Electronic supplementary information (ESI)

Methylene blue-containing liposomes as new photodynamic anti-bacterial agents.

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Preparation and characterization of liposomes

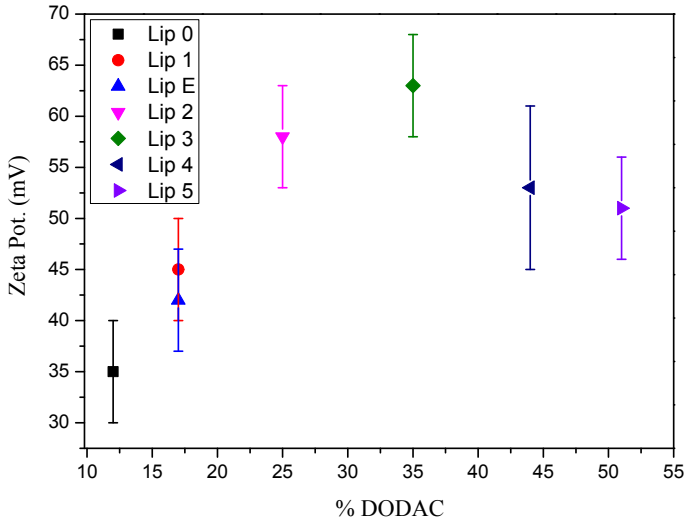

Fig. S1 Zeta Potential values measured for liposomes carrying different amounts of the cationic lipidic component DODAC (expressed as molar percentage %). Liposomes were diluted 1/300 in milliQ water from REV preparations.

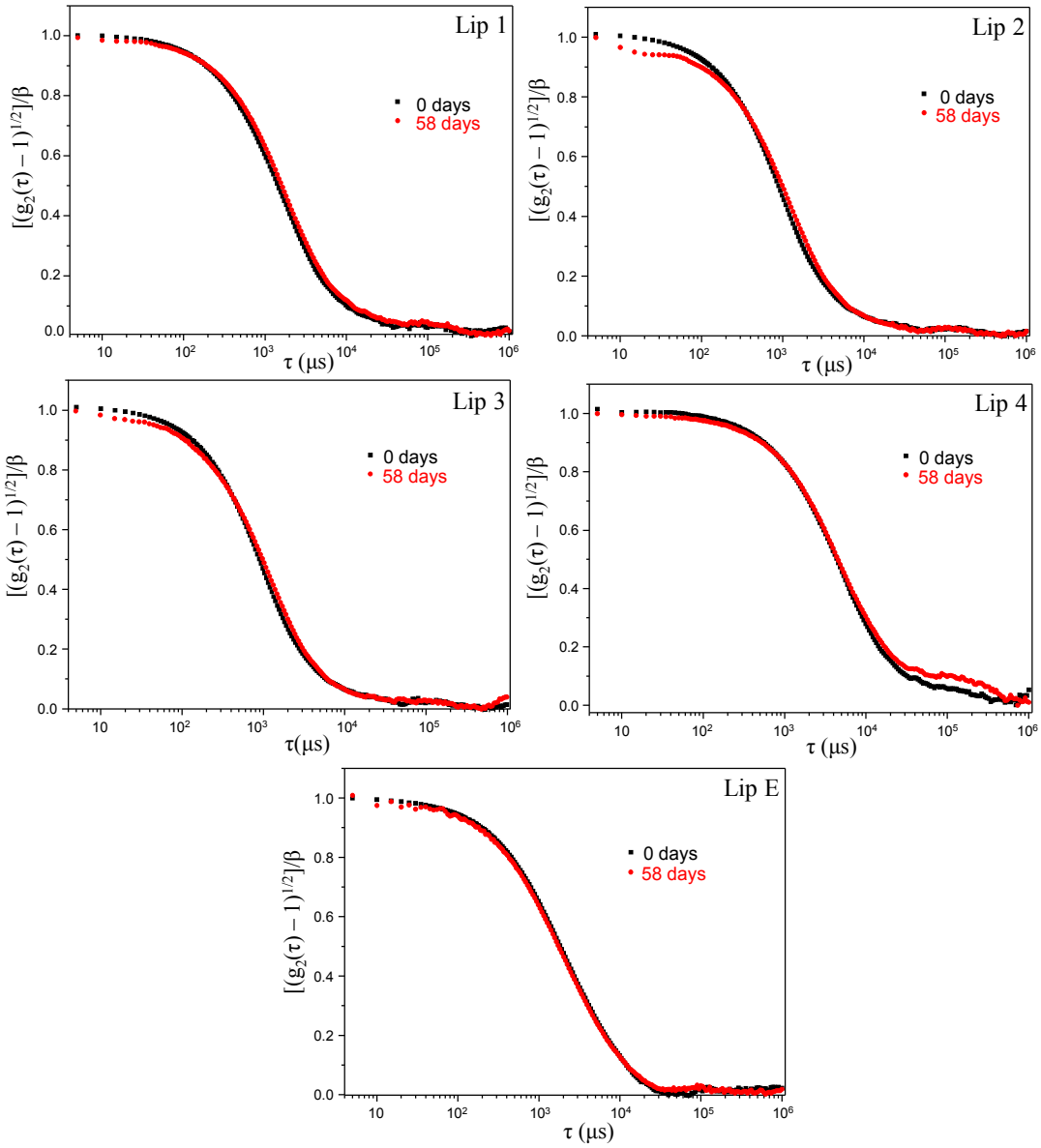

Fig. S2 Normalized autocorrelation functions of the scattered intensity measured at 90° for aqueous dispersion of Lip 1, Lip 2, Lip 3, Lip 4, and Lip E as a function of time (days) from their preparation (dilution 1/300 in milliQ water of liposome obtained by REV procedure).

Transmission electron microscopy of liposomes

Liposome preparations were examined by transmission electron microscopy (TEM) to verify their morphologies and sizes. Samples were analyzed employing the method described below.[1,2,3]

An aliquot of 10% w/v trehalose stock solution was added to the liposome suspensions to give a final trehalose concentration of 2% w/v. Samples were vortexed before deposition of a drop of the sugar-liposome solutions was placed onto a carbon-coated copper grid. After 1 hour the grid was washed with a drop of milli-Q water. Samples were dried overnight before examination with TEM. Images were acquired with a STEM CM12 Philips electron microscope. The TEM images observed for Lip 1, Lip 2, Lip 3, Lip 4 and Lip E are reported in figures S3, S4, S5, S6 and S7, respectively.

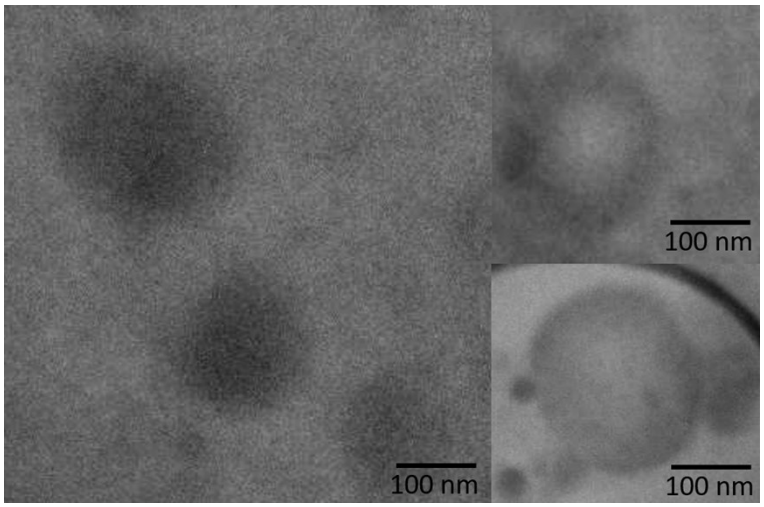

Fig. S3 Representative TEM micrographs of Lip 1 upon trehalose fixation.

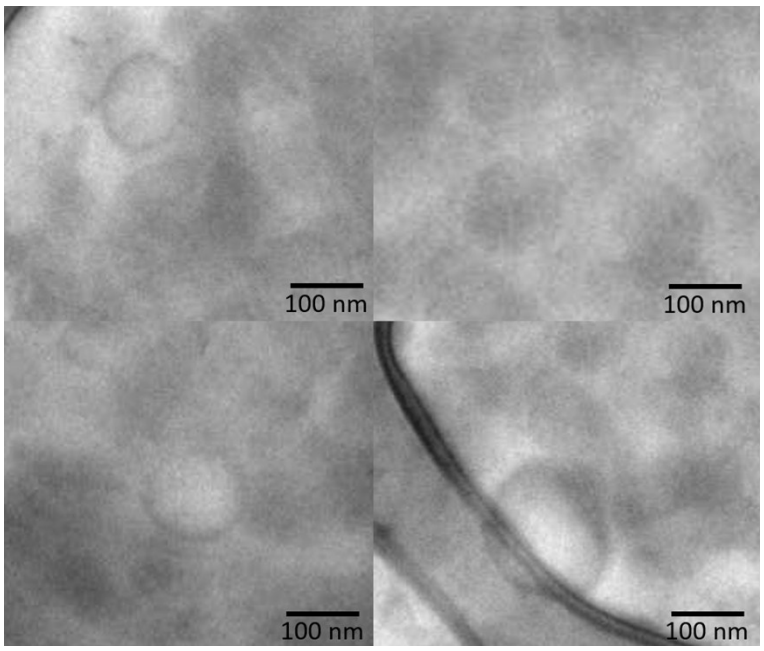

Fig. S4 Representative TEM micrographs of Lip 2 upon trehalose fixation.

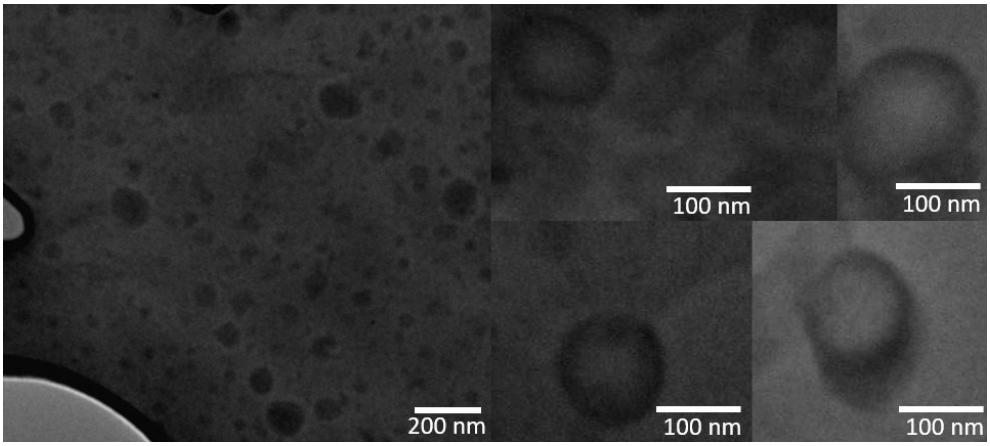

Fig. S5 Representative TEM micrographs of Lip 3 upon trehalose fixation.

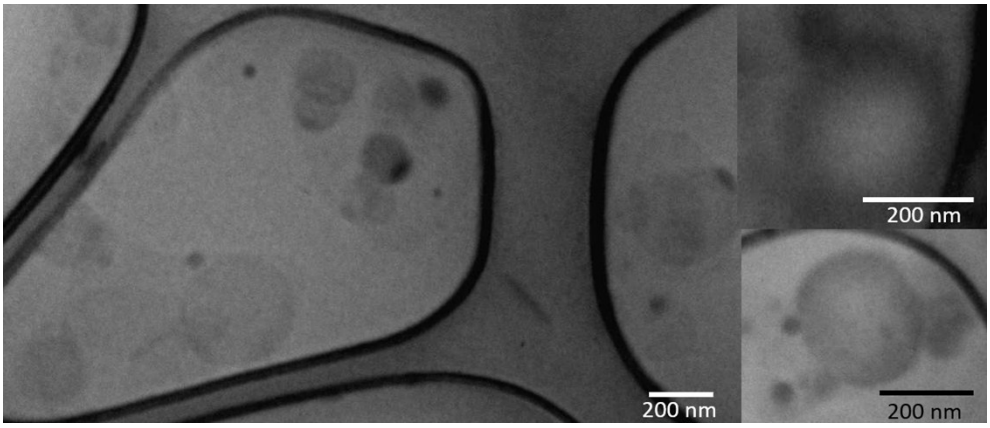

Fig. S6 Representative TEM micrographs of Lip 4 upon trehalose fixation.

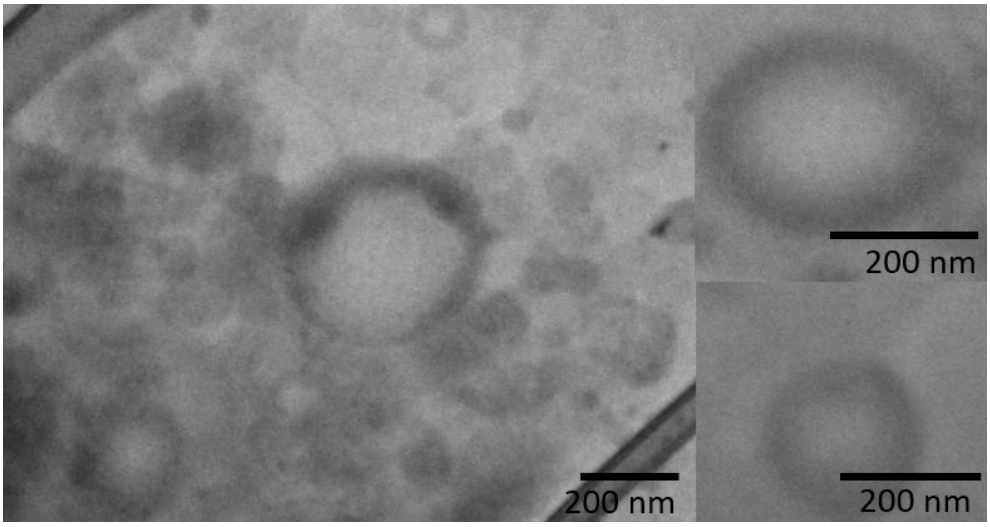

Fig. S7 Representative TEM micrographs of Lip E upon trehalose fixation.

Evaluation of Liposome Surface Charge Densities.

An estimate of the surface charge density ρ (C/nm^2) of the different liposomal formulation can be calculated from DLS and Zeta Potential data, considering liposomes as homogeneous spheres, as:

$$\rho = \frac{q}{4\pi R^2} = \frac{\varepsilon(1+\kappa R)\zeta}{R}$$

being q the charge of the entire sphere, R the radius of the liposomes, assumed to be equal to their hydrodynamic radius, obtained from DLS, ε the dielectric permittivity of the medium (water), κ the inverse Debye length, ς the surface potential, assumed to be equal to the measured zeta potential.

The calculated values at T = 298 K in water for the different liposomal formulations are reported in **Table 2** in the manuscript.

Methylene blue encapsulation.

The amount of MB encapsulated into liposomes was evaluated through the construction of a calibration curve, obtained by measuring the absorbance values at 664 nm of a 1:300 dilution of Lip E (taken as negative control reference), containing Triton X100 (1%) and increasing amounts of MB (from 1 to 100 μM) (see **Figure S9**). A molar extinction coefficient of 65860 M^{-1}cm^{-1} at 664 nm, slightly decreased with respect to that reported for MB in pure water[4-5] was found.

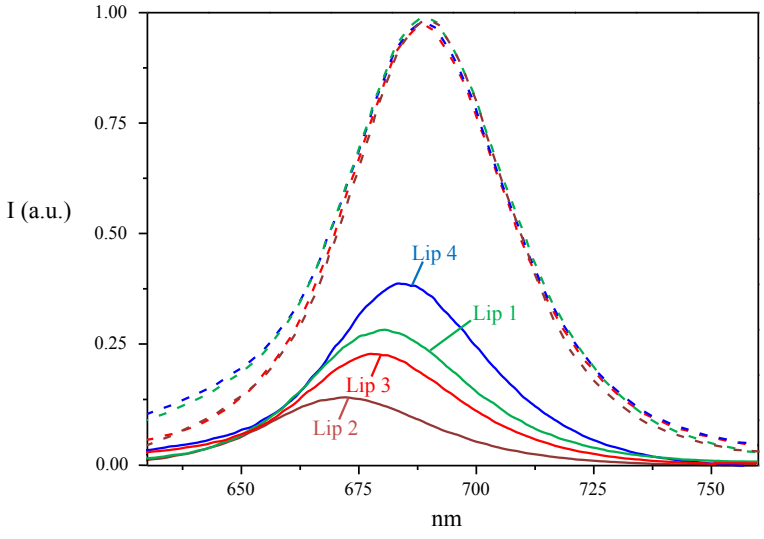

Fig. S8 Fluorescence spectra of MB-containing liposome preparations before (continuous lines) and after (dashed lines) treatment with Triton X100. Samples were diluted 1:300 in water in order to contain the same MB concentration [MB(Lip)], previously calibrated by means of UV-vis measurements. Spectra are normalized with respect to the highest fluorescence intensity registered upon release of MB from the liposomal pool. [MB(Lip)] = 1 x 10^{-5} M, $\lambda_{exc.}$ = 610 nm, final concentration of Triton X100 1%.

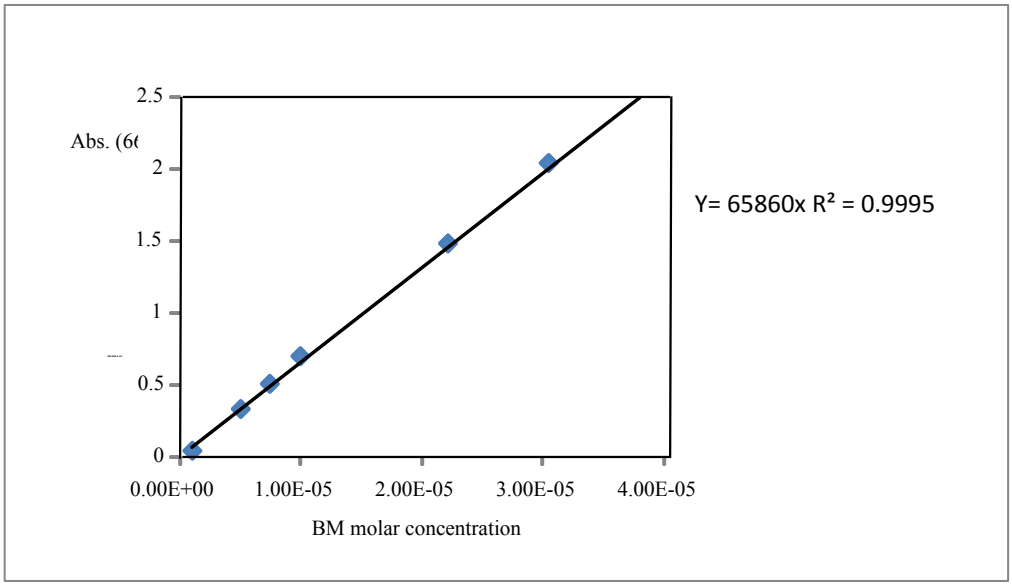

Fig. S9 Calibration curve obtained by measuring the absorbance values at 664 nm of samples containing increasing MB concentrations. Each sample contained the lipid composition of a reference liposome, Lip E, diluted 1:300 and added of Triton X100 (1% final molar concentration).

Bactericidal efficacy of photo-activated MB-containg liposomes towards *E. Coli*

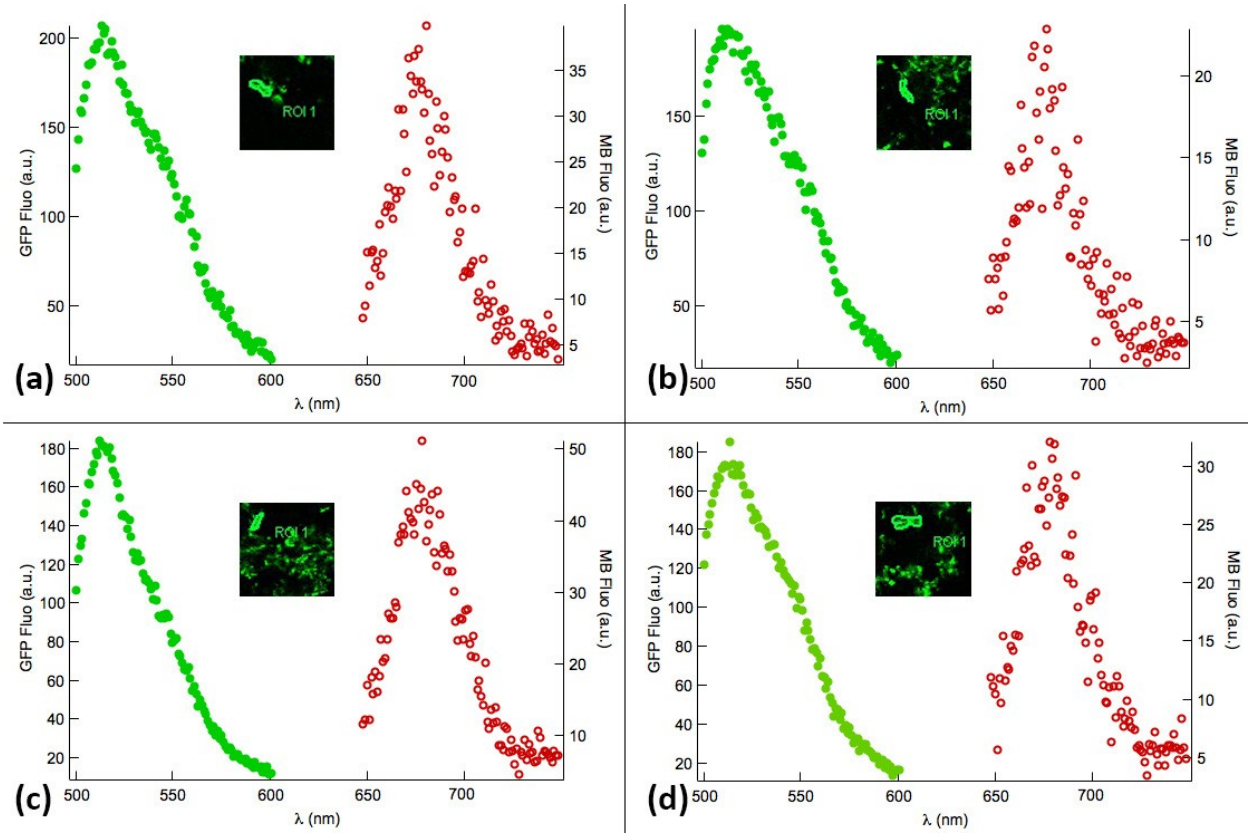

Fig. S10 Spatially resolved fluorescence emission spectra acquired for *E. coli* S-17-1 strains carrying pHC60 plasmid upon incubation with (a) Lip 1, (b) Lip 2, (c) Lip 3, (d) Lip 4 MB-loaded liposomes. In order to verify the efficient encapsulation of MB-loaded liposomes inside *E. coli* bacteria, the emission spectra were acquired in ROIs corresponding to the sole bacterial cytoplasm (see the confocal microscopy images reported in the graphs); the samples were excited at 488 nm (green filled circles), to acquire fluorescence emission of GFP from bacterial cytoplasm (GFP fluo) and at 633 nm (red empty circles), to acquire fluorescence emission of MB from the same area. For the four formulations a significant fluorescence emission of MB is detected from *E. coli* bacterial cytoplasm, confirming the efficient uptake of MB-loaded liposomes by the bacteria.

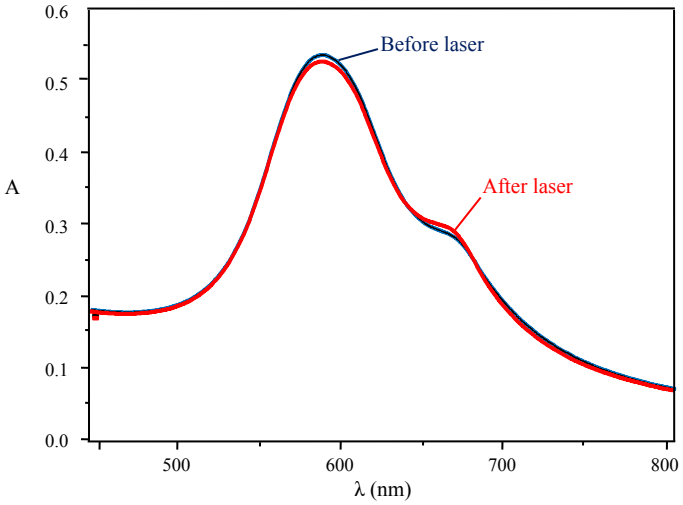

Fig. S11 UV-visible spectra of an aqueous dispersion of MB-containing liposomes (Lip 3) before (blue line) and after (red line) 30 seconds of irradiation with laser (λ = 630 nm, 150 mW). Liposomes were diluted (1/300) from the REV preparation in milliQ water.

Static biofilm of *E. coli* S17-1 pHC60 cells was prepared by growing a pre-inoculum ($OD_{600\ nm}$ = 0.2) in 250 µl M9 broth (Sigma-Aldrich, Milan, Italy) supplemented with 10 mg/L tetracycline in a 96-well microtiter for 24 h at 37°C, as described in literature.[6] To assess the ability of liposomes to interact (impregnate) bacterial biofilms, 200 µl of M9 broth were removed from wells and appropriate volumes of MB solution 0.02% w/v or liposome suspensions were added at a final molar concentration of MB of 0.02% w/v. After 10 minutes of incubation planctonic cells were removed by pipetting out the solution and washing three times each well with 200 µl of PBS buffer (NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4$10 mM, KH_2PO_4 1.8 mM, pH=7.4). Adhesion of MB was then measured as absorbance of each well at λ = 600 nm; the obtained results are reported in **Figure S12**.

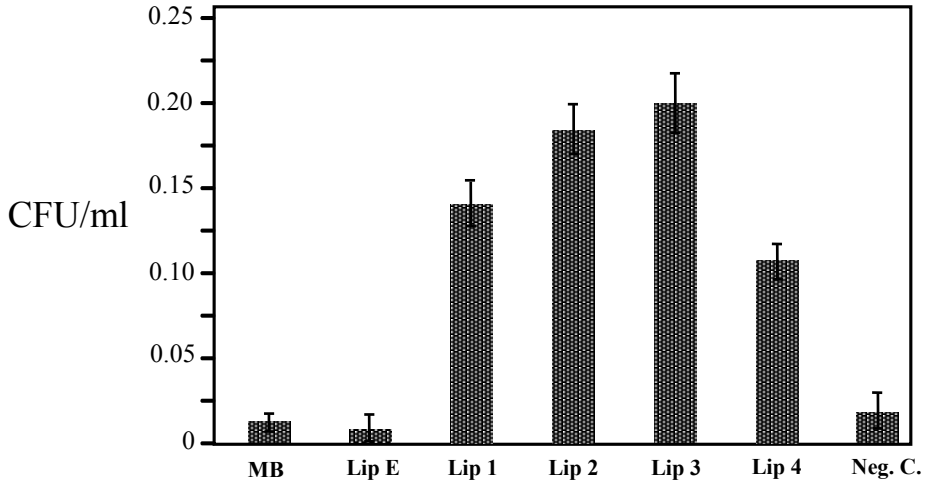

Fig. S12 MB-containing liposome abilities to penetrate the biofilm of *E. coli* S17-1 pHC60 compared to MB 0.02% w/v in water. Means are related to three technical replicates, Neg. C. = negative control.

References

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