Supplementary material

for

Enhanced clearing of *Candida* biofilms on 3D urothelial cell *in-vitro* model by lysozyme-functionalized Fluconazole-loaded shellac nanoparticles

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Contents

Characterization of Free Lysozyme, Lysozyme-coated shellac NPs, and Lysozyme-Coated Fluconazole-Loaded shellac NPs

Malvern Zetasizer Nano ZS was used to measure the zeta potential and particle size of the modified shellac NPs. The refractive index (RI) was set to 1.512, which is the RI of shellac. 1 mL of the samples was added to a quartz cuvette and tested three times at 25^{o}C. The data was collected as a mean of three separate tests.

Preparation of the Fluconazole-loaded Shellac NPs and encapsulation efficiency test

The encapsulation of the antifungal agent, Fluconazole, was achieved by pH drop. The mixture of 0.2 wt% shellac, 0.25 wt% P407, and 0.02 wt% Fluconazole was fully soluble at pH 10 after sonication. The NPs precipitated after the pH was lowered to 4, which intercalated the Fluconazole inside their shellac cores. The NPs were collected by centrifugation and used as 1 × stock nanosuspension. The encapsulation efficiency of the NPs was detected by a UV-visible spectrometer at 260 nm as a function of time and pH. The pH of the stock 1 × nanosuspension was changed by dropwise addition of 0.25M HCl and 0.25M NaOH, and then the solution was pelletized to collect the supernatant for encapsulation test. The remaining Fluconazole in the supernatant was regarded as nonencapsulated.

Fluconazole-loaded Shellac NPs' encapsulation efficiency

The design of the NPs used in the current work is based on shellac core and P407. The fabrication of the current NPs was studied in the work done by Al-Obaidy et al. [1,2] and Weldrick et al.[3] The optimal precipitation conditions for such shellac NPs were found at pH 5.5, which was used for our following experiments. The primary purpose of the current work is to check the possibility of using the biofilm-infected 3D urothelial cell model for testing antimicrobial nanotherapeutics; the NPs were tested for their ability to act as nanocarriers for the antifungal agent. The results obtained from the average hydrodynamic diameter and zeta-potential analysis of the produced shellac NPs as a function of Fluconazole concentration at pH 5.5 are shown in Figure 5. The increase of the Fluconazole concentration had only a very minor effect on the average nanoparticle hydrodynamic diameter, ranging between 68 nm to 79 nm.

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As can be seen from Figure S5F, the pH had only a minor effect on the Fluconazole encapsulation efficiency. All the set groups yielded an encapsulation efficiency higher

than 70 %. The release kinetics of the Fluconazole from the NPs 1 × stock suspension was measured at pH 5.5 as a function of time. Figure S5 shows that the Fluconazole released about 50 % after 15 h and approximately 70 % after 25 h. This release kinetics makes it suitable for treating the biofilms.

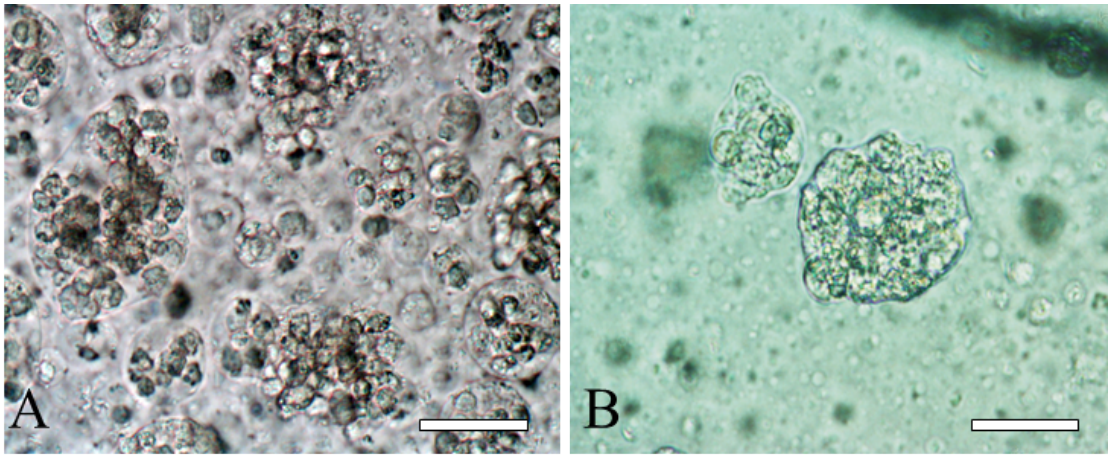

Figure S1. Microscopy observation of the clusteroids encapsulated in the 5.5 wt% DEX/5.5 wt% PEO w/w Pickering emulsion before (A) and after (B) shrinking with more concentrated (11 wt%) PEO solution. The bar is 50 μm.

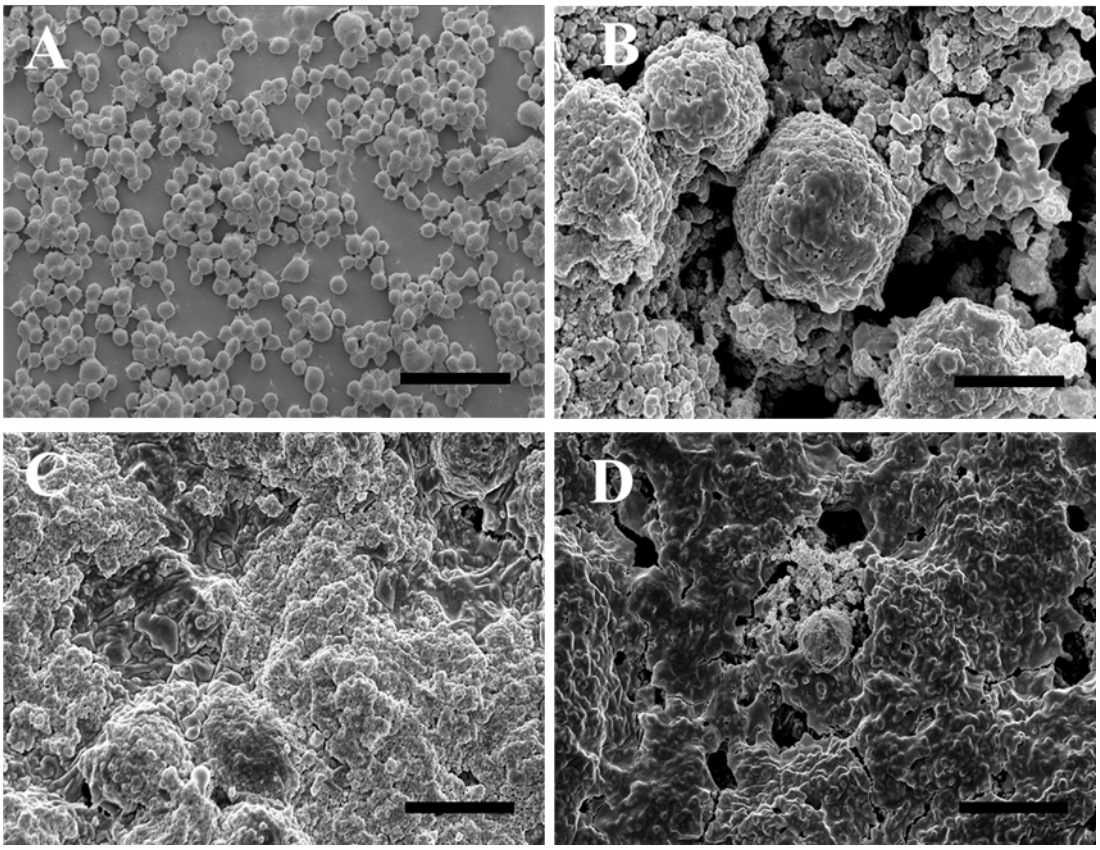

Figure S2. Original SEM observation of (A) an individual ECV 304 cell layer without any fungal infection or treatment by 4 × Lysozyme-coated Fluconazole-loaded shellac NPs stock suspension. (B) Individual ECV 304 clusteroids layer without any fungal infection or Shellac-Fluconazole NPs treatment. (C) ECV 304 clusteroid layer infected with a *C. albicans* biofilm. D: ECV 304 clusteroid layer infected with *C. albicans* biofilm imposed by the treatment of 4 × Shellac-Fluconazole NPs stock solution. The bar is 100 μm for (A, B, C,) and 200 μm for (D). The 1 × stock suspension of the Shellac-Fluconazole-Lysozyme NPs is 0.2 wt% Shellac, 0.25 wt% P407, 0.2 wt% Lysozyme and 0.02 wt% Fluconazole. The cell concentration used in the experiments was 1×10^5/mL.

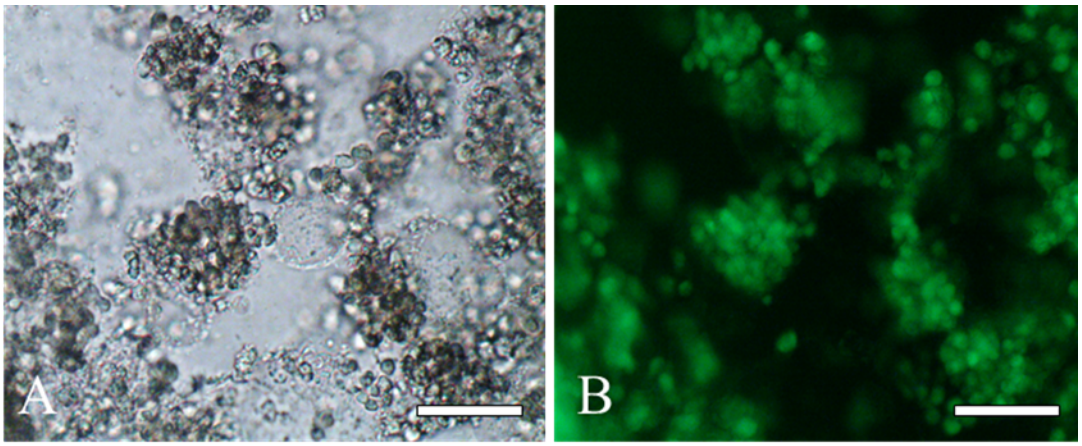

Figure S3. Collected clusteroids (A) by diluting the w/w Pickering emulsion template with FDA staining (B) showing the viability of the clusteroids. The bar is 50 μm.

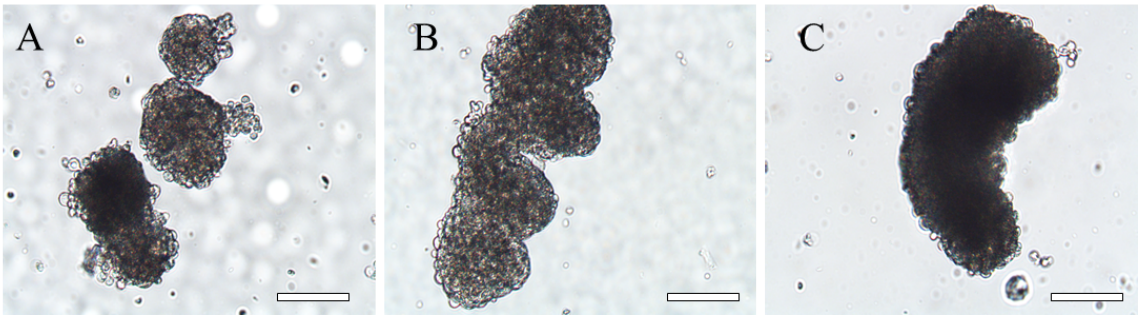

Figure S4. Microscopy observation showing the ECV304 clusteroids fusion progress on day 3 (A),5 (B),7 (C). The bar is 100 μm

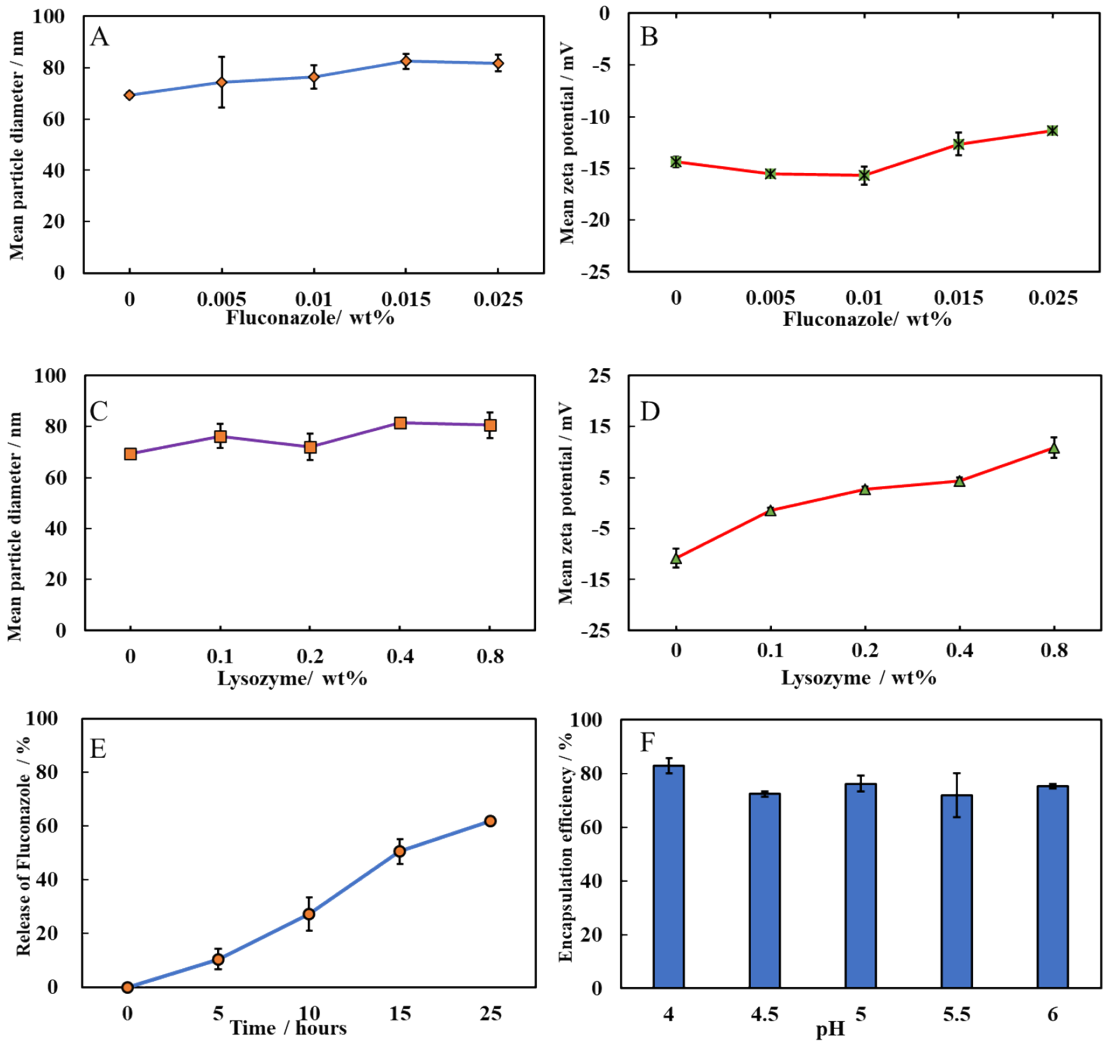

Figure S5. (A) Mean particle diameter of 0.2 wt% Shellac-0.25 wt% P407-Fluconazole nanoparticles measured at pH 5.5 (acetate buffered saline) with various concentrations of Fluconazole. (B) Mean particle zeta potential of 0.2 wt% Shellac-0.25 wt% P407-Fluconazole nanoparticles versus the Fluconazole concentration. (C) Mean particle diameter vs. Lysozyme concentration of the 0.2 wt% Shellac-0.25 wt% P407-0.02 wt% Fluconazole-Lysozyme nanoparticles measured using a Malvern Zetasizer Nano ZS at 25 °C. Each value represents a triple replicate with ±S.D. (D) ζ-potential of 0.2 wt% Shella-0.25 wt% P407-0.02 wt% Fluconazole-Lysozyme nanoparticles immobilized with different concentrations of the Lysozyme at pH 5.5 (adjusted with acetate buffered saline) measured immediately after preparation. (E) Release of Fluconazole from 0.02 wt% Fluconazole-loaded 0.2 wt% Shellac NPs stabilized by 0.25 wt% P407 and coated with 0.2 wt% Lysozyme at different time points. (F) Encapsulation efficiency of 0.02 wt% Fluconazole-loaded 0.2 wt% Shellac NPs stabilized by 0.25 wt% P407 and coated with 0.2 wt% Lysozyme nanoparticles. The encapsulation efficiency was tested immediately after the NPs were prepared using a UV-visible spectrometry set at 260 nm wavelength. The solid lines are guides to the eye.

References

1. S.S.M, Al-Obaidy, G.M. Greenway and V.N. Paunov, *Nanoscale Adv*., 2019, **1**, 858-872.
2. S.S.M. Al-Obaidy, A.F Halbus, G.M. Greenway and V.N. Paunov, J. *Mater. Chem. B*, 2019, **7**, 3119-3133.
3. P. J. Weldrick, M. J. Hardman and V. N. Paunov, *Adv. Nanobiomed. Res.,* 2021, **1**, 2000027.