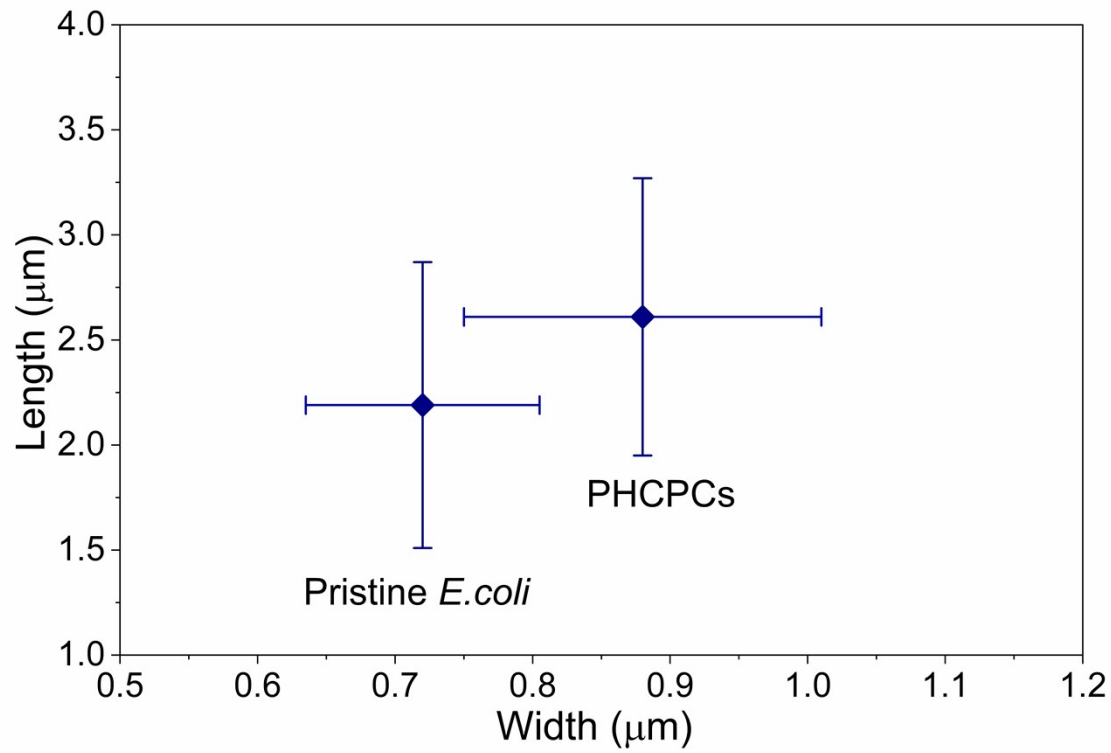

Figure S3. The sizes (length and width) of pristine *E. coli* and PHCPCs were determined from SEM images (N = 50). Pristine *E. coli* exhibited 2.19 ± 0.68 μm in length and 0.72 ± 0.084 μm in width. In contrast, the PHCPCs measured 2.61 ± 0.66 μm in length and 0.88 ± 0.13 μm in width.

Refences

[S1] Zhang, F., Xiong, X., Li, Z. et al. RHEB neddylation by the UBE2F-SAG axis enhances mTORC1 activity and aggravates liver tumorigenesis. *EMBO J* **44**, 1185–1219 (2025). https://doi.org/10.1038/s44318-024-00353-5