

Supporting Information

A Mineralized Cell-based Functional Platform: Construction of Yeast cell with biogenetic Intracellular Hydroxyapatite Nanoscaffold

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Experimental Section

Materials. All chemicals used in the study are of analytical grade and were used without further purification. Anhydrous calcium chloride (CaCl_2 , analytical grade) and sodium phosphate tribasic dodecahydrate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$) was purchased from Chemical Reagent Company of Tianjin. Maltose was purchased from Beijing Spiritualizing Star Biotechnology Company Limited, China. Yeast was purchased from ANGEL YEAST CO., Ltd.. Doxorubicin Hydrochloride (DOX-HCl, Beijing Huafeng United Technology Co., Ltd., China) was used as the model drug to examine the pH-responsive anticancer drug carrier. Tetracycline hydrochloride (Baoman Biotechnology Co., Ltd., Shanghai, China) could coordinate with calcium ions to induce a blue fluorescence under UV light (360 - 370 nm), and it was used as a fluorescence dye to detect the position of HAP nanoparticles. Ultrapure water was used in the whole experiment.

Characterizations of nHAP mineralized yeast cells (nHAP@yeasts). The morphologies of the mineralized yeast cells and native cells were characterized by a JSM-6390LV scanning electron microscope (SEM). High-resolution TEM (HRTEM) analysis was conducted on JEM-2100 transmission electron microscope equipped with an energy-dispersive spectrometer (EDS) operated at 200 kV. The broken mineralized yeast cells were resuspended in double distilled water and then drying on a piece of ultra-thin carbon coated copper grid. The composite phases of the intracellular nanoparticles were identified by X-ray diffraction (XRD) on a Bruker D8 & Advance X-ray powder diffractometer with graphite monochromatized $\text{Cu K}\alpha$ ($\lambda=0.15406$ nm). Fluorescent images were acquired using TCS NT confocal laser scanning microscope (CLSM, Leica, Germany). The TG analysis was performed on an EXSTARTG/DTA 6300 instrument. The FT-IR

spectra were recorded on a Bio-Rad FTS-40 Fourier transform infrared spectrometer in the wavenumber range of 4000-400 cm^{-1} . The spectra were collected at 2cm^{-1} resolution with 128 scans by preparing KBr pellets with a 3:100 “sample to-KBr” ratio. A TU-1900 UV/Vis spectrophotometer was adopted to obtain the absorbance of DOX, and further determine the concentration of the DOX solution.

Confocal Laser Scanning Microscopy (CLSM). Tetracycline hydrochloride was used as a fluorescence dye to detect the position of nHAP inside the mineralized yeast cells because it could coordinate with calcium ions to induce a blue fluorescence under UV light (360-370 nm). The mineralized yeast cells and control yeast cells were put into the tetracycline hydrochloride aqueous solution for 12 h, respectively. Then the systems were washed using triply distilled water by centrifugation. The concentration of the tetracycline hydrochloride in the media was 2.5×10^{-2} g/L. Images were acquired using a Leica TCS NT confocal laser scanning microscope (Germany).

Protein Characterization. To identify the proteins in the yeast cells responsible for synthesis of HAP nanoparticles, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 5 % (w/v) polyacrylamide gel in 1M Tris-HCl buffer (pH = 6.8) stacking gel and a 12 % (w/v) polyacrylamide in 1.5 M Tris-HCl buffer (pH = 8.8) resolving gel. nHAP were harvested after the mineralized cells were crushed by Ultrasonic cell crusher and washed with double distilled water. And the samples (40 μL) were mixed with 10 μL 5x SDS-PAGE loading buffer (1M Tris-HCl, 50 % glycerol, 10 % SDS, 25 % (v/v) β -mercaptoethanol, 1 % bromophenol blue) and heated at 100°C for 5 min, followed by loading 20 μL of the resulting solution on the gel. Experiments were performed at 120 V for 1.5 h. Proteins in SDS-PAGE gels were visualized with Coomassie brilliant blue staining for 13 h. The gels were destained (AcOH / Methanol / double-

distilled H₂O = 2 : 1: 17). Matrix assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) was conducted on a MALDI TOF/TOF 5800 mass spectrometer (AB SCIEX, USA) to determine the type of proteins.

In-gel proteolytic digestion and identification. Protein slices in fresh CCB-stained gel were excised and plated into a 96-well microtitre plate. Excised slices were firstly destained twice with 200 µL of 50 mM NH₄HCO₃ and 50 % acetonitrile and then dried twice with 200 µL of acetonitrile. Afterwards, the dried pieces of gels were incubated in ice-cold digestion solution (trypsin 0.01 µg/µL and 20 mM NH₄HCO₃) for 20 min and then transferred into a 37 °C incubator for digestion overnight. Finally peptides in the supernatant were collected after extraction twice with 200 µL extract solution (1 % formic acid in 50 % acetonitrile). The peptide solution described above was dried under the protection of N₂.

The 0.8 µL matrix solution (5 mg/mL α -cyano-4-hydroxy-cinnamic acid diluted in 0.1 % TFA, 50 % ACN) was pipetted to dissolve it. Then the mixture was spotted on a MALDI target plate (AB SCIEX). MS analysis of proteins was performed on an AB SCIEX 5800 TOF/TOF. The UV laser was operated at a 400 Hz repetition rate with wavelength of 355 nm. The accelerated voltage was operated at 20 kV, and mass resolution was maximized at 1600 Da. Myoglobin digested with trypsin was used to calibrate the mass instrument with internal calibration mode. All acquired spectra of samples were processed using TOF/TOF Explorer TM Software (AB SCIEX) in a default mode. The data were searched by GPS Explorer (V3.6) with the search engine MASCOT (2.3). The search parameters were as follows: the database NCBIInr-yeast, trypsin digest with one missing cleavage, MS tolerance was set at 100 ppm, MS/MS tolerance of 0.8 Da. Proteins with protein score confidence intervals (C.I.) above 95 % (protein score > 64) were considered

confident identifications.

Viability measurement of nHAP@yeasts. The living cells show achromaticity because of the integrity of a plasma membrane and the ability of a viable cell could exclude the trypan blue dye. In contrast, the whole body of the dead yeast cells stained by the trypan blue would show blue. The nHAP@yeasts suspension at a high concentration (ca. 150 cells/mL) was prepared. One drop of the nHAP@yeasts suspension was mixed with one drop of the trypan blue solution (0.8 mM in PBS) on clean glass slide. After about 3 min, the stained nHAP@yeasts was observed by light microscope.

Proliferation test of nHAP@yeasts. The nHAP@yeasts and control yeast cells were cultured in YPD medium (2% peptone and 2% glucose) at 30°C with shaking at 150 rpm, respectively. The culture densities of the nHAP@yeasts and control yeast cells were measured by turbidity (OD_{600}) and then observed by light microscope.

Flow cytometry analysis. For cells viability and death assay, flow cytometry was further performed using a COULTER Epics XL flow cytometry system (Beckman Coulter, Inc., CA, USA) in sextuplicate and the percentages of cells viability were recorded. The cells were washed with cold PBS, fixed with ethanol (70 %, 3 mL) and precooled to -20 °C, respectively. Then the fixed cells were centrifuged and washed with cold PBS. The cells were resuspended in 195 μ L of binding buffer (10 mM), then 5 μ L of Annexin V-FITC and 10 μ L of propidium iodide (PI) were added and incubated in the dark for 30 min. The stained cells were filtered through 41 μ m Nylon net filters, analyzed and assayed by flow cytometry. The signals from the yeast cells were measured at 488 nm on a FSC channel, at 515-545 nm on a FL1-H channel, and at 564-606 nm on a FL2-H channel.

To demonstrate the specific targeting of DOX-nHAP@yeasts-FA toward different cell lines, fluorescence measurements were obtained by flow cytometry analysis. After treatments with 80 μg of DOX-nHAP@yeasts, DOX-nHAP@yeasts-FA and the equivalent free DOX for 6 h in the culture bottle, HepG2 cells were detached by incubation with 0.25% trypsin for 5 min and centrifuged, then washed by cold PBS twice. A laser at 486 nm was used as the excitation source and the signals from the autofluorescence of DOX were measured at 560-650 nm. The untreated cells were used as control group. In flow cytometry tubes, about 10^6 cells treated with the above products subjected to flow cytometric analysis.

HepG2 and V79-4 Cells culture. Human hepatocellular carcinoma HepG2 cells were cultured in DMEM medium containing with heat-inactivated FBS (10 %), penicillin (100 units·mL⁻¹), streptomycin (100 μg ·mL⁻¹), amphotericin B (fungizone, 0.25 μg ·mL⁻¹) and sodium bicarbonate (2 mg·mL⁻¹) in a humidified 5 % CO₂ atmosphere at 37 °C. V79-4 Chinese hamster lung cells (ATCC No. CCL-93) were cultured in DMEM medium supplemented with 10 % heat-inactivated FBS, Penicillin (100 units/mL), Streptomycin (100 μg /mL), amphotericin B (fungizone, 0.25 μg /mL) and sodium bicarbonate (3.7 mg/mL) in a humidified incubator at fully humidified atmosphere at 37 °C, 5 % CO₂ and 95 % room air. To determine the cellular uptake and distribution of DOX released from the DOX-nHAP@yeasts-FA, HepG2 cells and V79-4 were seeded onto a 24-well flat-bottomed plates with microslides covering the bottom and incubated with DOX-nHAP@yeasts-FA at 37 °C for 6 h. Tetracycline hydrochloride (with a concentration of 0.1 g/L) was then added into the plates and incubated for 5 h. The distributions of DOX and

nHAP inside the cells from the DOX-nHAP@yeasts-FA were observed using a confocal laser scanning microscope.

MTT colorimetric assay. HepG2 cells and V79-4 were seeded separately in the wells of sterile 96-well flat-bottomed culture microplates and acclimated for 24 h. Then the samples (native yeasts, nHAP@yeasts, nHAP@yeasts-FA, DOX-nHAP@yeasts, DOX-nHAP@yeasts-FA, and free DOX) was added with different concentrations (from 0.78 to 200 $\mu\text{g}/\text{mL}$) respectively, and incubated for 72 h. Afterwards, freshly prepared MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 20 μL , 5 $\text{mg}\cdot\text{mL}^{-1}$, Sigma-Aldrich, Co., MO, USA) in filtered PBS was added to each well of the control and the treated cells, and incubated for another 5 h. The medium was carefully removed by centrifugation, followed by adding 150 μL of DMSO to each well to dissolve the dark blue crystals completely. A microplate reader was used to measure the absorbance of the solution in each well at the wavelength of 570 nm. The extent of cell proliferation was reflected by the average value of absorbance. The results were reported as mean cell viability \pm standard deviation (SD) based on the calculation of the triplicate samples.

Establishment of tumor-bearing animal models. The BALB/c nude mice (8 weeks old; 20-25 g) were provided by Department of Human Anatomy, Xinxiang Medical University, Henan, P. R. China. The BALB/c nude mice were inoculated with hepatocellular carcinoma cells (5×10^6) in the back through subcutaneous injection for tumor induction. Tumor lengths were measured by a caliper. When the tumors grew up to about 10 mm in length, the model mice were used in the experiments.

Results and discussion

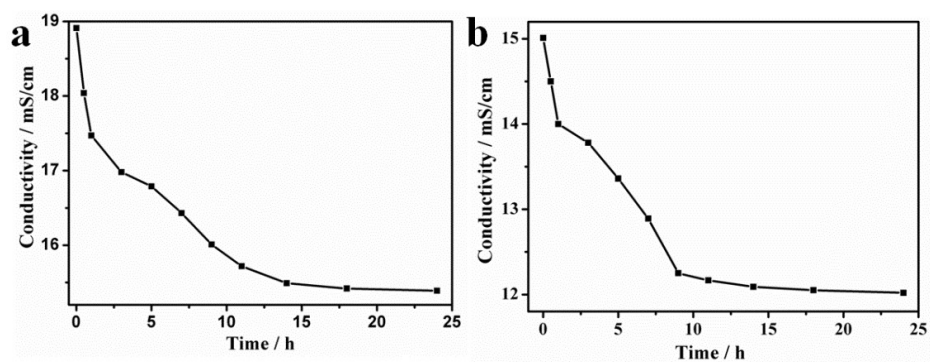


Figure S1. The time-dependent electro-conductivity of the CaCl_2 (a) and Na_3PO_4 (b) solution reacted with yeast cells, respectively.

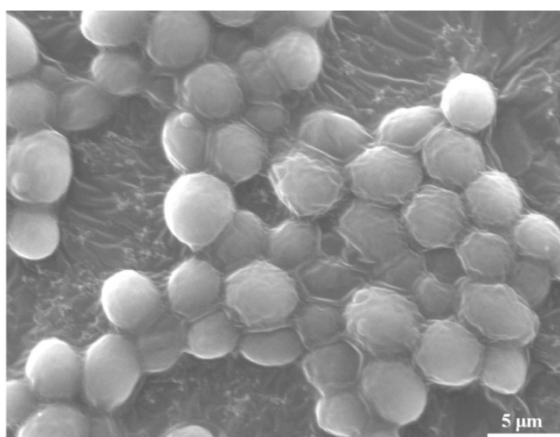


Figure S2. The typical scanning electron micrograph of the native yeast cells.

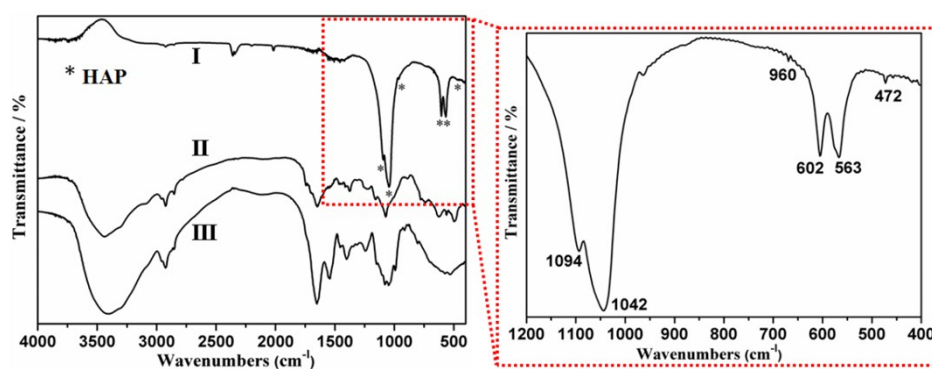


Figure S3. The FT-IR spectra of the nHAP@yeasts crushed (I), the nHAP@yeasts (II) and control native yeast cells (III).

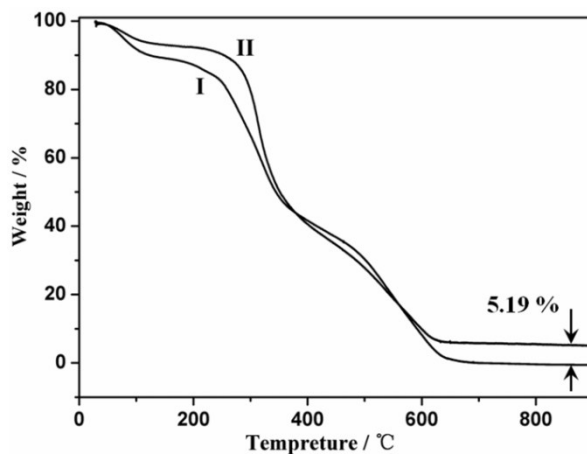


Figure S4. Thermo-gravimetric (TG) analyses of I) the nHAP@yeasts. II) native yeast cells.

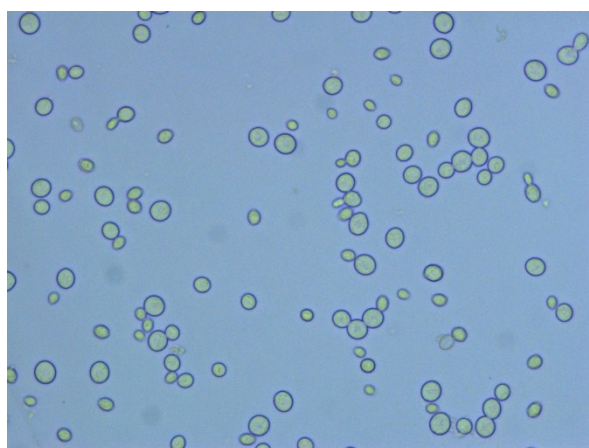


Figure S5. The nHAP@yeasts are proliferating after incubation for 50 h in YPD broth.

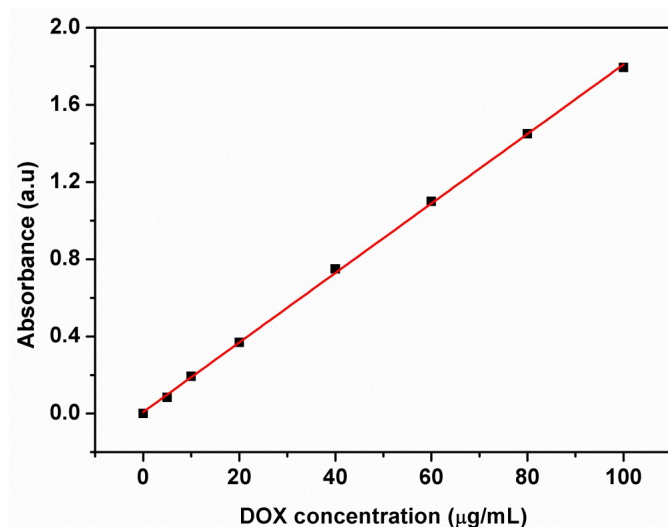


Figure S6. Absorbance at 480 nm vs. free DOX concentration (optical path = 1 cm). Solid line is the liner fit using the analysis tool in Origin software and the $R^2 = 0.9999$.

| Table S1. dependent | Sample | pH | Zeta potential (mv) | The time- electro- |
|------------------------|--------------------|-------|---------------------|-----------------------|
| | Native yeast cells | 5.9 | -17.9 | |
| nHAP@yeasts | | -14.6 | | |
| PDDA | 6.2 | 28.9 | | |
| FA | 6.0 | -26.3 | | |
| DOX | 5.8 | 1.09 | | |
| DOX-nHAP@yeasts | 6.3 | -12.1 | | |
| DOX-nHAP@yeasts@PDDA | 6.6 | 23.1 | | |
| DOX-nHAP@yeasts-FA | 6.7 | -9.5 | | |

conductibility of DOX-nHAP@yeasts in the solution of PDDA (a) and DOX-nHAP@yeasts@PDDA in the solution of FA.

The results of the electro-conductibility shows that the DOX-nHAP@yeasts wrapped by folic acid (DOX-nHAP@yeasts-FA) could be synthesized by the covering of PDDA (Table S1).

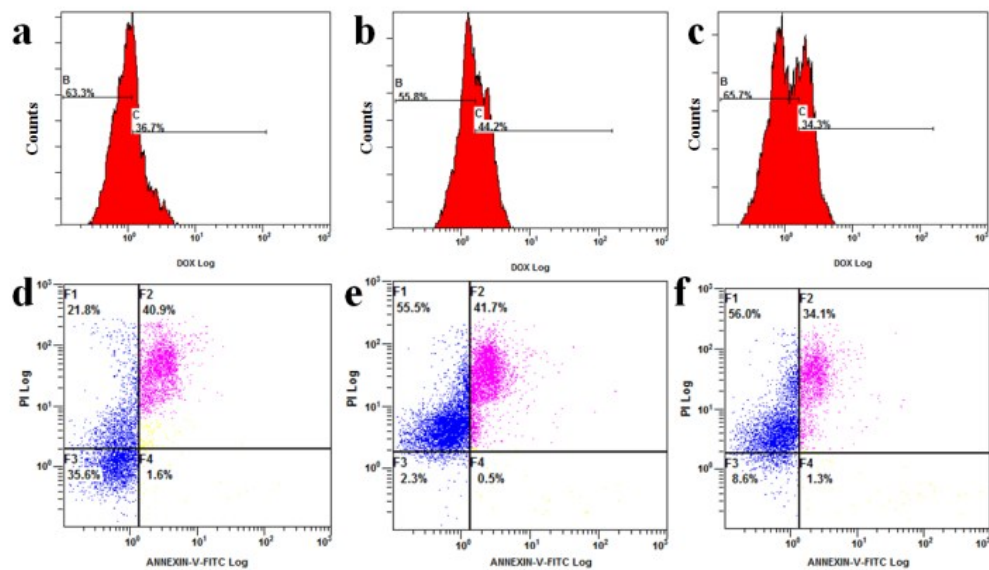


Figure S7. Flow cytometry analysis of quantification of DOX levels uptake into HepG2 cell and evaluation of the death pathways of HepG2 cells treated with DOX-nHAP@yeasts (a and d), DOX-nHAP@yeasts-FA (b and e) and free DOX (c and f) at the equivalent DOX concentration (60 $\mu\text{g}/\text{mL}$) for the same incubation time period (48 h). F1, F2, F3, and F4 zones represent necrosis, apoptosis, normality and early apoptosis, respectively.

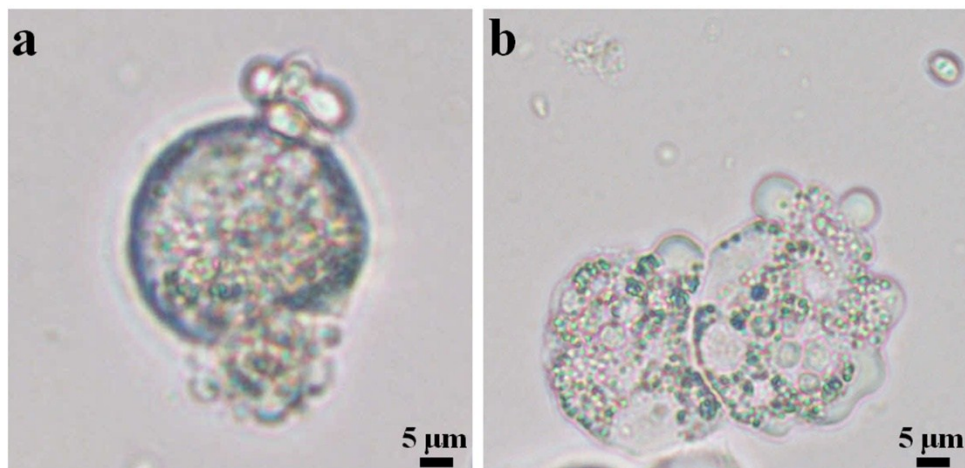


Figure S8. The light micrographs of HepG2 after incubation with DOX-nHAP@yeasts for 5 h and 15 h, respectively.