

Supplementary Materials: Cytoprotection of Probiotic *Lactobacillus acidophilus* with Artificial Nanoshells of Nature-Derived Eggshell Membrane Hydrolysates and Coffee Melanoidins in Single-Cell Nanaencapsulation

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Experimental Section

Materials. Sodium chloride (NaCl, 99.5% Daejung Chemicals), sodium hydroxide (NaOH, 95%, Junsei), calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\geq 99.0\%$, Sigma-Aldrich), sodium carbonate (Na_2CO_3 , $\geq 99.5\%$, Sigma-Aldrich), iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, $\geq 98.0\%$, Sigma-Aldrich), poly(sodium 4-styrenesulfonate) (PSS, Mw $\sim 70,000$, Sigma-Aldrich), polyethylenimine, branched (PEI, average Mw $\sim 25,000$, Sigma-Aldrich), tannic acid (TA, Sigma-Aldrich), hydrogen chloride solution (HCl, 35%, Junsei), ethanol (95.0%, Samchun Chemicals), acetone (99.5%, Samchun Chemicals), dimethyl sulfoxide (DMSO, $\geq 99.0\%$, Sigma-Aldrich), fluorescein diacetate (FDA, Sigma-Aldrich), propidium iodide (PI, Sigma-Aldrich), SYTOTM 9 green fluorescent nucleic acid stain (Thermo Fisher Scientific), pepsin from porcine gastric mucosa (≥ 400 units/mg, Sigma-Aldrich), *S. cerevisiae* (ATCC 18824 from the Korean Collection for Type Cultures (KCTC)), *L. acidophilus* (ATCC 4356 from the American Type Culture Collection (ATCC)), *L. brevis* (ATCC 14869 from the ATCC), yeast-extract-peptone-dextrose (YPD) broth (Duchefa Biochemistry), YPD agar (Duchefa Biochemistry), *Lactobacilli* MRS broth (BD), *Lactobacilli* MRS agar (BD), silica particles, amino-functionalized (diameter: $3.92\ \mu\text{m}$, Microparticles GmbH), buffer solutions (pH 1, 2, 3, 4, 5, 6, and 7, Samchun Chemicals), silver foil (Ag, 99.9%), aluminum foil (Al, 99.997%, Alfa Aesar), copper foil (Cu, 99.95%, Alfa Aesar), nickel (Ni, 99.5%, Alfa Aesar), tin foil (Sn, 99.8%, Alfa Aesar), stainless steel (SS, Fe:Cu:Ni; 70:19:11 wt%, Alfa Aesar), and titanium foil (Ti, 99.5%, Alfa Aesar) were used as received. Polycarbonate (PC), polyethylene (PE), polypropylene (PP), polyurethane (PU), and polytetrafluoroethylene (PTFE) substrates were purchased at an internet market (ENGP, Korea). Deionized (DI) water ($18.3\ \text{M}\Omega\ \text{cm}$) from Milli-Q Direct 8 (Millipore) was used.

Characterizations. The ellipsometric thickness was measured with an Elli-SE spectroscopic ellipsometer (Ellipso Technology). At least five independent points of each sample were measured, and average value (with at least 20 measurements) were recorded. Field-emission scanning electron microscopy (FE-SEM) images were obtained with an Inspect F50 microscope (FEI) with an accelerating voltage of 5 kV or 10 kV, after sputter-coating with platinum. Atomic force microscopy (AFM) image was obtained with a NanoWizard 4 XP Bioscience AFM (JPK), in the QI mode using an HQ:NSC15/Al BS tip (MikroMasch). X-ray photoelectron spectroscopy (XPS) spectra were acquired with a Sigma Probe (Thermo VG Scientific). Fourier-transform infrared (FT-IR) spectra were recorded with a nitrogen-purged Thermo Nicolet Nexus FT-IR spectrophotometer. Static water contact angle measurements were performed using a Phoenix 300 goniometer (Surface Electro Optics Co.) equipped with a video camera. The static contact angle of a $3\text{-}\mu\text{L}$ water droplet was measured at three different locations on each sample, and average value were recorded. The confocal laser-scanning microscopy (CLSM) images were carried out with an LSM 700 (Carl Zeiss). The ζ -potential was measured with a Zetasizer Nano ZS (Malvern). The cell density was measured with a microplate reader (SpectraMax iD5, Molecular Devices).

One-Step Formation of ESMH-CM Films and Shells. The stock solution of ESMHs or CMs was made to the final concentration of 2 mg/mL in a NaCl solution (50 mM). Prior to use, gold

substrates were cleaned with ethanol and acetone. The cleaned gold substrates were immersed in a 1:1 mixture of the ESMH and CM stock solutions (500 μ L each), stirred at 120 rpm for 3 h, washed with DI water, and dried under a stream of argon gas. The same protocol was employed for other flat substrates. CaCO_3 particles were prepared by mixing PSS (4 mL, 2 mg/mL in DI water), an aqueous solution of Na_2CO_3 (48 μ L, 1 M), and an aqueous solution of CaCl_2 (96 μ L, 1 M) under vigorous stirring for 40 s, incubating for 7 min, and calcinating at 450 $^\circ\text{C}$ for 2 h. ESMH-CM shells were formed on the resulting CaCO_3 particles with a 1:1 mixture of the ESMH and CM stock solutions (500 μ L each).

Single-Cell Nanoencapsulation (SCNE). A single colony of *S. cerevisiae*, picked from the YPD agar plate, was cultured for 30 h in a YPD broth medium at 33 $^\circ\text{C}$. After washing with DI water, *S. cerevisiae* were immersed for 3 h in a 1:1 mixture of the ESMH and CM stock solutions (500 μ L each) and washed with DI water three times. The same protocol was employed for *L. acidophilus* and *L. brevis*, after culturing for 24 h in an MRS broth medium at 33 $^\circ\text{C}$. For viability assay of *S. cerevisiae*, 5 μ L of the FDA stock solution (10 mg/mL in acetone) and 2 μ L of an aqueous PI stock solution (1 mg/mL) were added to a *S. cerevisiae* suspension (1 mL), and the resultant was incubated for 15 min at 33 $^\circ\text{C}$. SYTO 9 was used instead of FDA for *L. acidophilus* and *L. brevis*. To a cell suspension (1 mL) were added 2 μ L of the SYTO 9 stock solution (3.34 mM in DMSO) and 2 μ L of the PI stock solution (20 mM in DMSO), and the resultant was incubated for 20 min at 33 $^\circ\text{C}$. To form the ESMH-CM $[\text{Fe}^{3+}]$ shell, *L.acidophilus*@ESMH-CM or *L.brevis*@ESMH-CM was immersed in an aqueous solution of FeCl_3 (10 mM) for 30 min. The $t_{-2.0}^{\text{OD}_{600}}$ values were calculated based on the results of cell culture in the MRS broth medium. In short, 1 mL of an aqueous cell suspension (*L. acidophilus*, *L.acidophilus*@ESMH-CM, or *L.acidophilus*@ESMH-CM $[\text{Fe}^{3+}]$, $\text{OD}_{600} = 0.15$) was added to 150 mL of the MRS broth medium (final $\text{OD}_{600} = 0.001$) and incubated at 33 $^\circ\text{C}$. The 100 μ L of the culture mixture was picked at the predetermined time, and the cell density was measured at 600 nm with a microplate reader. Linear fitting of $\ln(\text{OD}_{600})$, from -4.0 to 1.0 , with incubation time (in hour) gave $t_{-2.0}^{\text{OD}_{600}}$, the time for $\ln(\text{OD}_{600})$ of -2.0 .

Cytoprotection. (a) *PEI*: *S. cerevisiae* and *S.cerevisiae*@ESMH-CM were suspended in 1 mL of an aqueous PEI solution (0.5, 1, 10, or 50 mg/mL) for 30 min, and the cell viability was measured by the FDA-PI assay. (b) *TA*: *S. cerevisiae* or *S.cerevisiae*@ESMH-CM were suspended in 1 mL of an aqueous TA solution (1, 5, 10, 25, or 50 mg/mL) for 1 h, and the cell viability was measured by the FDA-PI assay. (c) *SGF*: The SGF was made to contain 0.2% (w/v) NaCl and pepsin (3 mg/mL), with pH adjustment to 2 by 1 M HCl . The cells were suspended in the SGF solution and incubated at 37 $^\circ\text{C}$ for 2 h, and the cell viability was measured with SYTO 9 and PI.

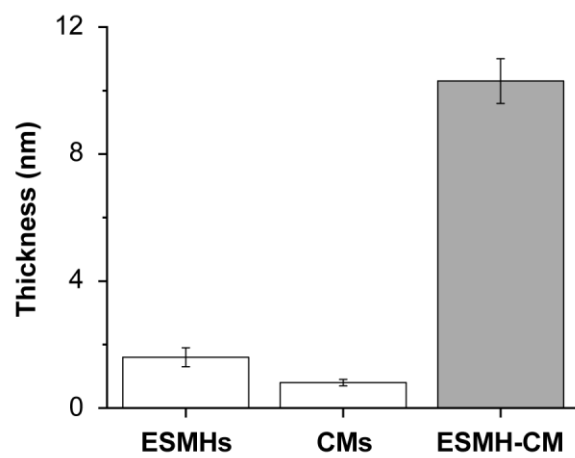


Figure S1. Graph of film thickness after 3 h of incubation (ESMHs, CMs, and ESMH-CM).

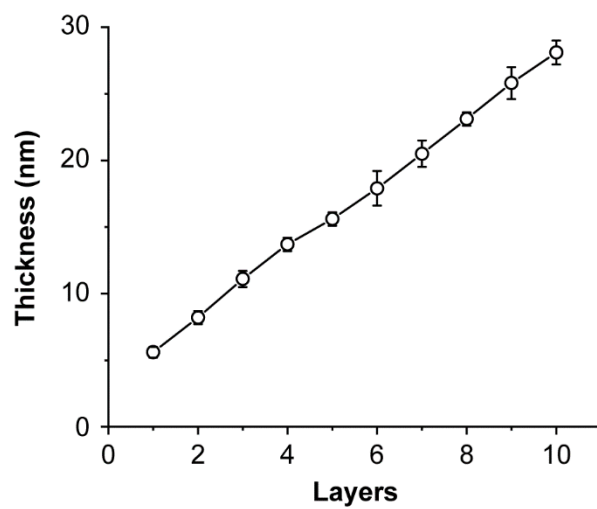


Figure S2. Thickness of $[\text{ESMH-CM}]_n$ films versus number of depositions. Each deposition was done for 10 min in a freshly made, 50-mM NaCl solution of ESMHs and CMs ($[\text{ESMH}] = [\text{CM}] = 1 \text{ mg/mL}$).

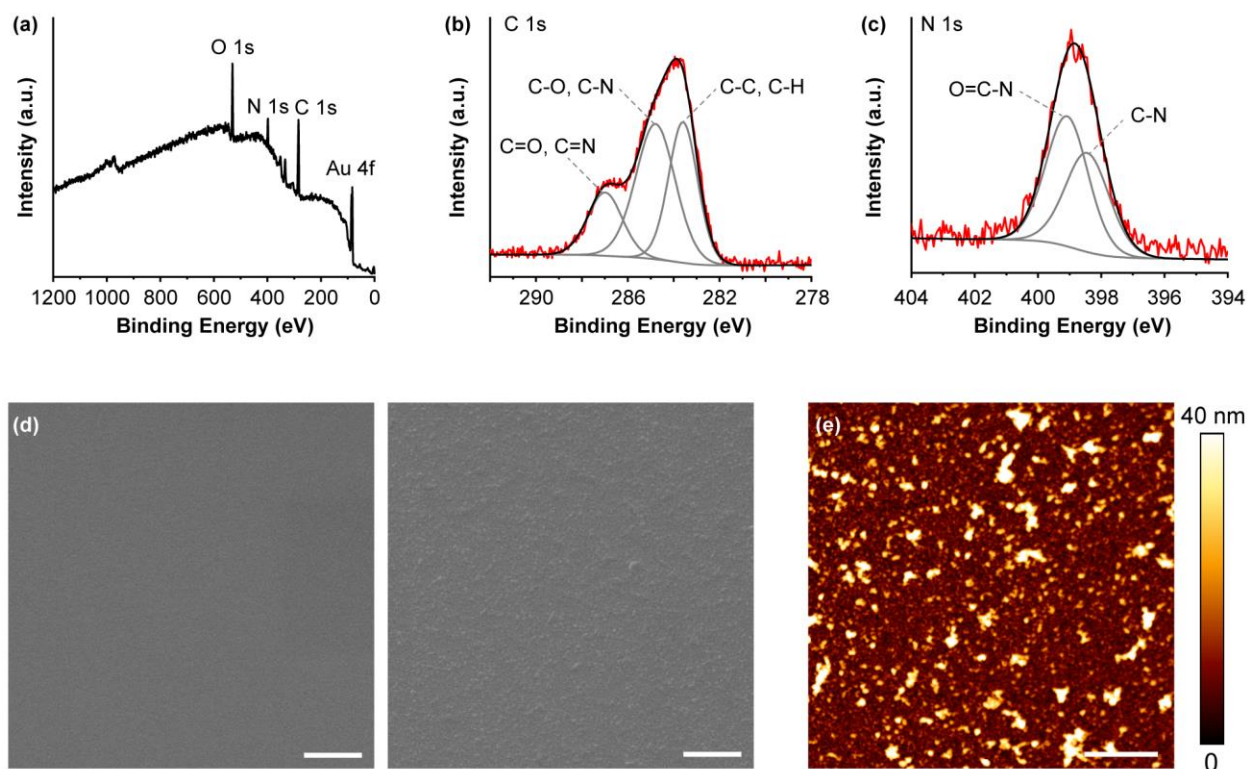


Figure S3. Characterizations of ESMH-CM films on gold substrates ($[\text{ESMH}] = [\text{CM}] = 1$ mg/mL; $[\text{NaCl}] = 50$ mM; 3 h of incubation): (a) XPS spectrum. (b-c) Deconvoluted XPS spectra for C 1s and N 1s. (d) FE-SEM images (left: bare gold substrates, right: ESMH-CM films on gold substrates). (e) AFM image. Scale bar: 1 μm .

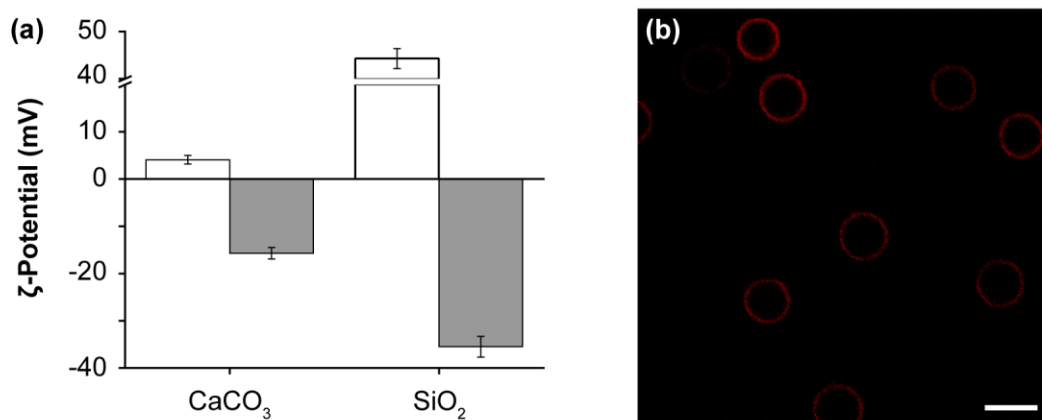


Figure S4. (a) ζ -Potential changes (white) before and (gray) after ESMH-CM-shell formation on CaCO_3 and SiO_2 particles. (b) CLSM image of ESMH_TAMRA-CM shells on SiO_2 particles. Scale bar: 5 μm .

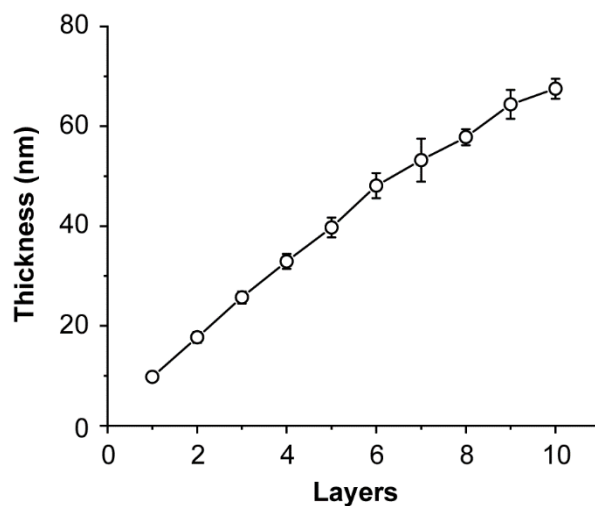


Figure S5. Thickness of $[\text{ESMH-CM}]_n$ films versus number of depositions. Each deposition was done for 3 h in a freshly made, 50-mM NaCl solution of ESMHs and CMs ($[\text{ESMH}] = [\text{CM}] = 1 \text{ mg/mL}$).