Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2024

Electronic Supplementary Information

Biphasic Water-Oil Systems for Functional Augmentation of Probiotic *Lactobacillus acidophilus* Nanoencapsulated in Luteolin-Fe^{3+} Shells

*Duc Tai Nguyen,[1] Sang Yeong Han,[1] Filip Kozlowski,[2] Gulaim A. Seisenbaeva,[2] Vadim G. Kessler,[2] Beom Jin Kim,[3] and Insung S. Choi[1,]**

[1] Center for Cell-Encapsulation Research, Department of Chemistry, KAIST, Daejeon 34141, Korea
[2] Department of Molecular Sciences, Swedish University of Agricultural Sciences, P.O. Box 7015, 75007 Uppsala, Sweden
[3] Department of Chemistry, University of Ulsan, Ulsan 44776, Korea

Table of Contents

- Experimental.
- **Figure S1.** Optical images of the biphasic system before and after vortex.
- **Figure S2.** Young's moduli of *L. acidophilus* and *L.acidophilus*@LUT-Fe^{3+}.
- **Figure S3.** AFM image, FT-IR, XPS, and UV-vis absorbance spectra of hollow LUT-Fe^{3+} capsules.
- **Figure S4.** FE-SEM images of various *L.acidophilus*@flavonoid-Fe^{3+}.
- **Figure S5.** FE-SEM images, EDX mapping, CLSM images and viability test of *S.cerevisiae*@LUT-Fe^{3+}.
- **Figure S6.** Viability at pH 2 and long-term viability of naïve and SCNEd *L. acidophilus*.
- **Figure S7.** Optical images of the *L.acidophilus*@LUT-Fe^{3+} suspension and FE-SEM images of *L.acidophilus*@LUT-Fe^{3+} before and after incubation in GSH.
- **Figure S8.** ABTS assay of the supernatant collected from the cell suspension after incubation with H_2O_2.

Experimental

Materials. Luteolin (LUT, 98%, TCI), quercetin (QUE, 95%, Sigma-Aldrich), naringenin (NAR, 93%, TCI), myricetin (MYR, 97%, TCI), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, ≥98.0%, Sigma-Aldrich), sunflower seed oil (100% extract, Haepyo), Alexa Fluor 488-conjugated albumin from bovine serum (BSA-Alexa 488, Invitrogen), polyoxyethylene (20) sorbitan monooleate (Tween 80, TCI), polystyrene microparticle (PS, diameter: 3.97 µm, Microparticles GmbH), tetrahydrofuran (THF, HPLC grade, Junsei), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, 98%, Sigma-Aldrich), peroxidase from horseradish (HRP, 250 U/mg, Sigma-Aldrich), hydrogen peroxide (H_2O_2, 35.5%, Samchun), L-glutathione reduced (GSH, 98%, Sigma-Aldrich), α-amylase from porcine pancreas (10-11 U/mg, Sigma-Aldrich), hydrochloric acid (HCl, 37%, Daejung), lysozyme from hen egg white (>23000 U/mg, Roche), 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich), propidium iodide (PI, Sigma-Aldrich), SYTO™ 9 green fluorescent nucleic acid stain (SYTO 9, Thermo Fisher Scientific), fluorescein diacetate (FDA, Sigma-Aldrich), *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), L-ascorbic acid (99%, Sigma-Aldrich), BD GasPak™ EZ anaerobic gas generating pouch systems (BD GasPak™ supplies), dimethyl sulfoxide (DMSO, 99.9%, Sigma-Aldrich), ethanol (absolute, Supelco), potato starch covalently linked with Remazol Brilliant Blue R (Starch Azure, Sigma-Aldrich), acetone (99.5%, Samchun Chemicals), phosphate-buffered saline (PBS, pH 7.4, Welgene), and acetic acid (99.7%, Sigma-Aldrich) were used as received. Deionized (DI) water (18.3 MΩ·cm) from Milli-Q Direct 8 (Millipore) was used.

Single-Cell Nanoencapsulation (SCNE). A single colony of *L. acidophilus*, picked from an MRS agar plate, was cultured for 24 h in the MRS broth medium at 33 °C prior to SCNE. To 5 mL of an aqueous suspension of *L. acidophilus* (OD_{600} = 0.5) with $FeCl_3$ (0.5 mM) was added 5 mL of a sunflower oil solution of LUT (0.5 mM). The mixture was stirred for 60 min at 700 rpm at room temperature, transferred to a microtube, and centrifuged at 6000 *g* for 1 min to collect *L.acidophilus*@LUT-Fe^{3+}. The SCNEd cells were washed with an aqueous solution of Tween 80 (1 mg/mL) at least 3 times to remove oil residuals, and washed with DI water. For SCNE of *S. cerevisiae*, a single colony of *S. cerevisiae*, picked from an YPD agar plate, was cultured for 30 h in the YPD broth medium at 33 °C before SCNE. For the formation of hollow capsules, PS particles were coated using the same protocol as for *L. acidophilus.* For the removal of the PS core, the PS@LUT-Fe^{3+} in THF (1 mL) was vortexed for 1 min and centrifuged at 2000 *g* for 1 min, and the supernatant was removed. After repeating the process three times, THF was added, and the suspension was incubated for 3 h for PS removal.

Viability. To an aqueous suspension of naïve or *L.acidophilus*@LUT-Fe^{3+} (1 mL) were added 2 µL of the SYTO 9 stock solution (3.34 mM in DMSO) and 2 µL of the PI stock solution (20 mM in DMSO). The resultant was incubated for 20 min at 33 °C, and viability was assessed, after washing with DI water, by confocal laser-scanning microscopy (CLSM). For viability assay of *S. cerevisiae*, FDA (10 mg/mL in acetone) was used instead of SYTO 9. For the measurement of colony-forming units (CFUs), *L.acidophilus*@LUT-Fe^{3+} were incubated in an aqueous solution

of L-ascorbic acid (20 mM) for 15 min for shell degradation. The cell suspension (OD_{600} = 1) was 10-fold serially diluted (from 10^0 to 10^{-5}) in DI water, and 100 μL of the final diluted suspension (10^{-5}) was spread on an MRS agar plate and cultured for 24 h at 33 ºC. For long-term viability measurement, the cell suspension in DI water were put in a sealed tube, placed in an anaerobic pouching system, and keep at 4 ºC until the SYTO 9/PI viability test at predetermined time point.

Functional Augmentation. *(a) Lysozyme:* To 100 μL of *L.acidophilus*@LUT-Fe^{3+} or naïve cells (OD_{600} = 1) was added 900 μL of a lysozyme solution (1 mg/mL in PBS, pH 7.4). The mixture was incubated at 37 ºC in an incubator, and OD_{600} of the mixture was measured, with 100 μL of an aliquot, at the predetermined time points. *(b) DPPH:* To 1100 μL of ethanol were sequentially added 100 μL of *L.acidophilus*@LUT-Fe^{3+} or naïve cells (OD_{600} = 1) and 300 μL of a DPPH solution (600 μM in ethanol), and the UV absorbance was measured at 517 nm at the predetermined time points, after taking 100 μL of the supernatant. *(c) H_2O_2:* The naïve cells or *L.acidophilus*@LUT-Fe^{3+} (OD_{600} = 1 each) were incubated in DI solution of H_2O_2 (30 mM) for 10 min. After that, the supernatant was subjected to an ABTS (1 mM)/HRP(0.75 U/mL) assay to record the $ABTS^{+\bullet}$ absorbance at 414 nm. *(d) α-Amylase*: To 100 μL of a sample (*L.acidophilus*@LUT-Fe^{3+}, naïve *L. acidophilus*, free LUT, or free QUE) was added 200 μL of aqueous α-amylase solution (2 mg/mL). After 15 min of incubation at 37 ºC for 15 min, 200 μL of a Starch Azure suspension (pre-heated at 37 ºC, 20 mg/mL) was immediately added to the mixture, and the resulting mixture was incubated at 37 ºC. After 1 h of reaction, 100 μL of acetic acid (3 M) was added to the mixture to terminate the reaction, followed by filtering with membrane filters (pore size: 0.2 μm) to collect the supernatants. The UV-vis absorbance was measured at 595 nm, and the inhibition percentage was calculated by %Inhibition = 100 − (absorbance of a sample)/(absorbance of the control) × 100. In the case of the control, DI water was used in replacement of *L. acidophilus* suspension in the reaction mixture.

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 and energy-dispersive X-ray (EDX) spectroscopy elemental analysis were conducted with a Talos F200X (FEI) with a carbon-support-film copper mesh. Atomic force microscopy (AFM) images were taken in the QI mode with a NanoWizard4 BioAFM (JPK), and Young's modulus was calculated using the Hertz/Sneddon model (tip: HQ-NSC15/AI BS, MikroMasch). The ζ-potential was measured with a Zetasizer Nano ZS (Malvern). 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 to 11) were prepared, with one measurement conducted for each sample, except for the measurement of Young's moduli. For Young's moduli, two independent samples were prepared, with 6 measurements taken from individual bacteria in each sample (n = 12). Data are presented as mean ± SE and represented as white circles to denote the measured value from each independent experiment. Statistical significance was determined using an unpaired, two-sided Student's t-test (n = 10). IBM SPSS Statistics 26 and Origin 2019 were used to perform data analysis and graph generation.

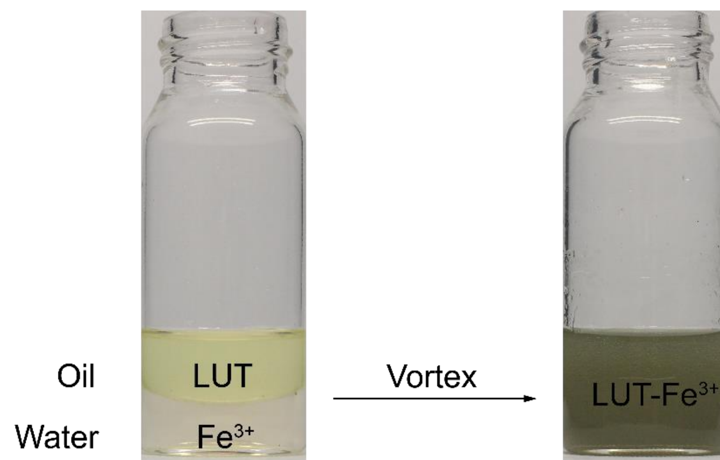

Figure S1. Optical images of the reaction mixture before and after vortex.

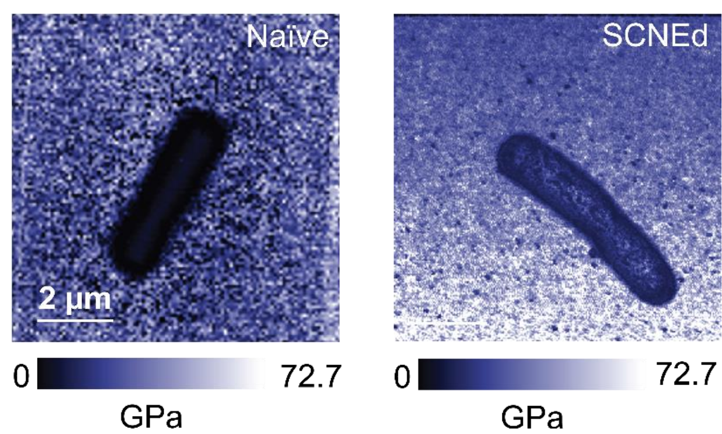

Figure S2. Young's moduli of naïve and SCNEd *L. acidophilus*.

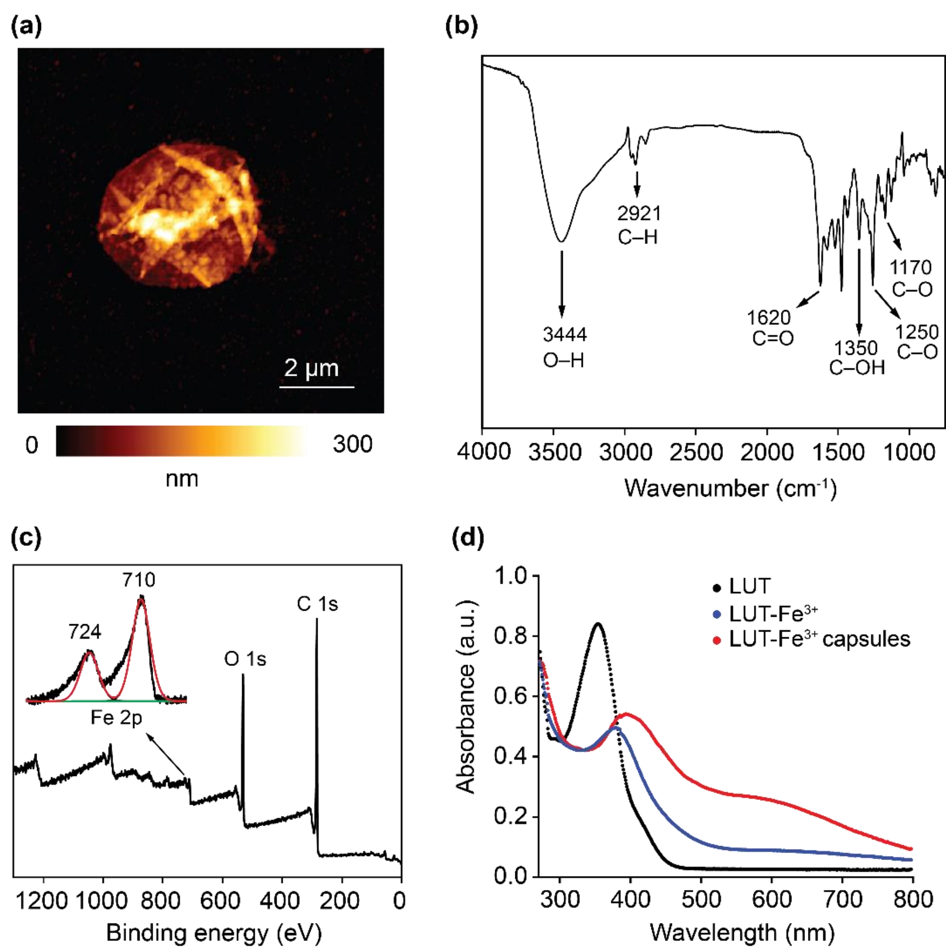

Figure S3. (a) AFM image of hollow LUT-Fe^{3+} capsules. (b-d) (b) FT-IR, (c) XPS, and (d) UV-vis absorbance spectra of hollow LUT-Fe^{3+} capsules.

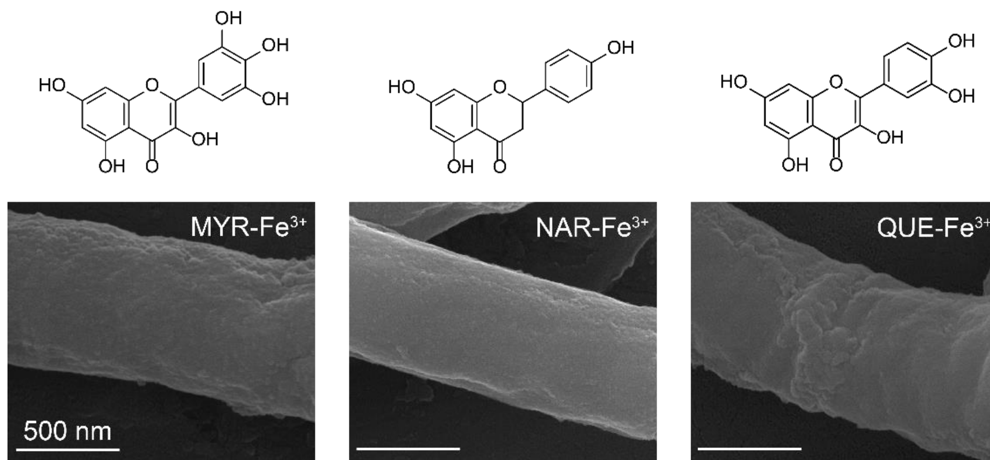

Figure S4. FE-SEM images of *L.acidophilus*@flavonoid-Fe^{3+}.

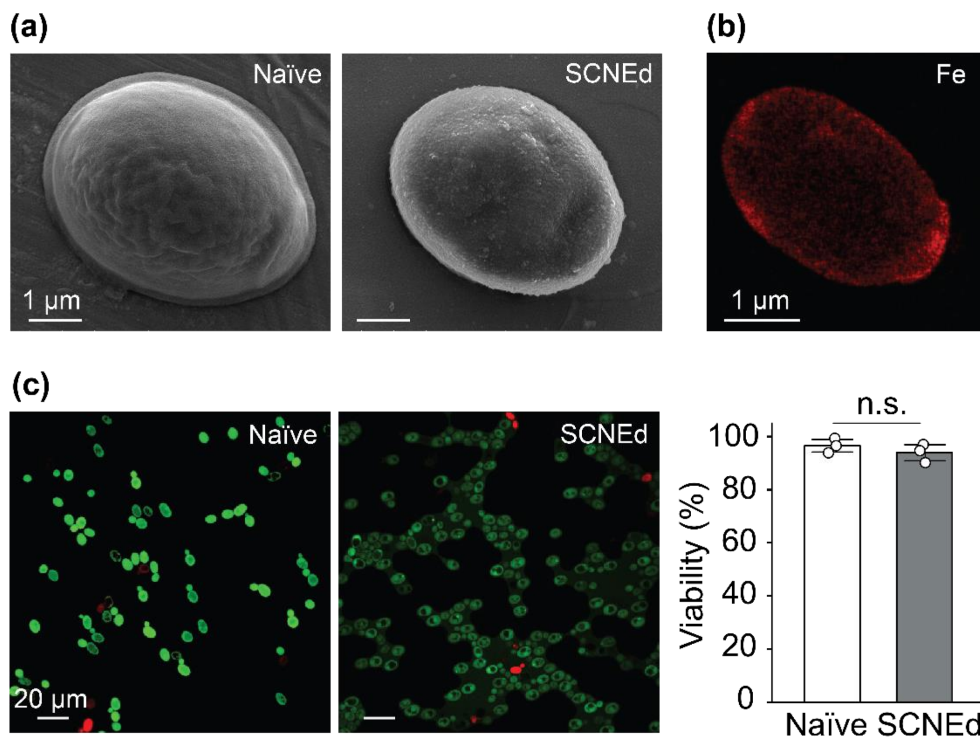

Figure S5. (a) FE-SEM images of naïve and SCNEd *S. cerevisiae*. (b) TEM-EDX Fe mapping of *S.cerevisiae*@LUT-Fe^{3+}. (c) Live/dead assay: CLSM images and viability of *S. cerevisiae* before and after SCNE. FDA (green): live and PI (red): dead. Data are represented as mean ± SE (unpaired, two-sided Student's *t*-test; *n* = 3; n.s.: not significant).

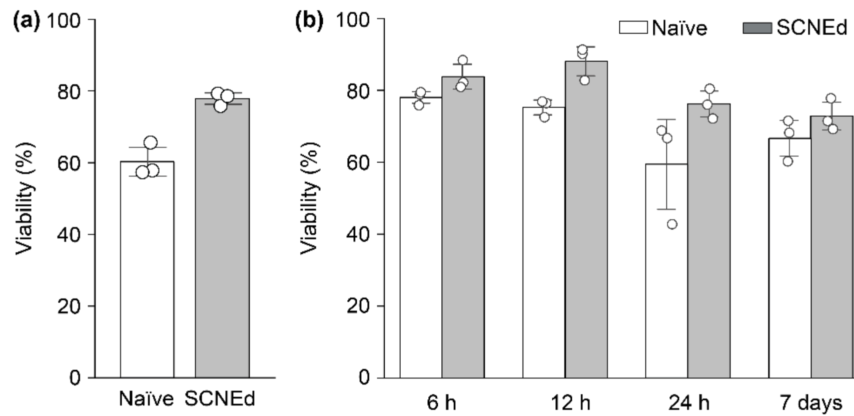

Figure S6. (a) Viability of naïve and SCNEd *L. acidophilus* at pH 2. (b) Long-term viability of naïve and SCNEd *L. acidophilus*.

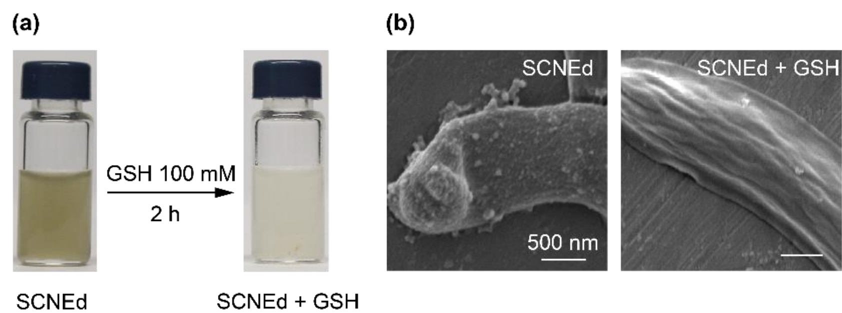

Figure S7. (a) Optical images of the *L.acidophilus*@LUT-Fe^{3+} suspension before and after incubation in 100-mM GSH. (b) FE-SEM images of *L.acidophilus*@LUT-Fe^{3+} before and after incubation in 100-mM GSH.

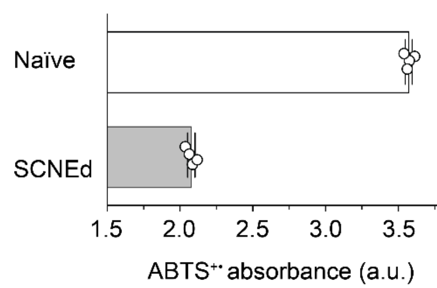

Figure S8. ABTS assay of the supernatant collected from the cell suspension after incubation with H_2O_2.