# Supplementary Material

# Biomineral-binding liposomes with dual antibacterial effects for preventing and treating dental caries

## Supplementary methods

*1 Acidogenicity*

The effect of Mag and FLC on the acidogenicity of *S. mutans* or *C. albicans* was evaluated by glycolytic pH drop analysis. *S. mutans* was inoculated into BHI broth (pH 7.4) and *C. albicans* into SDB (pH 7.4) containing 1% (w/v) glucose. CHG was used as the positive control and the culture medium as the negative control. Serial 2-fold dilutions of Mag and FLC at concentrations from 0.125 MIC to 2 MIC were then added, and the pH value was measured after 0, 4, 6, 8, 12, and 24 h at 37 °C. The acidogenicity of *S. mutans* treated with blank LPs, Mag-LPs, or PPi-Mag-LPs was examined by fixing the concentration of Mag at the MIC value. Similarly, the acidogenicity of *C. albicans* treated with blank LPs, Mag/FLC-LPs, or PPi-Mag/FLC-LPs was determined by fixing the concentrations of Mag and FLC at the synergistic MIC value.

*2 Acid resistance detection*

The tolerance of *S. mutans* and *C. albicans* to acidic conditions was assessed by determining their viability at different pH values. Briefly, *S. mutans* was inoculated into BHI broth and *C. albicans* into SDB at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, then they were incubated at 37 °C for 24 h. The growth rate of both microorganisms was determined by measuring the absorbance at 600 nm. Culture medium at pH 7.4 served as the control.

**Supplementary results**

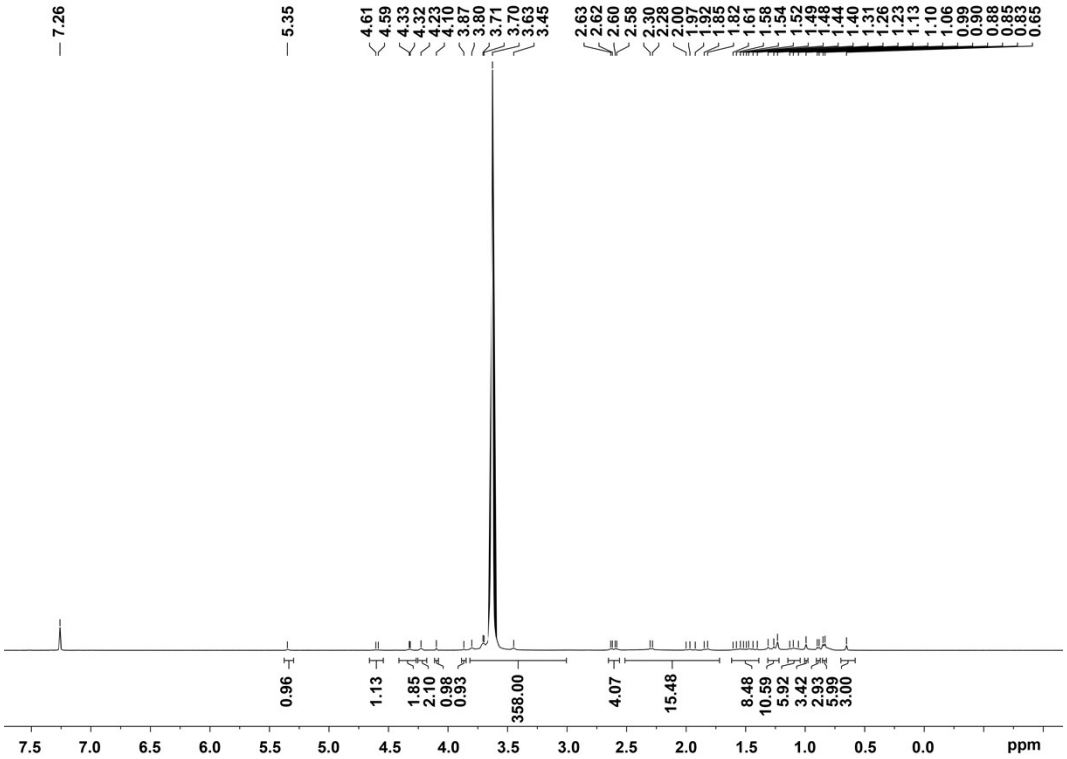


**Fig. S1.** $^{1}$H NMR spectrum of CHEMS-PEG$_{2000}$-BA. The compound CHEMS-PEG2000-BA was dissolved in deuterated chloroform, and the proton peak of CHEMS-PEG2000-BA was analyzed by NMR (400 MHz).

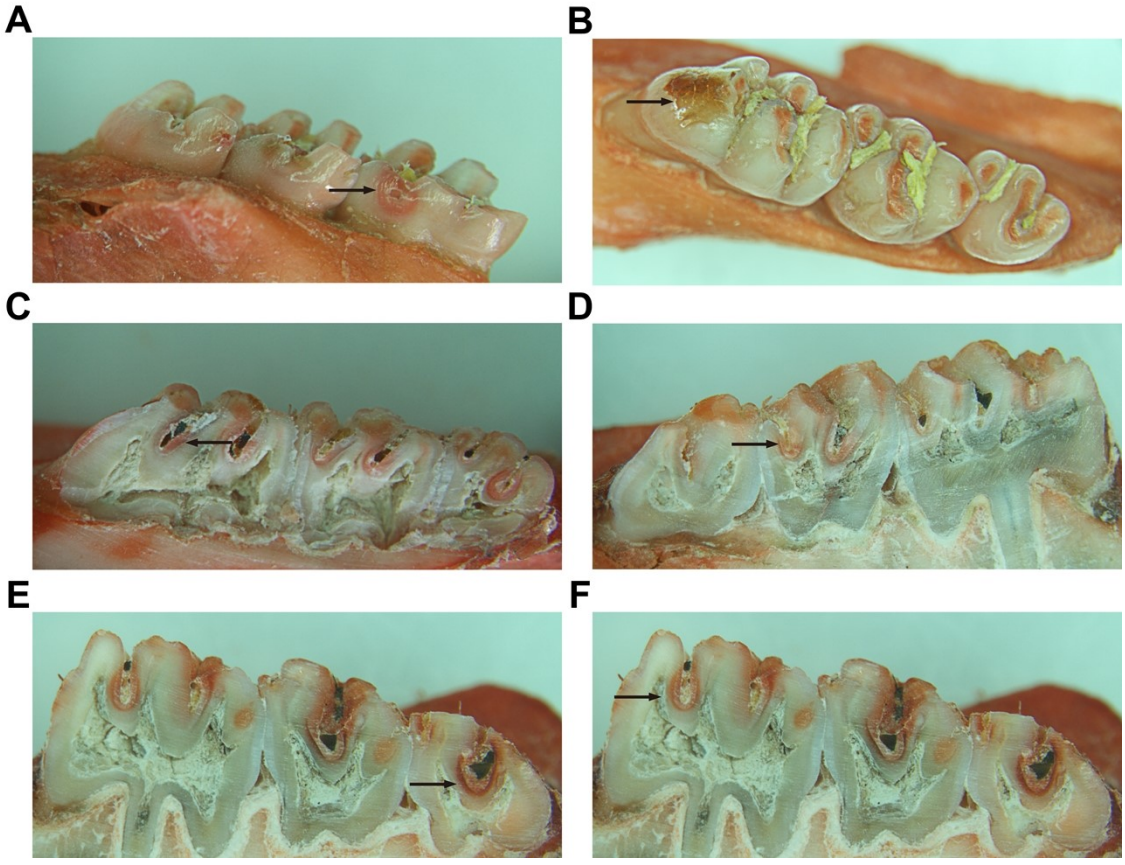


**Fig. S2.** Stereomicroscopic images of murexide-dyed molars collected from dental caries rats.

(**A-C**) Enamel caries on the (A) smooth, (B) proximal, and (C) sulcal molar surface. (**D**) Slight, (**E**) moderate, and (**F**) extensive dentin caries on the sulcal surface. Arrows indicate the respective descriptions.

***Acidogenicity***

The effect of Mag and FLC on the acidogenicity of *S. mutans* or *C. albicans* was evaluated by glycolytic pH drop analysis. *S. mutans* was inoculated into BHI broth (pH 7.4) and *C. albicans* into SDB (pH 7.4) containing 1% (w/v) glucose. CHG was used as the positive control and the culture medium as the negative control. Serial 2-fold dilutions of Mag and FLC at concentrations from 0.125 MIC to 2 MIC were then added, and the pH value was measured after 0, 4, 6, 8, 12, and 24 h at 37 °C. The acidogenicity of *S. mutans* treated with blank LPs, Mag-LPs, or PPi-Mag-LPs was examined by fixing the concentration of Mag at the MIC value. Similarly, the acidogenicity of *C. albicans* treated with blank LPs, Mag/FLC-LPs, or PPi-Mag/FLC-LPs was determined by fixing the concentrations of Mag and FLC at the synergistic MIC value.

Mag suppressed the acidogenicity of *S. mutans* in a concentration-dependent manner, reaching an inhibition rate as high as 92.07% (± 2.26) after 6 h of treatment at 1 MIC (**Fig. S3** A-B). When the concentration of Mag was set at 7.8 μg/mL, PPi-Mag-LPs inhibited the acidogenicity of *S. mutans* to a similar extent as free Mag (**Fig. S3** C-D). Similarly, synergistic treatment with Mag and FLC suppressed the ability of *C. albicans* to produce organic acids in a concentration-dependent manner, reaching a maximum inhibition rate of 94.26% (± 3.19) after 4 h of treatment at a synergistic concentration of 1 MIC (**Fig. S3** E-F). When the synergistic concentration of Mag and FLC was set at 15.6 μg/mL, PPi-Mag/FLC-LPs showed an inhibitory effect similar to that of the mixture of free Mag and FLC (**Fig. S3**G-H).

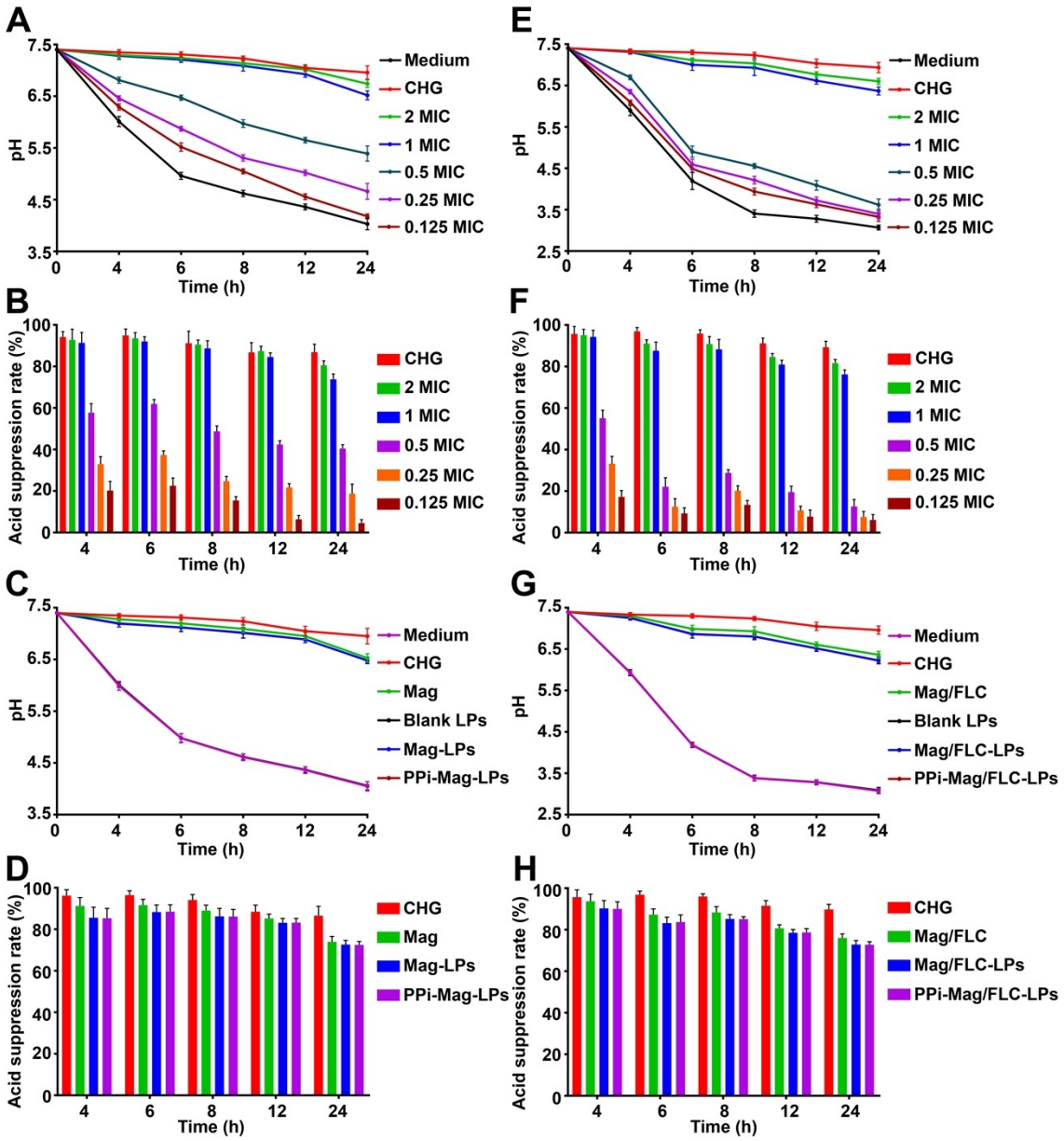


**Fig. S3.** Effects of formulations on the acidogenicity of *S. mutans* and *C. albicans*. (**A-B**) Inhibitory effect of Mag (0.125 MIC - 2 MIC) on the acidogenicity of *S. mutans*. (**C-D**) Inhibitory effect of different formulations (Mag, 7.8 μg/mL) on the acidogenicity of *S. mutans*. (**E-F**) Synergistic inhibitory effect of Mag and FLC (0.125 MIC - 2 MIC) on the acidogenicity of *C. albicans*. (**G-H**) Inhibitory effect of different formulations (Mag, 15.6 μg/mL; FLC, 15.6 μg/mL) on the acidogenicity of *S. mutans*. Data are shown as mean ± SD (n = 3). $^{****}P < 0.0001$. CHG, chlorhexidine gluconate; FLC, fluconazole; LPs, liposomes; Mag, magnolol; MIC, minimum inhibitory concentration; PPi, pyrophosphate ion.

### *Acid resistance*

The tolerance of *S. mutans* and *C. albicans* to acidic conditions was assessed by determining their viability at different pH values. Briefly, *S. mutans* was inoculated into BHI broth and *C. albicans* into SDB at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, then they were

incubated at 37 °C for 24 h. The growth rate of both microorganisms was determined by measuring the absorbance at 600 nm. Culture medium at pH 7.4 served as the control.

The growth of *S. mutans* and *C. albicans* was not affected at pH 7.0, 6.5 or 6.0, whereas significant inhibition was observed at pH 4.0, 4.5 and 5.0 relative to pH 7.4 (**Fig. S4**). In addition, the growth of *S. mutans* but not *C. albicans* was significantly inhibited at pH 5.5, suggesting that *C. albicans* is more acid-tolerant.

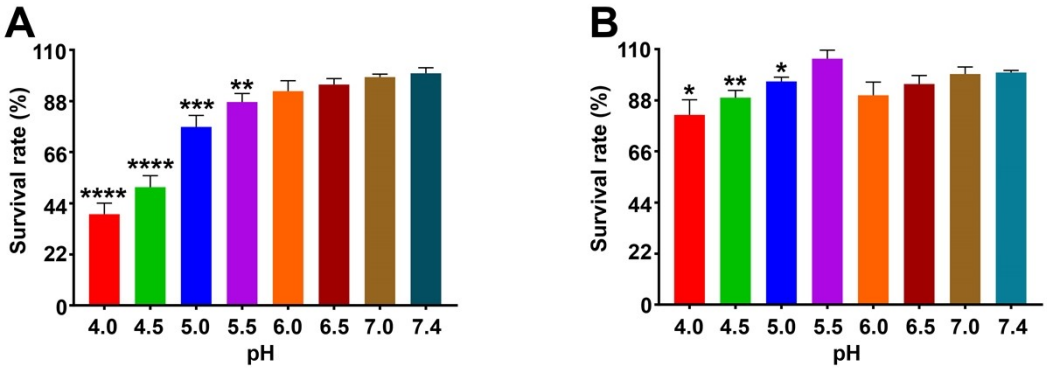


**Fig. S4.** Aciduricity of *S. mutans* and *C. albicans*. Survival rate of (**A**) *S. mutans* and (**B**) *C. albicans* at different pH values. Data are shown as mean ± SD (n = 5). $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$, $^{****}P < 0.0001$ *vs* pH 7.4.

**Table S1.** Actual values of independent variables at different levels.

| Independent variable | Level | | | | |
|---|---|---|---|---|---|
| | −1.732 | −1 | 0 | +1 | +1.732 |
| $X_1$ | 160.00 | 176.91 | 200.00 | 223.09 | 240.00 |
| $X_2$ | 0.12 | 0.20 | 0.31 | 0.42 | 0.50 |
| $X_3$ | 2.00 | 4.11 | 7.00 | 9.89 | 12.00 |

$X_1$, weight of phosphatidylethanolamine (mg); $X_2$, oil/aqueous ratio; $X_3$, duration of ultrasonication (min).

**Table S2.** Experimental results of the central composite design (n = 3).

| Run | $X_1$ | $X_2$ | $X_3$ | $Y_1$ | $Y_2$ | $Y_3$ |
|---|---|---|---|---|---|---|
| 1 | 176.91 | 0.20 | 9.89 | 44.03 | 35.95 | 260.10 |
| 2 | 176.91 | 0.20 | 4.11 | 58.92 | 50.26 | 248.36 |
| 3 | 200.00 | 0.50 | 7.00 | 38.05 | 30.97 | 234.83 |
| 4 | 223.09 | 0.42 | 9.89 | 62.16 | 53.05 | 255.27 |
| 5 | 200.00 | 0.31 | 7.00 | 88.91 | 76.89 | 218.07 |
| 6 | 200.00 | 0.31 | 7.00 | 89.02 | 78.45 | 216.46 |
| 7 | 176.91 | 0.42 | 4.11 | 68.74 | 59.49 | 248.36 |
| 8 | 200.00 | 0.31 | 2.00 | 86.51 | 75.82 | 281.20 |
| 9 | 200.00 | 0.31 | 7.00 | 86.42 | 76.17 | 217.16 |
| 10 | 240.00 | 0.31 | 7.00 | 58.71 | 50.08 | 249.97 |
| 11 | 223.09 | 0.42 | 4.11 | 75.24 | 62.85 | 240.53 |
| 12 | 160.00 | 0.31 | 7.00 | 49.67 | 40.45 | 234.63 |
| 13 | 176.91 | 0.42 | 9.89 | 53.05 | 46.82 | 264.16 |
| 14 | 223.09 | 0.20 | 9.89 | 50.16 | 42.86 | 252.26 |
| 15 | 200.00 | 0.31 | 12.00 | 35.36 | 29.39 | 269.93 |
| 16 | 200.00 | 0.31 | 7.00 | 88.15 | 78.21 | 218.33 |
| 17 | 223.09 | 0.20 | 4.11 | 65.13 | 54.24 | 243.86 |
| 18 | 200.00 | 0.13 | 7.00 | 44.53 | 37.73 | 226.20 |
| 19 | 200.00 | 0.31 | 7.00 | 87.74 | 76.53 | 216.73 |
| 20 | 200.00 | 0.31 | 7.00 | 87.97 | 76.46 | 218.60 |

$X_1$, weight of phosphatidylethanolamine (mg); $X_2$, oil/aqueous ratio; $X_3$, duration of ultrasonication (min); $Y_1$, encapsulation efficiency of magnolol (%); $Y_2$, encapsulation efficiency of fluconazole (%); $Y_3$, particle size (nm).

**Table S3.** Observed and predicted values of particle size and encapsulation efficiency under optimal formulation conditions (n = 3).

| Variable | Optimized value | Response | Predicted value | Observed value | Mean value (± SD) |
|---|---|---|---|---|---|
| $X_1$ | 215.4 | $Y_1$ | 88.38 | 88.21 | 87.66% (± 1.06) |
| | | | | 88.34 | |
| | | | | 86.44 | |
| $X_2$ | 0.32 | $Y_2$ | 76.89 | 74.82 | 75.53% (± 0.67) |
| | | | | 75.68 | |
| | | | | 76.14 | |
| $X_3$ | 6.06 | $Y_3$ | 222.84 | 222.6 | 220.37 nm (± 2.82) |
| | | | | 217.2 | |
| | | | | 221.3 | |

$X_1$, weight of phosphatidylethanolamine (mg); $X_2$, oil/aqueous ratio; $X_3$, duration of ultrasonication (min);

$Y_1$, encapsulation efficiency of magnolol (%); $Y_2$, encapsulation efficiency of fluconazole (%); $Y_3$, particle size (nm).

**Table S4.** Stability of blank LPs, Mag/FLC-LPs, and PPi-Mag/FLC-LPs at 4 °C.

| Sample | Days | Particle size (nm) | Polydispersity index | EE (Mag) | EE (FLC) |
|---|---|---|---|---|---|
| Blank LPs | 0 | 184.33 ± 1.98 | 0.143 ± 0.006 | - | - |
| | 5 | 184.57 ± 1.80 | 0.146 ± 0.005 | - | - |
| | 10 | 185.80 ± 2.19 | 0.148 ± 0.009 | - | - |
| Mag/FLC-LPs | 0 | 219.53 ± 1.80 | 0.122 ± 0.006 | 85.83 ± 1.08 | 74.40 ± 0.82 |
| | 5 | 221.33 ± 1.91 | 0.123 ± 0.006 | 85.78 ± 0.71 | 74.29 ± 1.15 |
| | 10 | 222.27 ± 2.48 | 0.125 ± 0.008 | 85.71 ± 0.60 | 74.17 ± 0.87 |
| PPi-Mag/FLC-LPs | 0 | 238.83 ± 2.33 | 0.164 ± 0.005 | 87.21 ± 1.06 | 75.17 ± 1.25 |
| | 5 | 239.47 ± 2.51 | 0.166 ± 0.005 | 86.91 ± 1.02 | 74.83 ± 0.90 |
| | 10 | 241.93 ± 2.27 | 0.170 ± 0.004 | 86.84 ± 0.75 | 74.57 ± 0.81 |

Data are shown as mean ± SD. EE, encapsulation efficiency (%); FLC, fluconazole; LPs, liposomes; Mag, magnolol; PPi, pyrophosphate ion.

**Table S5.** Stability of blank LPs, Mag/FLC-LPs, and PPi-Mag/FLC-LPs at 25 °C.

| Sample | Days | Particle size (nm) | Polydispersity index | EE (Mag) | EE (FLC) |
|---|---|---|---|---|---|
| Blank LPs | 0 | 184.33 ± 1.98 | 0.143 ± 0.006 | - | - |
| | 5 | 334.77 ± 3.01 | 0.456 ± 0.018 | - | - |
| | 10 | - | - | - | - |
| Mag/FLC-LPs | 0 | 219.53 ± 1.80 | 0.122 ± 0.006 | 85.83 ± 1.08 | 74.40 ± 0.82 |
| | 5 | 414.50 ± 3.40 | 0.676 ± 0.031 | 72.80 ± 0.84 | 63.70 ± 0.76 |
| | 10 | - | - | 56.21 ± 0.95 | 48.73 ± 1.09 |
| PPi-Mag/FLC-LPs | 0 | 238.83 ± 2.33 | 0.164 ± 0.005 | 87.21 ± 1.06 | 75.17 ± 1.25 |
| | 5 | 451.53 ± 2.95 | 0.707 ± 0.049 | 71.52 ± 0.71 | 60.78 ± 0.83 |
| | 10 | - | - | 52.10 ± 0.51 | 43.82 ± 0.69 |

Data are shown as mean ± SD. EE, encapsulation efficiency (%); FLC, fluconazole; LPs, liposomes; Mag, magnolol; PPi, pyrophosphate ion.

**Table S6.** Long-term stability of blank LPs, Mag/FLC-LPs, and PPi-Mag/FLC-LPs at 4 °C.

| Sample | Months | Particle size (nm) | Polydispersity index | EE (Mag) | EE (FLC) |
|---|---|---|---|---|---|
| Blank LPs | 0 | 183.73 ± 2.06 | 0.144 ± 0.004 | - | - |
| | 1 | 185.90 ± 2.34 | 0.154 ± 0.005 | - | - |
| | 2 | 188.40 ± 2.86 | 0.173 ± 0.011 | - | - |
| | 3 | 192.03 ± 3.25 | 0.204 ± 0.018 | - | - |
| Mag/FLC-LPs | 0 | 219.33 ± 2.17 | 0.121 ± 0.006 | 85.86 ± 1.02 | 74.38 ± 1.39 |
| | 1 | 223.17 ± 2.66 | 0.132 ± 0.004 | 85.64 ± 1.04 | 74.21 ± 1.08 |
| | 2 | 227.67 ± 3.45 | 0.156 ± 0.014 | 84.73 ± 0.82 | 73.53 ± 0.88 |
| | 3 | 291.53 ± 3.71 | 0.304 ± 0.017 | 73.93 ± 1.43 | 62.27 ± 1.78 |
| PPi-Mag/FLC-LPs | 0 | 239.27 ± 2.11 | 0.163 ± 0.006 | 86.92 ± 0.73 | 74.98 ± 0.92 |
| | 1 | 243.63 ± 2.68 | 0.177 ± 0.007 | 86.65 ± 0.64 | 74.58 ± 1.18 |
| | 2 | 248.07 ± 3.32 | 0.201 ± 0.015 | 85.30 ± 1.04 | 73.06 ± 1.30 |
| | 3 | 315.97 ± 3.20 | 0.315 ± 0.012 | 71.30 ± 2.21 | 60.69 ± 1.46 |

Data are shown as mean ± SD. EE, encapsulation efficiency (%); FLC, fluconazole; LPs, liposomes; Mag, magnolol; PPi, pyrophosphate ion.