Electronic Supplementary Information

Ultrafast, Cytocompatible Mineralization of Calcium Phosphate in Formation of Stratified Nanoshells of Artificial Spores

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Experimental

Materials. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, 99%, Sigma), potassium phosphate tribasic (K_3PO_4, 98%, Junsei), tannic acid (TA, Sigma-Aldrich), Alexa Fluor 488-conjugated albumin from bovine serum (BSA-Alexa 488, Invitrogen), Alexa Fluor 647-conjugated albumin from bovine serum (BSA-Alexa 647, Invitrogen), deoxyribonucleic acid sodium salts from salmon testes (DNA), Hoechst 33342 (trihydrochloride, trihydrate,10 mg/mL, solution in water, Invitrogen^{TM}), lysozyme from hen egg white (>23000 U/mg, Roche), lyticase (from Arthrobacter luteus, Sigma-Aldrich), D-(+)-glucose (≥99.5%, Sigma-Aldrich), glucose oxidase (GOx, from *Aspergillus niger*, Sigma-Aldrich), horseradish peroxidase (HRP, from *Amoracia rusticana*, Sigma-Aldrich), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥98.0%, Sigma-Aldrich), propidium iodide (PI, Sigma-Aldrich), fluorescein diacetate (FDA, Sigma-Aldrich), calcein (mixture of isomers, TCI), glycerol (99%, Sigma-Aldrich), polystyrene microparticle (PS, diameter: 3.97 μm, Microparticles GmbH), *Lactobacillus acidophilus* (*L. acidophilus* ATCC 4356 from the American Type Culture Collection (ATCC)), *Lactobacilli* MRS broth (BD), *Lactobacilli* MRS agar (BD), *Saccharomyces cerevisiae* (*S. cerevisiae* ATCC 18824 from the Korean Collection for Type Cultures (KCTC)), yeast-extract-peptone-dextrose (YPD) broth (Duchefa Biochemistry), YPD agar (Duchefa Biochemistry), phosphate-buffered saline (PBS, pH 7.4, Welgene), and ethylenediaminetetraacetic acid (EDTA, ≥98%, Sigma-Aldrich) were used as received. Deionized (DI) water (18.3 MΩ·cm) from Milli-Q Direct 8 (Millipore) was used.

Preparation of *a*-preCaP solutions. To 600 μL of DI water were sequentially added 200 μL of an aqueous $CaCl_2$ solution (24 mM) and 200 μL of an aqueous K_3PO_4 solution (16 mM). For biomolecule incorporation into CaP shells, the appropriate volume of an aqueous biomolecule stock solution was added to the $CaCl_2$ solution (800 μL) before the addition of the K_3PO_4 solution, in the preparation of an ***a*-preCaP** solution. The concentrations and volumes of the biomolecule stock solutions used in the immobilization experiments are provided in the table below:

Biomolecule	Concentration (mg/mL)	Volume (μL)
BSA-Alexa 647	5	1
BSA-Alexa 488	1	0.5
DNA	1	10
GOx	1	5
		2.5 (for the mixture of GOx and HRP)
HRP	1	5
		2.5 (for the mixture of GOx and HRP)

Formation of CaP Shells. *(a) TA-Ca^{2+} priming:* PS particles were used as received. A single colony of *L. acidophilus*, picked from an MRS agar plate, was cultured in the MRS broth medium at 33 °C for 24 h. A single colony of *S. cerevisiae* was picked from a YPD agar plate and cultured in a YPD broth with shaking at 30 °C for 30 h. To a pellet of PS particles or cells, 1000 μL of a TA stock solution (6 mM) and 500 μL of the $CaCl_2$ stock solution (24 mM) were

added sequentially. After 60 min of incubation, the TA-Ca^{2+}-primed PS particles or cells were purified by centrifugating at 3000 *g* and washing with DI water once. *(b) Formation of CaP nanoshells:* The TA-Ca^{2+}-primed PS particles or cells were mixed with 1000 μL of the ***a*-preCaP** solution, gently shaken, and immediately purified by centrifugating at 3000 *g* and washing with DI water twice. The second layer was formed by repeating the processes of TA-Ca^{2+} priming and CaP shell formation, with 30 min of incubation in the TA-Ca^{2+} mixture for the second layer. *(c) Formation of S.cerevisiae@CaP by the conventional method:* To 600 μL of TA-Ca^{2+}-primed *S. cerevisiae*, 200 μL of the $CaCl_2$ stock solution (24 mM) was added, followed by the dropwise addition of 200 μL of the K_3PO_4 stock solution (16 mM) at the rate of 20 μL/min with gentle shaking. The resulting cells were collected by centrifugating at 3000 *g* and washing with DI water. *(d) Immobilization efficiency of BSA-Alexa 647:* After forming CaP nanoshells in the ***a*-preCaP** solution containing 5 μg/mL BSA-Alexa 647 (1001 μL), the PS@CaP or *S.cerevisiae*@CaP pellet was collected by centrifugating at 3000 *g* and washing with DI water twice, and resuspended in 1001 μL of an aqueous EDTA solution (100 mM, pH 8) for shell degradation. After 1 min of incubation, the supernatant was collected by centrifugating at 3000 *g*, and the UV-Vis absorbance was measured at 647 nm. The immobilization efficiency was calculated with the UV-Vis absorbance of the ***a*-preCaP** solution that contained EDTA (100 mM, pH 8) and BSA-Alexa 647 (5 μg/mL) as a reference.

Viability Assay. The 5 μL of an FDA stock solution (10 mg/mL in acetone) and 2 μL of a PI stock solution (1 mg/mL in DI water) were mixed with 1 mL of a *S. cerevisiae* or *S.cerevisiae*@CaP suspension in DI water for 15 min at 30 °C, while shaking. The viability was assessed, after washing with DI water, by confocal laser-scanning microscopy (CLSM). For the viability assay of *L. acidophilus* and *L.acidophilus*@CaP, 1 μL of an SYTO 9 stock solution (3.34 mM in DMSO) and 2 μL of the PI stock solution (20 mM in DMSO) were used.

Cytoprotection and Enzymatic Reactions. *(a) Protection against lysozyme:* To 100 μL of *L.acidophilus*@CaP or naïve *L. acidophilus* (OD_{600} = 1, in DI water) was added 900 μL of a lysozyme solution (1 mg/mL in PBS buffer, pH 7.4). The mixture was incubated at 37 °C in an incubator, and the OD_{600} values were measured with 100 μL of aliquots taken at the predetermined time points. *(b) Protection against lyticase:* A lyticase stock solution was prepared by dissolving lyticase (2 mg) in a mixture of 500 μL of glycerol and 500 μL of PBS (50 mM, pH 7.4). To 500 μL of *S.cerevisiae*@CaP or naïve *L. acidophilus* (OD_{600} = 1, in DI water) were sequentially added 500 μL of PBS and 20 μL of the lyticase stock solution. The mixture was incubated at 37 °C in an incubator, and the OD_{600} values were measured with 100 μL of aliquots taken at the predetermined time points. *(c) Enzymatic reactions:* All the samples (*S.cerevisiae*@$CaP_{[HRP]/[GOx]}$, *S.cerevisiae*@$CaP_{[GOx]/[HRP]}$, *S.cerevisiae*@$CaP_{[GOx\_HRP]/[GOx\_HRP]}$, and naïve *S. cerevisiae*) were adjusted to an OD_{600} of 1 in DI water. To 200 μL of each sample were sequentially added 200 μL of DI water, 200 μL of ABTS (4 mM, in DI water), and 200 μL of glucose (100 mM, in DI water). The UV-Vis absorbance of the reaction mixture was measured at 414 nm every 2 min over a 4-hour period, using 100 μL aliquots in a 96-well plate, with a SpectraMax iD5 microplate reader (Molecular Devices) in kinetic mode. The plate was shaken for 5 sec, before each measurement, with double-orbital shaking mode at medium intensity. Five aliquots were used from each sample. The reaction rate was estimated based on the initial slope of the time-lapse absorbance curves.

Characterizations. Field-emission scanning electron microscopy (FE-SEM) imaging was performed with an FEI Inspect F50 microscope (FEI) with an accelerating voltage of 5 kV, after sputter-coating with platinum. Transmission electron microscopy (TEM) imaging, selected area electron diffraction (SAED), and energy-dispersive X-ray (EDX) spectroscopy elemental analysis were conducted with a Talos F200X (FEI) with a carbon-support-film copper mesh. The ζ potential and dynamic light scattering (DLS) size were measured with a Zetasizer Nano ZS (Malvern). X-ray diffraction (XRD) analysis was conducted with a SmartLab (RIGAKU). CLSM imaging was performed with an LSM 700 (Carl Zeiss), and UV-Vis absorbance was measured with a SpectraMax iD5 microplate reader (Molecular Devices) in a 96-well plate.

Statistical Analysis. Multiple independent samples ($n = 3$) were prepared, with one measurement performed for each sample, except for the ABTS enzymatic reaction experiments, where five measurements were taken per sample, and the average was considered as one data point. Data are presented as mean ± SD. Statistical significance was determined using an unpaired, two-sided Student's *t*-test ($n = 3$). Data analysis and graph generation were performed using IBM SPSS Statistics 26 and Origin 2019.

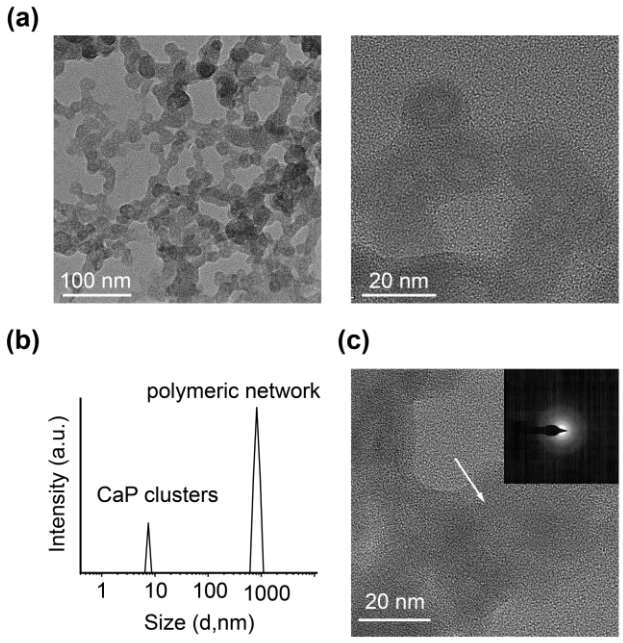

Figure S1. (a) TEM images of ***a*-preCaP**. (b) DLS measurement of ***a*-preCaP**. (c) SAED analysis of ***a*-preCaP.**

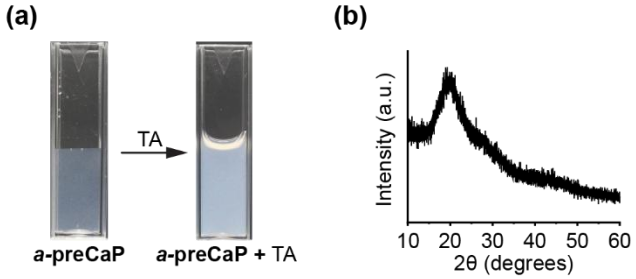

Figure S2. (a) Optical images of the aqueous ***a*-preCaP** solution before and after addition of TA. (b) XRD pattern of PS@CaP.

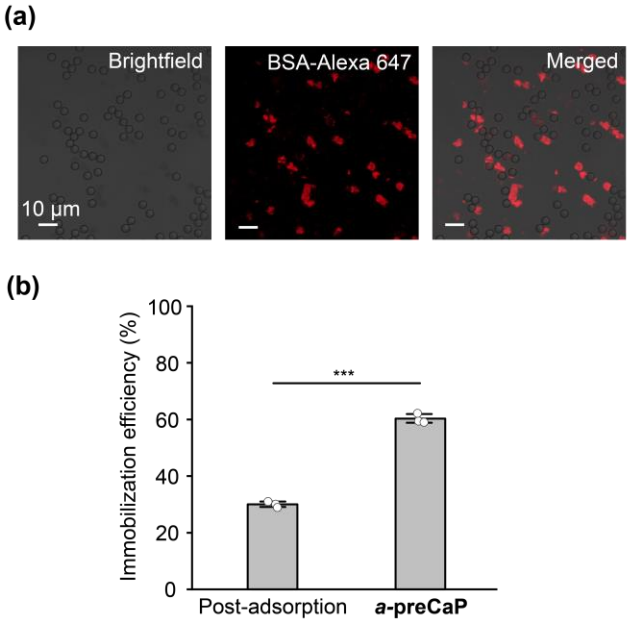

Figure S3. (a) CLSM images of homogenous ***a*-preCaP** precipitation with a high concentration of BSA-Alexa 647 (10 μg/mL). (b) Immobilization efficiency of BSA-Alexa 647 for the ***a*-preCaP** and post-adsorption protocols. Data are represented as mean ± SD (unpaired, two-sided Student's *t*-test; $n = 3$; ***$p < 0.001$).

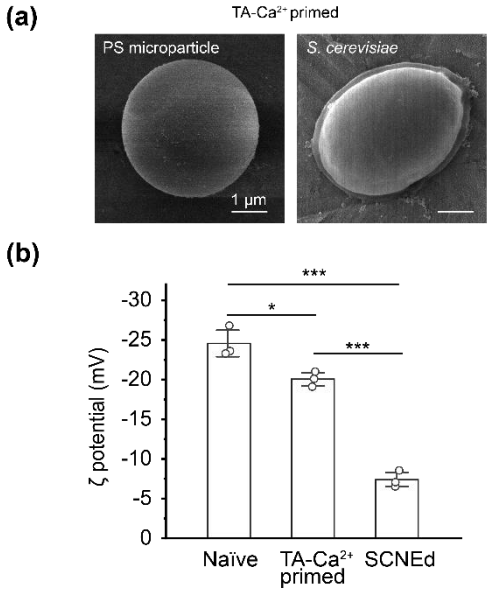

Figure S4. (a) FE-SEM images of PS microparticle and *S. cerevisiae* after TA-Ca^{2+} priming. (b) Zeta (ζ) potentials of naïve *S. cerevisiae*, TA-Ca^{2+}-primed *S. cerevisiae*, and *S.cerevisiae*@CaP. Data are represented as mean ± SD (unpaired, two-sided Student's *t*-test; $n = 3$; ***$p < 0.001$, *$p < 0.05$).

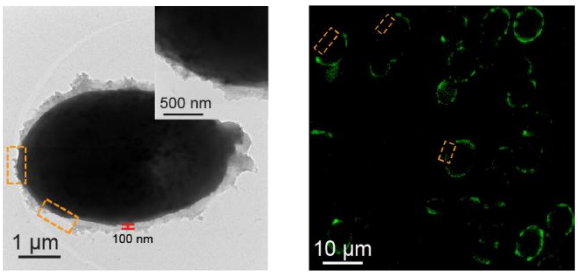

Figure S5. TEM and CLSM images of *S.cerevisiae*@CaP formed by the conventional titration protocol, showing CaP-defect sites (orange boxes).

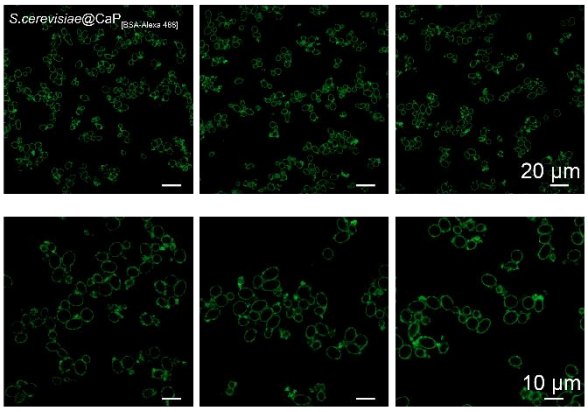

Figure S6. CLSM images of *S.cerevisiae*@$CaP_{[BSA\text{-}Alexa\ 488]}$ from 3 independent experiments.

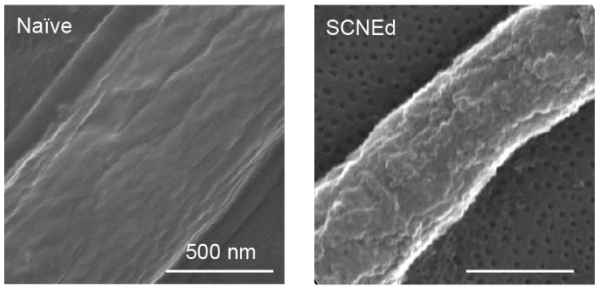

Figure S7. FE-SEM images of naïve and SCNEd *L. acidophilus*.

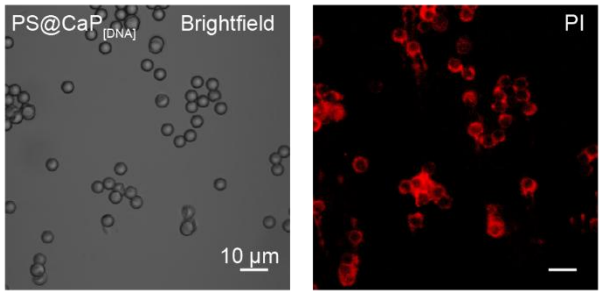

Figure S8. CLSM images of PI-stained PS@CaP$_{[DNA]}$.

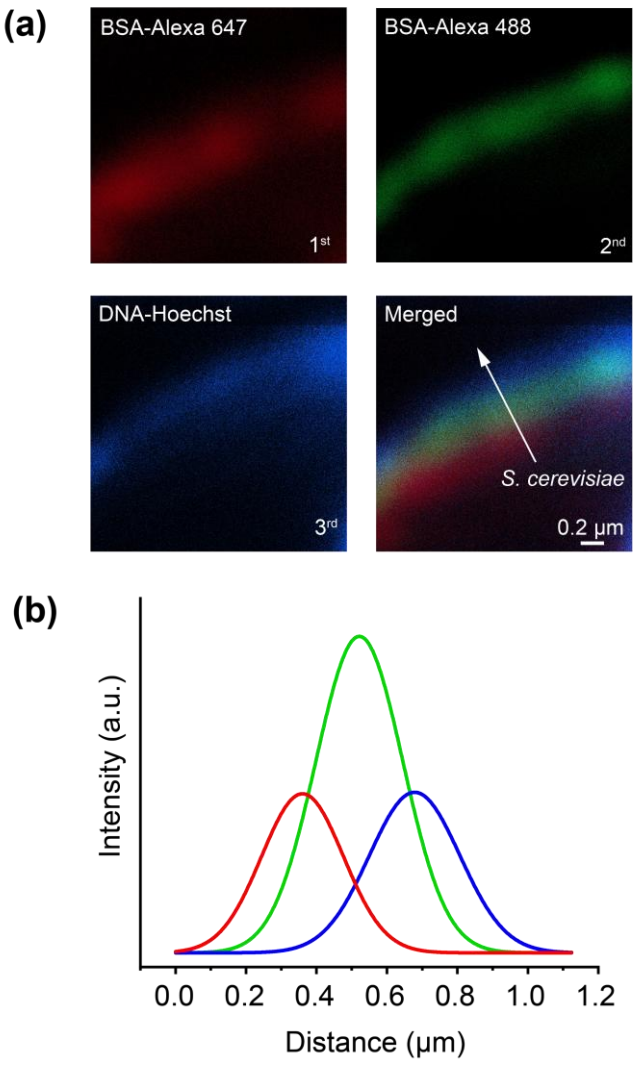

Figure S9. (a) CLSM images and (b) line profiles of *S.cerevisiae*@CaP$_{[BSA\text{-}Alexa\ 647]/[BSA\text{-}Alexa\ 488]/[DNA\text{-}Hoechst]}$.

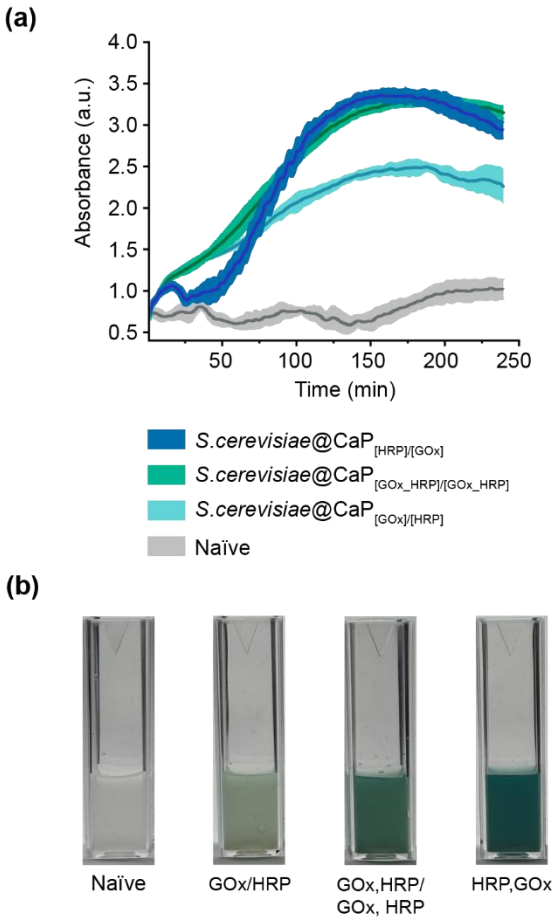

Figure S10. (a) Time-lapsed $ABTS^{\bullet+}$ absorbance during cascade reactions of GOx/HRP-embedded *S.cerevisiae*@CaP. (b) Optical images of ABTS assay reaction mixtures after 4 h.

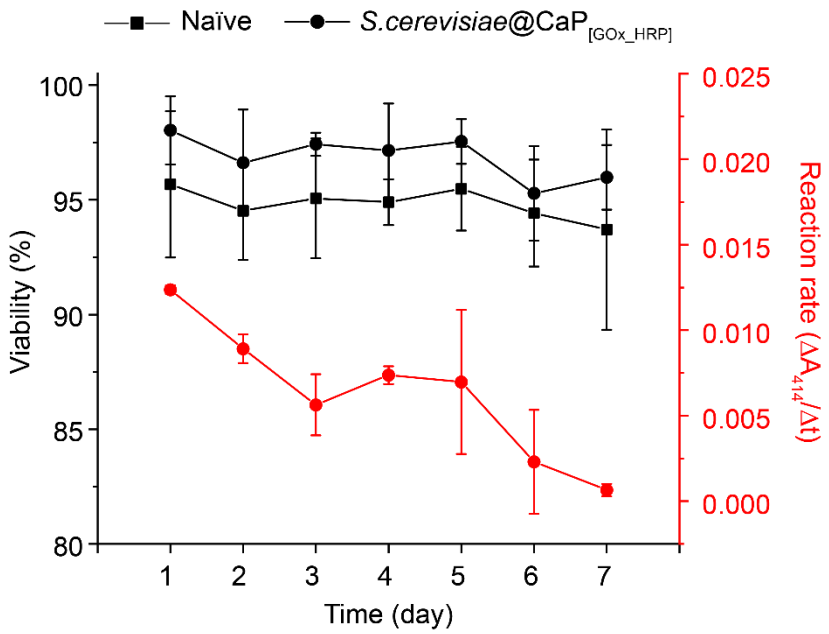

Figure S11. (black) Long-term viability of naïve and *S.cerevisiae*@$CaP_{[GOx\_HRP]}$ cells and (red) reaction rates for *S.cerevisiae*@$CaP_{[GOx\_HRP]}$ during storage at 4 ºC.