Supplementary Information

Polypeptide-based multilayer capsules with anti-inflammatory properties: exploring different strategies to incorporate hydrophobic drugs

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X-ray diffraction analysis

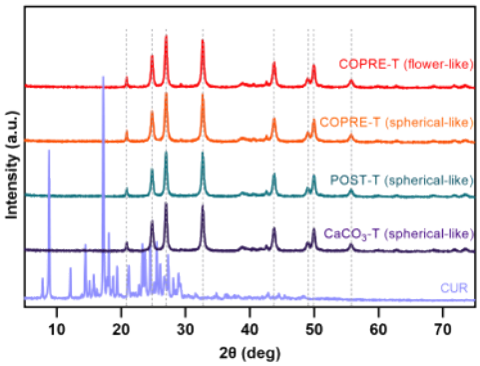

Figure S1. X-ray diffraction (XRD) pattern of curcumin (CUR) (light blue line), calcium carbonate ($CaCO_3$) microparticles ($CaCO_3$-T) (purple line), CUR-loaded $CaCO_3$ microparticles by post-encapsulation (POST-T) (blue line), CUR-loaded $CaCO_3$ microparticles by co-precipitation (COPRE-T) with their spherical-like (orange line), and flower-like (red line) shape. POST-T was obtained after dropwise addition of CUR (1 mg) dissolved in 0.5 mL of ethanol to pre-synthesized $CaCO_3$-T (30 mg) suspended in 0.5 mL of distilled water. Spherical COPRE-T was obtained by directly adding CUR (1 mg) dissolved in 0.3 mL of ethanol to 1.5 mL of a 0.2 M $CaCl_2$ solution. Flower-like COPRE-T was obtained by dropwise adding CUR (1 mg) dissolved in 1 mL of ethanol to 0.5 mL of a 0.2 M $CaCl_2$ solution.

Characterization of curcumin-conjugate poly(L-glutamic acid)

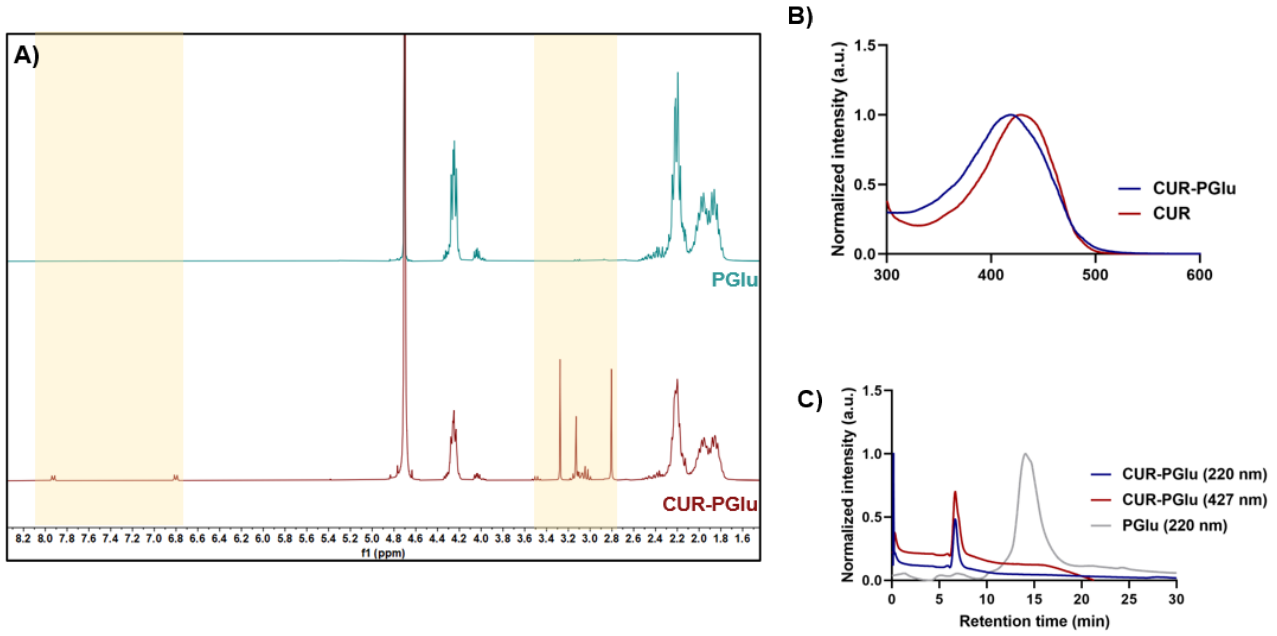

Figure S2. A) ^{1}H-NMR spectra of poly(L-glutamic acid) (PGlu) (blue line) and CUR-conjugate poly(L-glutamic acid) (CUR-PGlu) (red line) in deuterated water. B) UV-Vis spectra of curcumin (CUR) (red line) and CUR-PGlu (blue line). C) Chromatograms of PGlu (grey line) at 220 nm, CUR-PGlu at 220 nm (blue line) and at 427 nm (red line) acquired by high-performance liquid chromatography (HPLC) after the purification of the compound using a photo diode array (PDA) detector.

Scanning electron microscopy of LbL microcapsules with flower-like shape

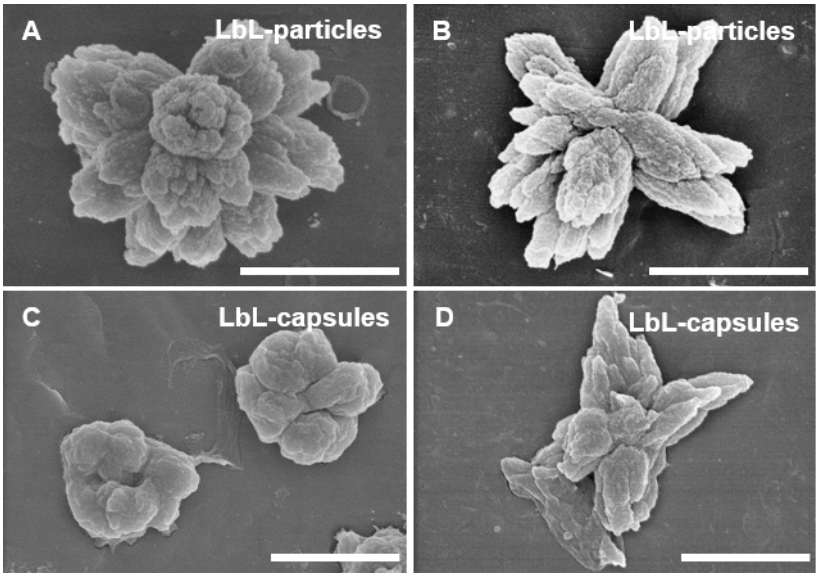

Figure S3. A-B) SEM images of layer-by-layer (LbL) particles synthesized from flower-like curcumin (CUR)-loaded templates. The flower-like templates were obtained *via* co-precipitation approach by direct (A) or dropwise (B) addition of CUR (1 mg) dissolved in 1 mL ethanol to 0.5 mL of 0.2 M $CaCl_2$. C-D) SEM images of LbL-capsules after the core removal from the systems synthesized by direct (C) and dropwise (D) addition of CUR during the synthesis of the template. Scale bar = 2 μm.

Characterization of unloaded LbL systems

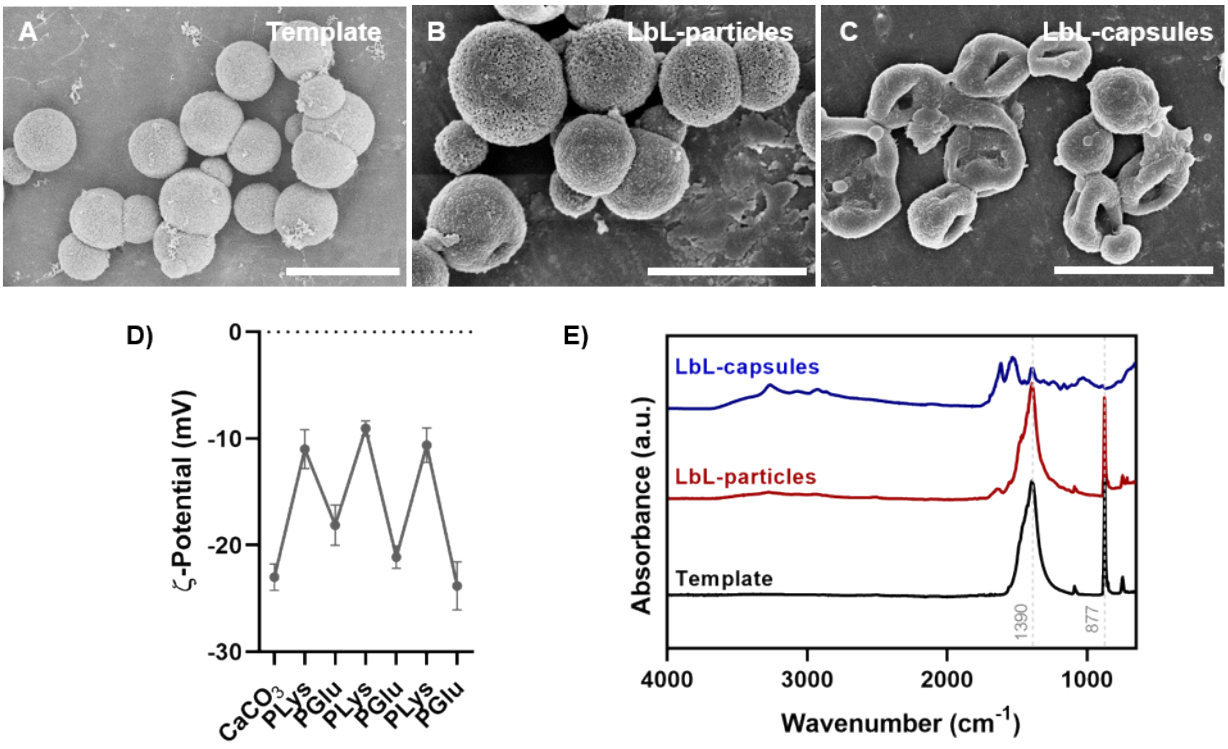

Figure S4. A-C) SEM images of unloaded calcium carbonate ($CaCO_3$) microparticles used as template (A), layer-by-layer (LbL)-particles (B), and LbL-capsules (C). Scale bar = 5 μm. D) ζ-potential measurements after the deposition of each polypeptide layer onto $CaCO_3$ microparticles. The values are reported as mean ± standard deviation (SD); (N = 3).

E) Attenuated total reflection – Fourier transform infrared spectroscopy (ATR-FTIR) spectra of template (black line), LbL-particles (red line), and LbL-capsules (blue line).

Stability studies of unloaded LbL-capsules

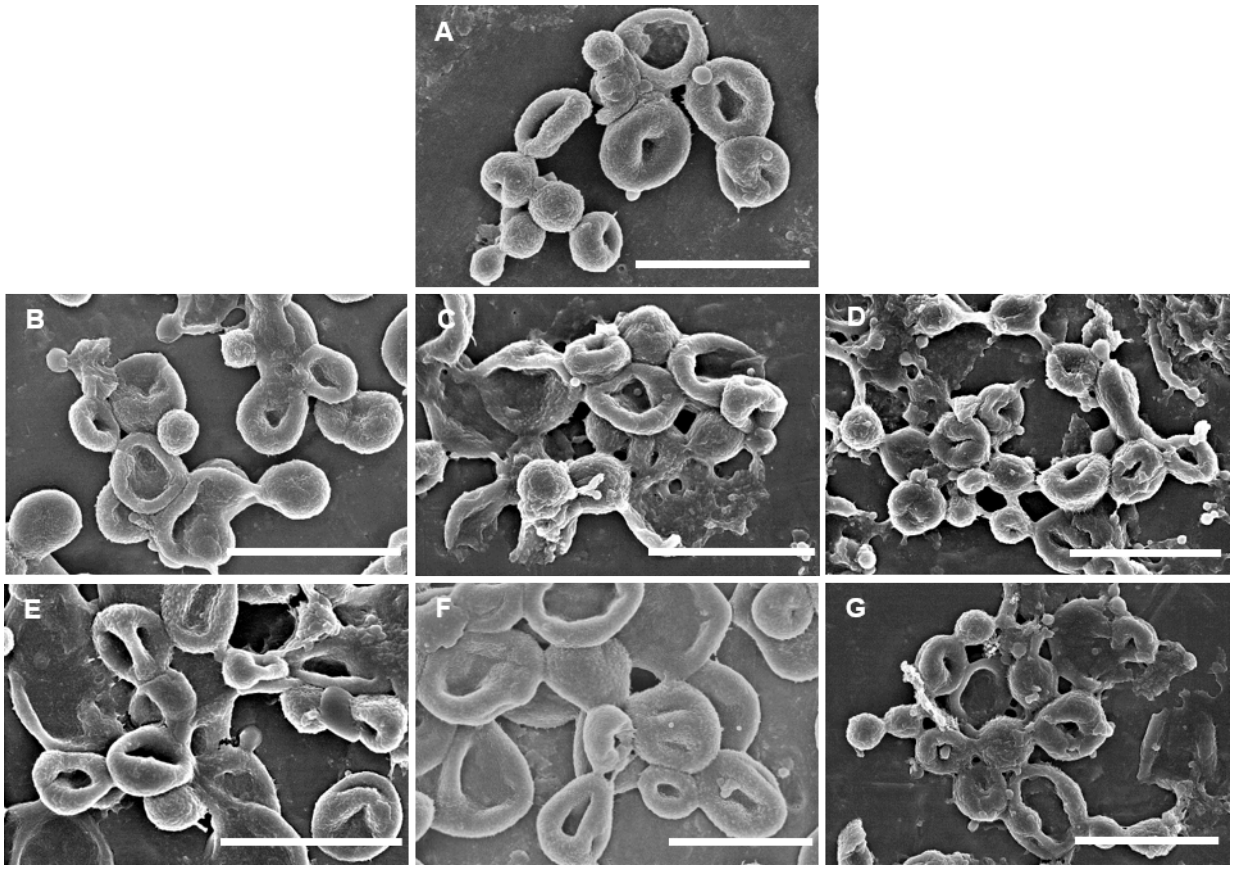

Figure S5. Stability studies of layer-by-layer (LbL)-capsules performed by incubation with tris(hydroxymethyl)aminomethane (TRIS) and/or sodium dodecyl sulfate (SDS) at pH 7.4 and 37 °C at two time points (6 h and 24 h). A-D) SEM images of the LbL-capsules after their synthesis taken as control (A), and after 6 h incubation with 0.1 M TRIS buffer (B), 0.1 M TRIS buffer + 0.1% SDS (C), and 0.1 M TRIS buffer + 0.5% SDS (D). E-G) SEM images of LbL-capsules after 24 h incubation with 0.1 M TRIS buffer (E), 0.1 M TRIS buffer + 0.1% SDS (F), and 0.1 M TRIS buffer + 0.5% SDS. Scale bar: 5 µm.

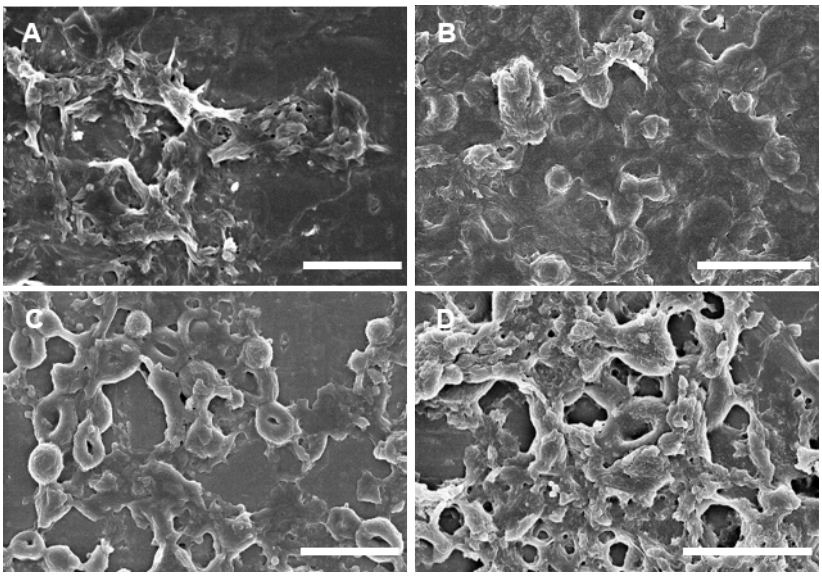

Figure S6. Stability studies of layer-by-layer (LbL)-capsules performed by incubation with tris(hydroxymethyl)aminomethane (TRIS), sodium dodecyl sulfate (SDS), and pronase at pH 7.4 and 37 °C at two time points (6 h and 24 h). A-B) SEM images of the LbL-capsules incubated with 0.1 M TRIS buffer + 0.1% SDS + 2 mg/mL pronase for 6 h (A) and 24 h (B). C-D) SEM images of the LbL-capsules incubated with 0.1 M TRIS buffer + 0.5% SDS + 2 mg/mL pronase for 6 h (C) and 24 h (D). Scale bar = 5 µm.

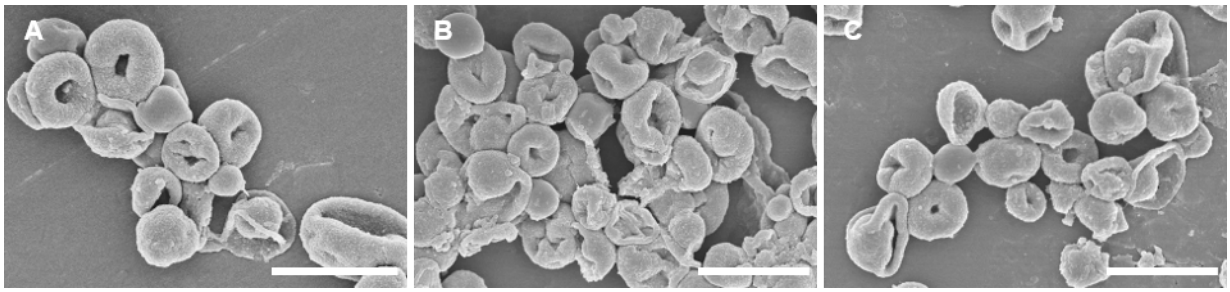

Figure S7. A) Micrograph of the layer-by-layer (LbL)-capsules after their synthesis. B-C) SEM images (LbL)-capsules incubated in artificial cerebrospinal fluid (ACSF) at 37 °C for 6 h (B) and 24 h (C). Scale bar = 5 µm.

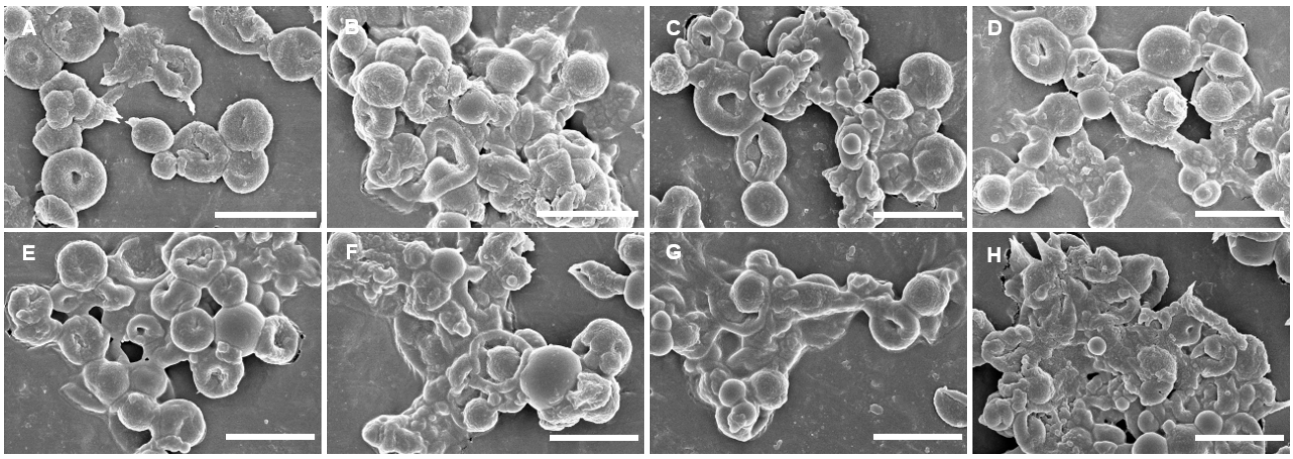

Figure S8. Stability studies of layer-by-layer (LbL)-capsules in artificial cerebrospinal fluid (ACSF) with 0.1% bovine serum albumin (BSA) in the presence or absence of matrix metalloproteinases 2 (MMP-2) at 37 °C for 6 and 24 h. A-D) SEM images of LbL-capsules incubated in 0.1% BSA ACSF (A), 0.1% BSA ACSF + 200 ng/mL MMP-2 (B), 0.1%

BSA ACSF + 500 ng/mL MMP-2 (C), and 0.1% BSA ACSF + 1000 ng/mL MMP-2 (D) for 6 h. E-F) SEM images of LbL-capsules incubated in 0.1% BSA ACSF (A), 0.1% BSA ACSF + 200 ng/mL MMP-2 (B), 0.1% BSA ACSF + 500 ng/mL MMP-2 (C), and 0.1% BSA ACSF + 1000 ng/mL MMP-2 (D) for 24 h. Scale bar = 5 µm.

Cell studies on THP-1 cells and BV-2 cells

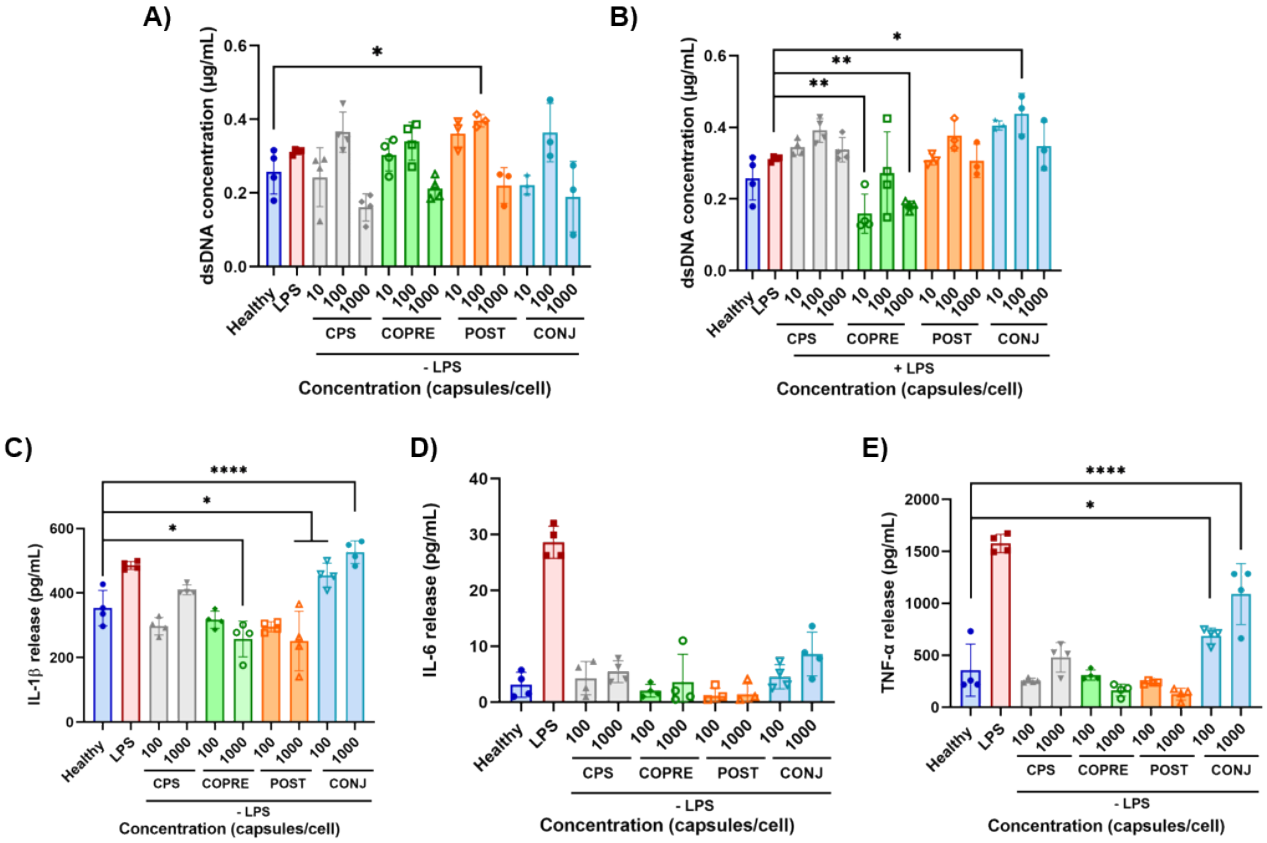

Figure S9. dsDNA content determined on THP-1 cells by Picogreen™ after 24 h of exposure to the layer-by-layer (LbL) capsules with and without curcumin (CUR) in absence (A) or presence of 100 ng/mL LPS treatment (B); (N = 4). CPS = unloaded LbL-capsules; COPRE = LbL-capsules synthesized after loading CUR into the template by co-precipitation approach; POST = LbL-capsules synthesized after loading CUR into the template by post-encapsulation approach; CONJ = LbL-capsules synthesized by loading CUR in the multilayer membrane. The values are reported as mean ± standard deviation (SD). Single ANOVA followed by Dunnet post-hoc test comparisons with the controls (healthy or LPS groups) was used (*$p < 0.05$; **$p < 0.01$). C-E) Effect on the cytokine release (i.e., tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6) from THP-1 derived macrophages treated with 100 or 1000 capsules/cell measured as the concentration in the supernatant (N = 4). The values are reported as mean ± SD. Single ANOVA followed by Dunnet post-hoc test comparisons with the controls (healthy or LPS groups) was used (*$p < 0.05$; **$p < 0.01$, ***$p < 0.0005$; ****$p < 0.0001$).

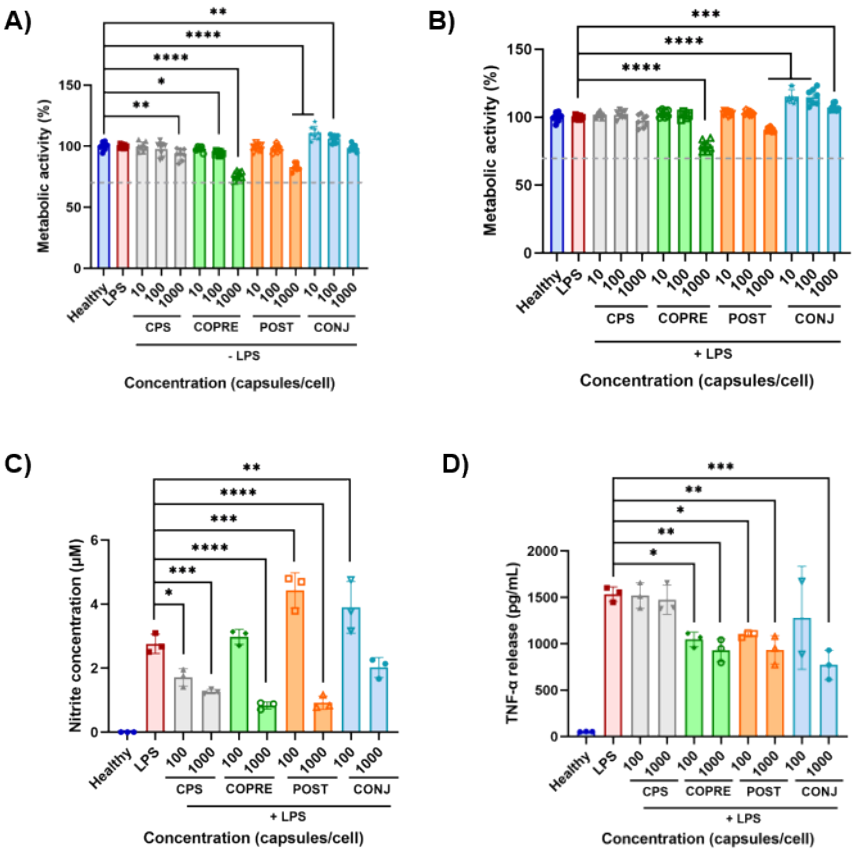

Figure S10. A-B) Metabolic activity of BV-2 cells without (A) and with (B) 20 ng/mL LPS treatment after 24 h incubation with of layer-by-layer (LbL)-capsules (i.e., 10, 100, and 1000 capsules/cell) containing curcumin (CUR) loaded by co-precipitation (COPRE), post-encapsulation (POST), and conjugation (CONJ) approaches, or in absence of CUR (CPS). Data obtained from N = 4. C) Determination of nitrite concentration released by BV-2 cells treated with 20 ng/mL LPS after incubation with CPS, COPRE, POST, and CONJ capsules at two different concentrations (i.e., 100 and 1000 capsules/cell) for 24 h. Data obtained from N = 3. D) Determination of tumor necrosis factor (TNF)-α released by BV-2 cells treated with 20 ng/mL LPS for 24 h after incubation with CPS, COPRE, POST, and CONJ capsules at two different concentrations (i.e., 100 and 1000 capsules/cell). Data obtained from N= 3. All the values are reported as mean ± standard deviation (SD). Single ANOVA followed by Dunnet post-hoc test comparisons with the controls (healthy or LPS groups) was used (*$p < 0.05$; **$p < 0.01$, ***$p < 0.0005$; ****$p < 0.0001$).

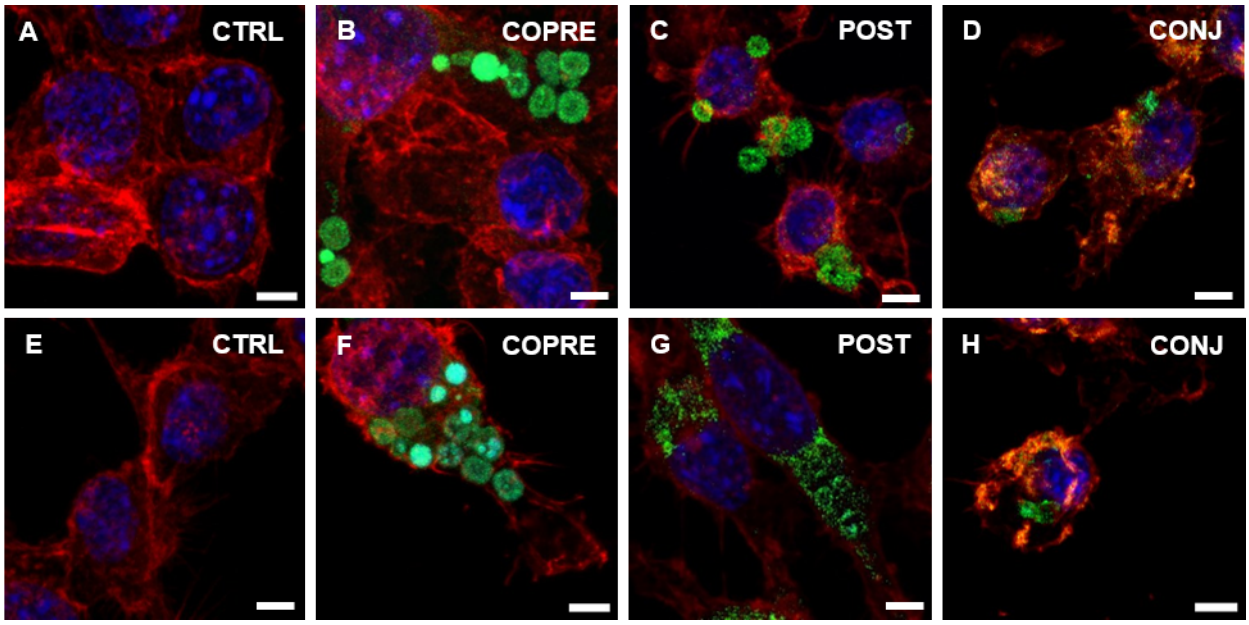

Figure S11. A-D) A zoom of the confocal images showing the uptake of COPRE (B), POST (D), and CONJ (F) capsules by resting BV-2 cells after 24 h of incubation with respect to the control (CTRL) (A). E-H) A zoom of the confocal images showing the uptake of COPRE (F), POST (G), and CONJ (H) capsules by activated BV-2 cells treated with 20 ng/mL LPS after 24 h of incubation with the capsules with respect to the control (CTRL) (E). The nuclei are stained in blue (DAPI), F-actin in red (Tetramethylrhodamine isothiocyanate (TRITC)-Phalloidin), while the green is attributed to the presence of curcumin (CUR). Scale bar: 5 µm.