Photoinactivation of *S. aureus* biofilms using porphyrin conjugates with green-synthesized TiO_2 immobilized on waste polystyrene

Nonkululeko Malomane[a], Nkgameleng K. Mokhonoana[a], Christian I. Nkanga[b], Ojodomo J. Achadu[c] Richard M. Moutloali[a] and Muthumuni Managa*[a]

[a]Institute for Nanotechnology and Water Sustainability (iNanoWS), Florida Campus, College of Science, Engineering and Technology, University of South Africa, Johannesburg 1710, South Africa

[b]Centre de Recherche en Nanotechnologies Appliquées aux Produits Naturels (CReNAPN), Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Kinshasa, B.P. 212, Kinshasa XI, Democratic Republic of the Congo

[c]School of Health and Life Sciences, and National Horizon Centre, Teesside University, Tees Valley, Middlesbrough TS1 3BX, UK

Correspondence: managme@unisa.ac.za (M. M.)

Methodology

Chemicals and Materials

Orange fruits (*Citrus sinensis*) were purchased from a local supermarket. Titanium tetraisopropoxide (TTIP) was used as the titanium precursor, pyrrole was distilled before use, isopropanol, propionic acid, 4-formylbenzoic acid, 4-bromophenylcarboxaldehyde, sodium hydroxide, indium (III) chloride, glacial acetic acid, sodium acetate, dimethyl formamide, tetrahydrofuran, methanol, ethanol and chloroform were purchased from Sigma Aldrich in South Africa. Expanded polystyrene cups were collected from waste bins at the University of South Africa, Florida campus and thoroughly washed and dried. Environmental *S. aureus* was isolated from water sampled from a pond at the University of South Africa Florida Campus.

Equipment

Fourier Transform Infrared spectroscopy (Perkin Elmer, FT-IR 100, Perkin Elmer Inc, USA) was used to determine the functional groups within the synthesized materials. The KBr-pellet method was used for the nanoparticles TP and TJ only scanning wavelengths from 400-4000 cm^{-1}, while the ATR method was performed on porphyrins and their conjugates in their free and immobilized forms. The X-ray diffraction (XRD) patterns were acquired with the Rigaku Smart Lab X-ray Diffractometer measurements with Cu-Kα (λ =0.154059 nm) radiation at 2θ/min scan speed at the College of Science, Engineering and Technology, in the department of Physics of the University of South Africa (UNISA). The sample was put on a slide and put into an XRD sample holder chamber for analysis and the X-ray generator operated at 40 kV and 30 mA, and the scanning range for the 2θ values were recorded from 10 to 80°. The surface morphology and elemental composition of the materials were determined using SEM-JEOL JSM IT 300 SEM/EDS Oxford X-MAXN Instruments Energy-dispersive X-ray spectroscopy (EDS) system at 10 kV. Samples were dispersed on a carbon tape and coated with gold (Au). Transmission electron microscopy (TEM) images were acquired in the electron microscope unit using JEOL-JEM 2100 operating at an accelerating voltage of 200 kV at the Counsel for Scientific and Industrial Research (CSIR). Samples sonicated for 30 minutes and dispersed on carbon coated Cu grids. Thermogravimetric analysis (TGA, PerkinElmer Pyris 1) was used to study the thermal stability of the synthesized materials. An approximate mass of 30 mg was weighed and placed in the TGA sample pan for the mentioned the materials were analysed from 50-900°C at a heating rate of 10°C/min under inert conditions. The liquid and solid-state UV-Vis absorption spectra were recorded using a PerkinElmer UV/Vis/NIR spectrometer Lambda. The solid-state UV-Vis spectrophotometry was used for TiO_2 nanoparticles and their conjugates in the range 300-800 nm.

Fluorescence emission spectra were recorded using Varian Eclipse spectrofluorometer. Fluorescence lifetimes were measured using a time-correlated single photon counting setup (TCSPC) (FluoTime 300, PicoquantR GmbH) with a diode laser (LDH–P–C-420, 420nm, 20 MHz repetition rate, 44 ps pulse width) in a manner described previously [15]. Thermo Scientific Genesys 10S UV-VIS Spectrophotometer was used for reading the absorbance of bacteria at 600 nm. Two plastic vials where prepared, the one with broth only was used as a blank and the other with bacteria in broth was used as the analyte. Thermo Scientific Varioskan LUX Multimode Microplate Readers was used for reading the absorbance of the bacteria and the biofilms in the microplates before and after inactivation. An LED-mounted irradiation chamber equipped with M415L4 LED with a continuous irradiance yielding a maximum of 15.6 µW/mm^2 was used.

Bacterial isolation

S. aureus bacteria were isolated from the water by shaking the bottle vigorously, followed by filtering a volume of 250 mL water using sterile nitrocellulose membrane paper (0.45 µm pore size) (Merck, South Africa) on the MERCK manifold to concentrate the bacteria. The membrane paper was then placed on mannitol salt agar plate and incubated for 24 h at 37°C. Presumptive *S. aureus* colonies, which appeared yellow in colour on mannitol salt agar, were then selected for further purification on a new mannitol salt agar plate by streaking until only pure yellow colonies appeared. Gram staining was used confirm the shape, arrangement and categorize the isolated presumptive *S. aureus*. The ability to degrade hydrogen peroxide into water and oxygen was tested using catalase test. *S aureus* ATCC 25923 were used as positive controls for on mannitol salt agar, morphology and biochemical tests**.** Pure S. *aureus* colonies were stored in 30% sterile glycerol solution at -20°C freezer for further analysis.

DNA extraction

The 30 % glycerol stock solutions of *S. aureus* previously stored at -20°C freezer were placed at room temperature to thaw. Then, a full loop of *S. aureus* stock was aseptically streaked on mannitol salt agar plate and incubated at 37°C for 24 hours to obtain fresh bacterial culture. A single colony was then taken and incubated for further 24 h at 37°C in nutrient broth. After incubation, 1.5 ml of the broth was added to 2 ml Eppendorf tubes and microcentrifuged at maximum speed for 2 minutes then the supernatant was discarded. The process was repeated twice to get a concentrated bacterial pellet. The bacterial pellet was washed and rinse with Phosphate-buffered saline (PBS) then microcentrifuged at the maximum speed for 2 mins before discarding the PBS. A volume of 250 µL PBS was added to the clean bacterial cells and the samples were pipet mixed. A volume of 250 µL bacterial cell in PBS were homogenized by pipet mixing. DNA extraction was done following the Quick-DNATM fungal/ Bacterial miniprep kit (Zymo Research, USA) protocol. The concentration and purity of the extracted DNA were quantified using EzDrop 1000 (BLUE-RAY BIOTECH).

Polymerase chain reaction, 16s rRNA Sequencing and Gel electrophoresis

The polymerase chain reaction was used to amplify 16s rRNA bacterial gene using 27 F (5'AGRGTTTGATCMTCMTGGCTCAG3') and 1492 R (5GGTTAGCCTTGTTACGACTT3') primers. PCR was performed using a Mycycler™ Thermal Cycler PCR system (Bio-Rad, T100 thermal cycler,) under the following conditions: an initial denaturation of 96 °C for 4 min; denaturation of 30 s at 96 °C, 2 min annealing at 57 °C, extension at 72 °C for 60 s and a final extension cycle of 72 °C for 10 min. A total reaction mixture of 25 µL consisting of 12.5 µL master mix (Thermo Scientific, Waltham, MA, USA), 1 µL each of forward and reverse primers (Inqaba Biotech, Mawniya, Nigeria), 7.5 µL PCR grade water, 1 µL of forward primer and 1 ul reverse primer and 3 µl of template DNA. 2% agarose gel was prepared using 1% TAE buffer and the gel was stained with 4 µL ethidium bromide. Gel Electrophoresis was carried out at 120 V for 60 min using a 1k bp DNA marker (Fermentas, Vilnius, Lithuania) was used to

estimate the DNA band. The gel was visualized on Bio-Rad ChemiDoc system. The samples were sent to Inqaba Biotech for DNA sequencing.

Results

SEM

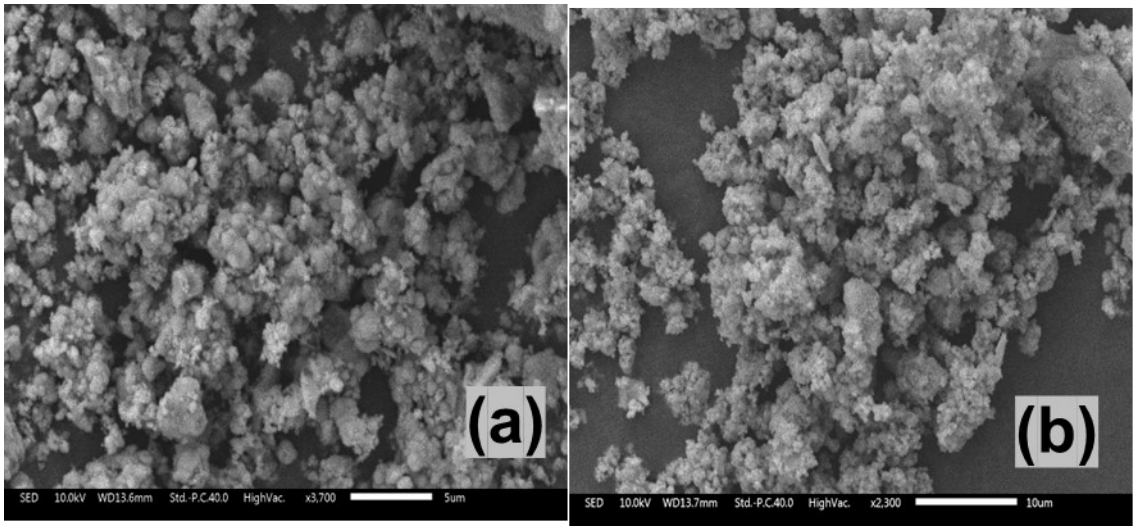

***Figure S1**: SEM micrograms for TJ (a) and **2**-TJ (b)*

EDX

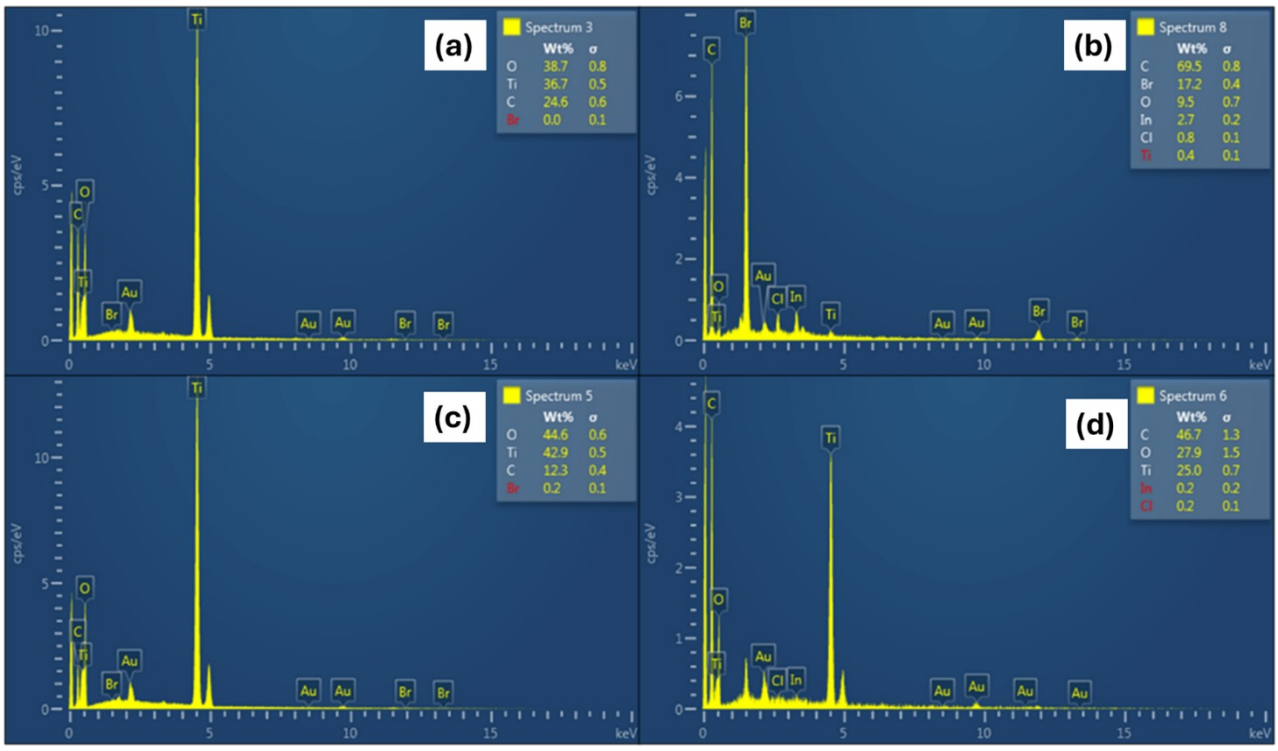

***Figure S2:** EDX Spectra of the free materials, TP, **2**, **1**-TP, and **2**-TP*

TCSPC

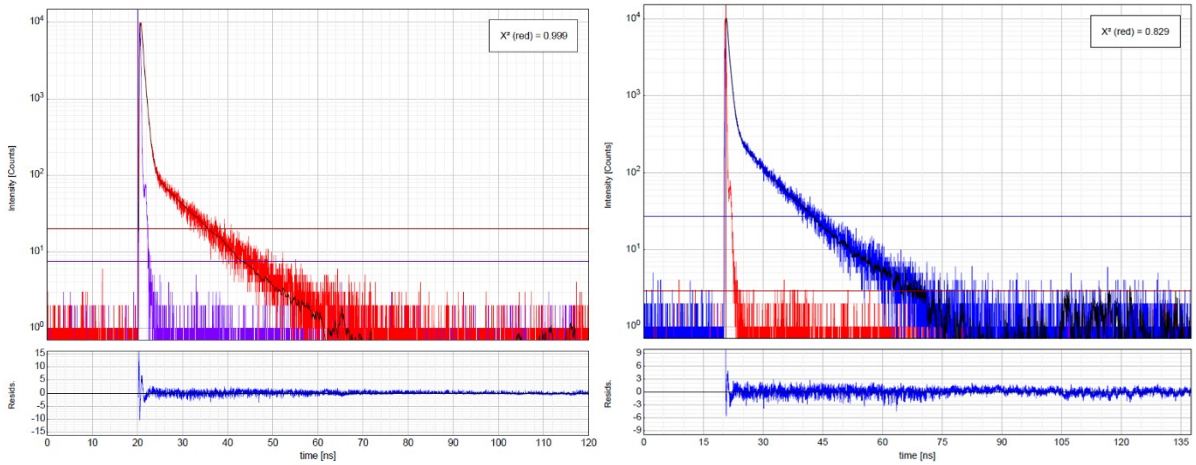

Figure S3: *TCSPC results for **1** and **1**-TJ showing fluorescence decay (red), X_2 fitting (black), and IRF curves in DMF*

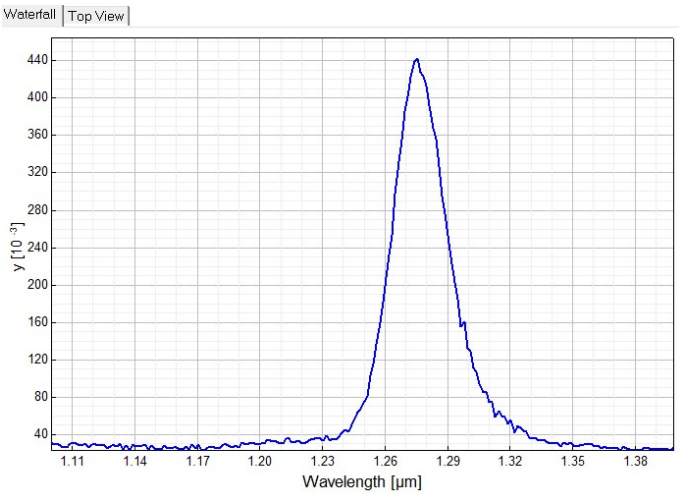

Figure S4*: Φ_Δ spectra of **2** as an example*

Bacterial Studies

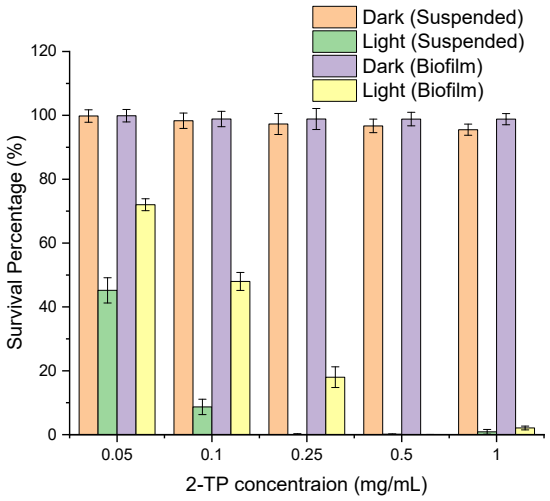

Figure S5*: Photosensitizer concentration optimization studies showing survival percentages of S. aureus suspended and biofilm bacteria inactivated using **2**-TP in the dark and under light for 30 minutes*