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Supplementary Information

Synergistic Antibacterial Action of Lignin-Squaraine Hybrid Photodynamic Therapy: Advancing Towards Effective Treatment of Antibiotic-Resistant Bacteria

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Materials and Methods

Chemicals and instrumentation

All reagents and HPLC grade solvents were purchased from Sigma-Aldrich, Alfa Aesar, and other commercial suppliers and used without further purification. 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) and 1,3-diphenylisobenzofuran (DPBF) were obtained from Sigma-Aldrich. Hoechst 33342, Propidium Iodide, and PF127 were obtained from Thermo Fisher Scientific. SQR29 was synthesized following our earlier report.[1] Poly(lignin/PEG/PPG urethane) (Lignin-PEG-PPG) was synthesised following an earlier report.[2] All absorbance and fluorescence measurements were done using Varioskan LUX spectrophotometer. The samples were irradiated with a Kessil PR160L-640nm-C lamp.

Encapsulation of SQR29 in Lignin-PEG-PPG

SQR29 (92.2 µL, 11.8 mM) and Lignin-PEG-PPG (80 mg) were added to H_2O (1 mL) and stirred at 60 °C for 2 h until all the reactants were dissolved to give a gel like solution. H_2O (2 mL) was further added, and the mixture was stirred in ice bath until the solution became less viscous. The mixture was left to stir at room temperature overnight for complete encapsulation. The concentration of SQR29 encapsulated in Lignin-PEG-PPG was measured by adding ethanol into the mixture and centrifuging down the lignin. The supernatant obtained contained SQR29, and its absorbance was measured. The encapsulation efficiency of LSQR29 was found to be 38%.

ROS Measurement

A solution of H_2DCFDA (0.24 mg, 1.0 mM) in MeOH (0.5 mL, 1.0 mM) was added to NaOH (2.0 mL, 0.01 M) and stirred at 37 °C for 30 minutes. The solution was neutralized with NaH_2PO_4 (10 mL, 25 mM)

and stored in the dark at -20 °C. H_2DCF (6.2 µL) was added to the samples (H_2O, Lignin-PEG-PPG, SQR29 and LSQR29, 93.8 µL) in a 96 well plate. Fluorescence intensity (Ex = 475 nm, Em = 520-530 nm) was measured before (T = 0 mins) and after (T = 30 mins) irradiation with lamp (640 nm, 0.662 W cm^{-2}). The fold change of the average fluorescence intensity measured before and after irradiation was calculated to determine the amount of ROS produced.

Quantum yield of ROS

A solution of 1,3-diphenylisobenzofuran (DPBF) (100 µM) in DMSO was added to SQR29 (9 µM) or methylene blue (9 µM) (standard). To measure the ROS generation, absorbance spectra of the sample and standard solutions were recorded for different exposure times by using a 640 nm lamp with an average power of 0.1 W cm^{-2}, during 3 mins with irradiation intervals every 30 s. The ROS production quantum yield (Φ_Δ) was calculated by using equation 1 and 2.[3]

$$\Phi_\Delta = \Phi_\Delta^{std} \frac{k}{k^{std}} \frac{I^{std}}{I} \qquad (1)$$

$$\frac{I^{std}}{I} = \frac{(1-10^{Astd})}{(1-10^{A})} \qquad (2)$$

in which, Φ_Δ^{std} is the ROS quantum yield of methylene blue (0.49), k and k^{std} are the photo-oxidation kinetic constants for SQR29 and methylene blue (standard), respectively, and A and A^{std} are the absorbances of SQR29 and methylene blue, respectively.[4]

Confocal microscope setup and data analysis

Confocal fluorescence imaging was performed with a Carl Zeiss LSM 800 microscope (Germany) equipped with oil immersion objective lenses (Plan-APOCHROMAT 60×, NA = 1.4). For a multi-colour imaging, the following filter sets were used: Hoechst 33342 (Ex = 405 nm; Em = 410-470 nm) and Propidium Iodide (Ex = 561 nm; Em = 550-617 nm). Two-dimensional images were acquired with an exposure time of 30 msec. ImageJ software (National Institutes of Health) was used to analyze the captured confocal images.

Bacterial culture

Bacterial strains used are *Escherichia coli* (K 12 JM 109, New England Biolabs, Ipswich, U.S.A.), and *Staphylococcus aureus* (NCTC® 8530™). For culture, the bacteria were inoculated in Luria-Bertani (LB) broth. Bacteria suspensions were grown in a shaking incubator overnight at 37 °C, and the optical densities were measured at 600 nm (OD_{600}). Bacterial samples that reached OD_{600} values of 1.0–1.5 were collected and used for the assay and bacterial concentration was determined for each species through CFU counting on plates done in triplicates.

Live/Dead bacterial cell imaging

Bacteria sample in PBS (approx. 10^9 CFU) was added to Lignin-PEG-PPG (8% w/w), SQR29 (65 μM) and LSQR29 (65 μM) respectively and the assay solution was mixed thoroughly and left to incubate in the dark at 37 °C for 30 min. The solution was irradiated under 640 nm lamp (0.662 W cm^{-2}) for 30 min. The supernatant was removed, and the pellet was resuspended in fresh PBS (10 μL) to concentrate the bacteria. Bacteria samples were then stained with Hoechst solution (1 mg mL^{-1}) and

propidium iodide (5 μM) for 15 min. Thereafter, confocal fluorescence imaging was performed to calculate the antibacterial rate based on equation 3.

$$\text{Antibacterial rate}\ (\%) = \left(\frac{\text{No.of dead bacteria}}{\text{No.of total bacteria}}\right) \times 100\% \qquad (3)$$

Plate counting assay

Bacteria sample in LB culture medium (approx. 10^4 CFU) was added to Lignin-PEG-PPG (8% w/w), SQR29 (65 μM) and LSQR29 (65 μM) respectively and the assay solution was mixed thoroughly and left to incubate in the dark at 37 °C for 30 min. The solution was irradiated under 640 nm lamp (0.662 W cm^{-2}) for 30 min. Bacteria samples were then seeded in an LB plate and cultured for 24 h before counting the number of the bacterial colonies. The antibacterial rate (%) was determined using the reported methods as defined in equation 4.[5]

$$\text{Antibacterial rate}\ (\%) = \left(1 - \frac{\text{CFU}_{\text{experimental group}}}{\text{CFU}_{\text{control group}}}\right) \times 100\% \qquad (4)$$

Cell culture and scratch assay

3T3-L1 (ATCC® CL-173™) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with newborn calf serum (NBCS, 10%) and penicillin-streptomycin (1%). The cells were grown and kept at 37 °C under 5% CO_2 environment. Cells were grown on glass-based dishes for confocal observation.

The *in vitro* scratch assay was conducted according to the previously reported protocol.[6] For this purpose, 3T3-L1 cells (10^6 cells mL^{-1}) were seeded with complete DMEM media in a 6-well plate and incubated at standard conditions. As soon as the cell growth reached the uniform monolayer, a scratch

was made with a sterilized pipette tip, followed by cell washing with DMEM (without FBS) to remove excess detached cells. Subsequently, the Lignin-PEG-PPG (8% w/w), SQR29 (65 μM) and LSQR29 (65 μM) (in FBS-free DMEM) were respectively added to the cells. In the control group, cells were remained untreated. After 0, 24, and 48 h of treatment, images were taken with bright field microscopy (Olympus, CKX41, Japan) using the capturing software (Q-Capture Pro 7). Furthermore, the initial and final width of the scratch were measured with ImageJ software. The wound closing rate was calculated with the following equation 5.[6]

$$\text{Wound closing rate } (\%) = \frac{\text{Original width} - \text{Final width}}{\text{Original width}} \times 100\% \qquad (5)$$

Antioxidant assay

DPPH solution (60 μM) in MeOH was added to Lignin-PEG-PPG (8% w/w), SQR29 (65 μM) and LSQR29 (65 μM) respectively. The solutions were incubated in the dark for 1 h while shaking. The DPPH free radical content was measured by monitoring the absorbance changes at 517 nm at each time point (1, 2, 4, 6, 24 h). All the samples were prepared and tested in triplicate. The antioxidant property is represented as the inhibition by measuring the decrease in absorbance compared with control solutions based on equation 6.[2]

$$\text{Inhibition } (\%) = \frac{\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{DPPH}}} \times 100\% \qquad (6)$$

Cell cytotoxicity assay

Cell cytotoxicity was evaluated using MTT assay. The mitochondrial dehydrogenase enzyme in living cell reduces MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to form formazan crystals. 3T3 cells were seeded in a 96-well plate at a density of 2.5×10^4 cells mL^{-1} in DMEM

supplemented with NBCS (10%) and penicillin-streptomycin (1%) and grown at 37 °C under 5% CO_2 environment. After 48 hours, the cells were washed with 100 µL phosphate buffered saline (PBS) in each well. The cells were then incubated with 150 µL unsupplemented DMEM mixed with Lignin-PEG-PPG (8% w/w), SQR29 (65 µM) or LSQR29 (65 µM) for varying incubation duration (4, 24, 48 h). Upon completion of incubation, 10 µL MTT solution was added into each well and incubated at 37 °C under 5% CO_2 condition. After 2 hours incubation, 200 µL DMSO was added into each well to dissolve the formed purple formazan crystals. The absorbance of the formazan crystal was then measured at 570 nm while background absorbance was measured at 630 nm. The corrected signal from each treated well was then normalized to that from the control well to obtain relative percentage cell viability.

References

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Supplementary Table and Figures

Compound	k (min^{-1})	ϕ_Δ
SQR29	0.36	0.199

Table S1: Photo-oxidation rate constants and reactive oxygen species (ROS) quantum yield of SQR29.

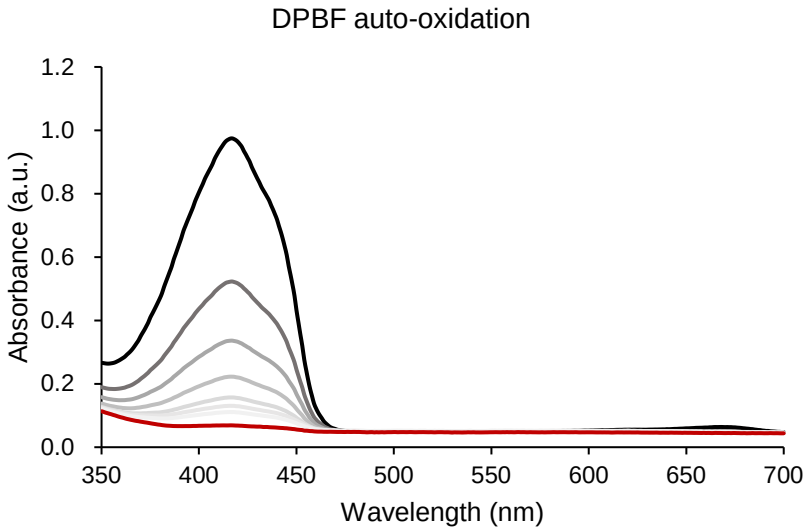

Figure S1: Photo-oxidation of DPBF by 640 nm lamp in the absence of derivatives.

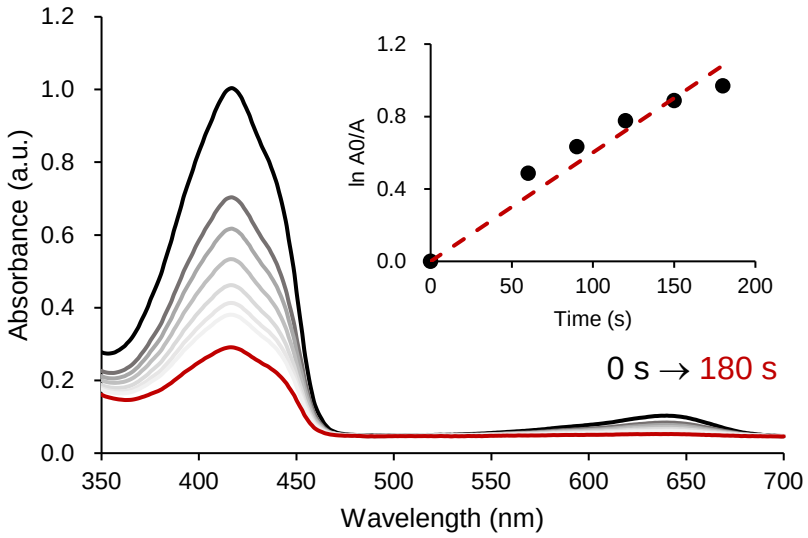

Figure S2: Photo-oxidation of DPBF by 640 nm lamp in the presence of SQR29. Insert: first-order kinetic profile.

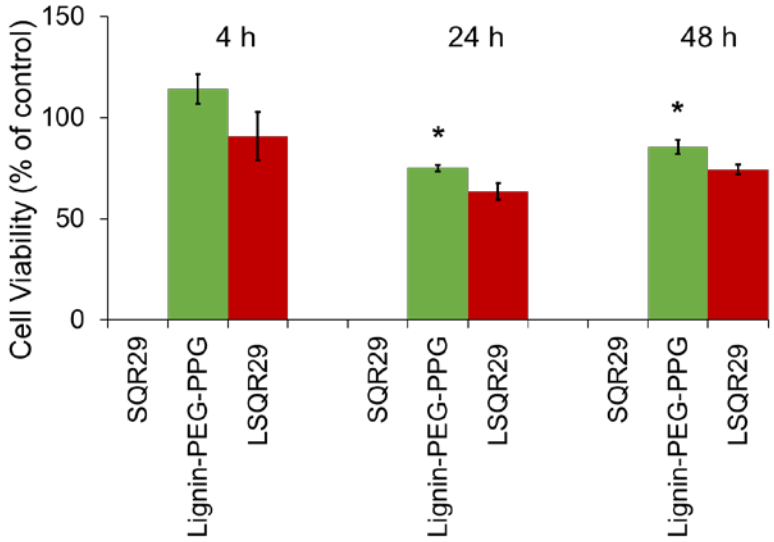

Figure S3. MTT Cytotoxicity assay of SQR29, Lignin-PEG-PPG, and LSQR29, *p < 0.05 versus LSQR29. All samples were measured in triplicate.

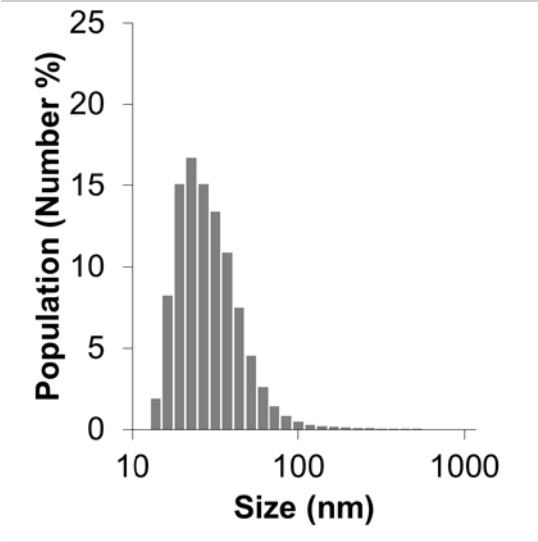

Figure S4. Particle size distribution of LSQR29.

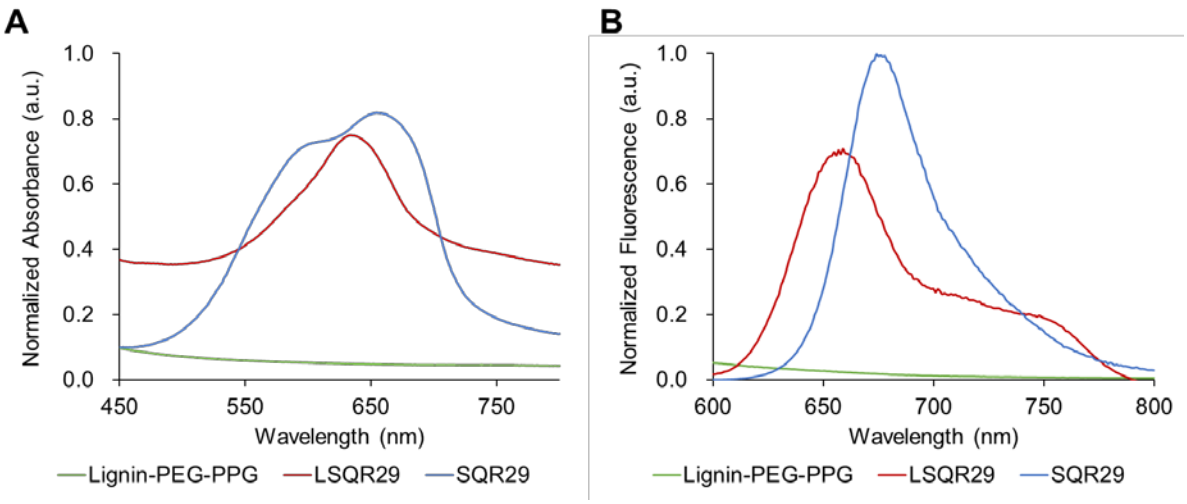

Figure S5. (A) Absorption and (B) emission spectra of the SQR29, Lignin-PEG-PPG and LSQR29.