Supplementary Data for Publication

Synthesis of Eucalyptus/Tea Tree Oil Absorbed Biphasic Calcium phosphate-PVDF Polymer Nanocomposite Films: A Surface Active Antimicrobial System for Biomedical Application

Biswajoy Bagchi[1,δ], Somtirtha Banerjee[1], Arpan Kool[1], Pradip Thakur[1,2], Suman Bhandary[3], Nur Amin Hoque[1] , Sukhen Das[1+]*

[1]Physics Department, Jadavpur University, Kolkata-700032, India.
2Department of Physics, Netaji Nagar College for Women, Kolkata-700092, India.
[3]Division of Molecular Medicine, Bose Institute, Kolkata-700054, India.
[+]Present Address: Department of Physics, Indian Institute of Engineering Science and Technology, Shibpur, Howrah, West Bengal-711103, India.
[§]Present Address: Fuel Cell and Battery Division, Central Glass and Ceramic Research Institute, Kolkata-700032, India.

*Corresponding author's email id: sdasphysics@gmail.com
Contact: +919433091337

Antimicrobial activity of EU and TTO treated films on E .coli and S. aureus by acridine orange/ethidium bromide (AO/EB) dual staining

Live/dead cell characterization of EU/TTO film treated bacterial cultures was also done to visualize the viability under fluorescence microscope (). The treated culture suspensions after 12 and 24 hours of incubation were collected by centrifugation (5000 rpm, 20 mins). The cell pellets were resuspended in PBS. The staining solution was prepared by mixing equal parts of acridine orange (5mg/mL) and ethidium bromide (3mg/mL) in ethanol. 20µL of the staining solution is then mixed with 10µL of the resuspended solution and incubated for 15 minutes at 37°C. 10µL of this solution was then placed on a glass slide and covered with cover slip to observe under fluorescence microscope. The microscope was operated using 495 nm and 515 nm filter. The dead cells are stained red by ethidium bromide while live cells appear green.

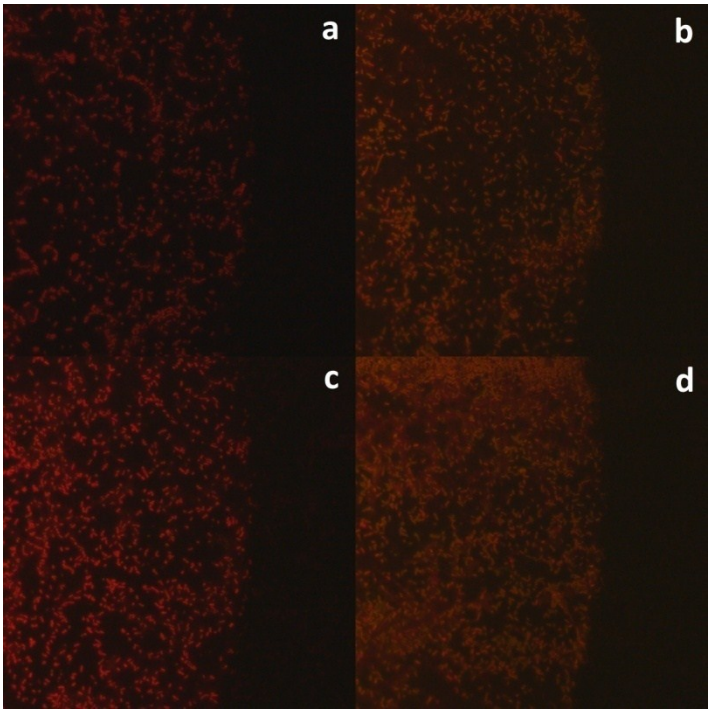


Figure 1: Fluorescence microscopy images of E. coli after treatment with EU film a) for 12 hrs, b) 24 hrs and TTO film c) for 12 hrs and d) 24 hrs

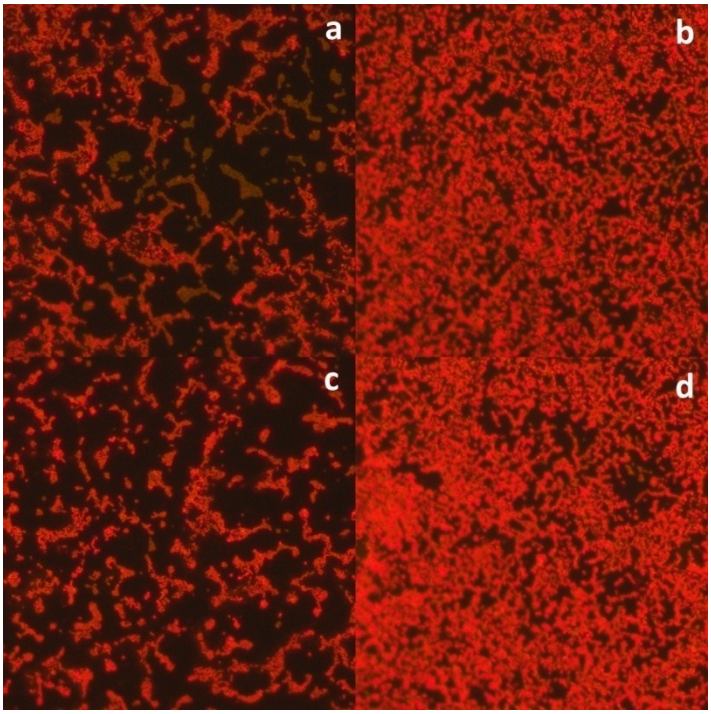


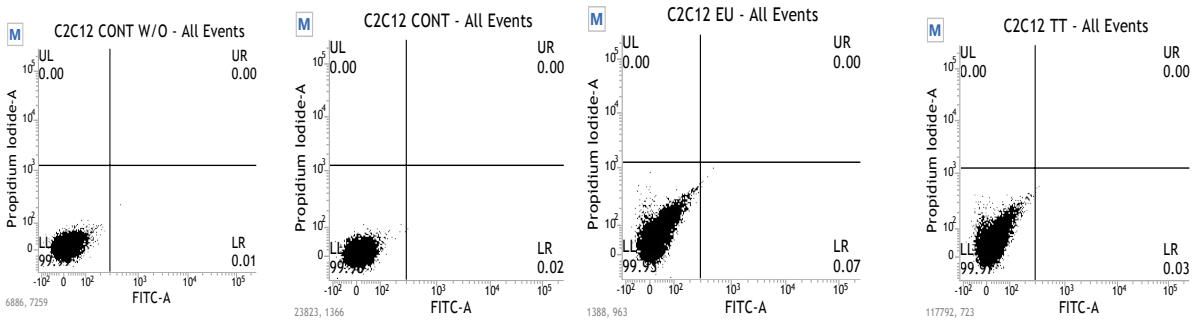
Figure 2: Fluorescence microscopy images of S. aureus after treatment with EU film a) for 12 hrs, b) 24 hrs and TTO film c) for 12 hrs and d) 24 hrs

Treated bacterial cultures were subjected to viability assay by ethidium bromide/acridine orange (EB/AO) staining. The principle of EB/AO method employs a viability stain in which AO diffuses into all cells and EB is not able to diffuse across a cell membrane unless it is compromised (leading to necrosis) or the cell is apoptotic. Cells with only AO present inside fluoresce green, cells that are dead or have compromised membranes absorb EB which dominates over AO and results in the cells fluorescing red. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin[1]. Figure 1a and b shows fluorescence images of E. coli treated with EU for 12 and 24 hrs respectively. Similarly, 1c and d shows E. coli treated with TTO for the same duration. The images show huge density of dead cells, consisting of mostly necrotic cells, although the rod shaped morphology can be visualized. This is because a cell can be dead without any apparent membrane deformation by slow leakage of vital fluids mediated by essential oils. In case of EU (Figure 2a and b) and TTO (Figure 2c and d) treated S. aureus, similar images were observed with almost 98% dead cells with apoptotic body formations. These results agree well to that obtained with plate counting and FESEM indicating potent antimicrobial action of EO and TTO films.

Biocompatibility studies on mouse myoblast by Flow cytometry of Annexin V-FITC/PI-stained cells:

Annexin V-Fluorescein isothiocyanate (FITC) staining was done in conjunction with Propidium Iodide (PI) to distinguish apoptotic and live cells from dead cells. During early apoptosis, phosphatidylserine is translocated to the outer membrane surface and Annexin V-FITC stains cells with this accessible phosphatidylserine. PI stains cells with permeable membranes, thus distinguishing cells with permeabilized membranes from those with healthy membranes. Following FACS, fluorescence of PI was plotted over Annexin V-FITC fluorescence. Healthy cells have no or low FITC and PI fluorescence. Early apoptotic cells have high FITC fluorescence (bound phosphatidylserine) but low PI fluorescence (intact membranes). Late apoptotic cells have high FITC fluorescence and high PI fluorescence. Necrotic or dead cells have low FITC fluorescence but high PI fluorescence[2].

To determine the proportion of cell death due to treatment with EU and TTO in tandem with untreated controls we have performed flow cytometry using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen ™, San Jose, CA). Briefly, EU (2.88% v/v) and TTO (2.94% v/v) treated cells were harvested and pelleted by centrifuging at 1000×g for 5 min at room temperature. After washing twice with cold PBS, cells were resuspended in 1× binding buffer at a concentration of $1 \times l0^6$ cells/ml. Then 5 µl of FITC-Annexin V and 5 µl PI was added to the cell suspension. Cells were gently vortex and incubated for 15 min at 25°C in the dark. Again 400 µl of 1X binding buffer was added to each tube and analyzed by flow cytometry by FACScan (Becton-Dickinson) using Cell Quest software (Becton-Dickinson, San Jose, CA) within 1 hr.



Control with out staining | Control with staining | Treated with EU | Treated with TT

Figure 2: FITC-annexin V/PI flow cytometric data of C2C12 cells cultured for 24 hrs with and without EU (2.88% v/v) and TTO (2.94% v/v) treatment.

The lower left quadrant of the cytograms shows the viable cells, which exclude PI and are negative for FITC-Annexin V binding. The upper right quadrant represents the non-viable, necrotic cells, positive for FITC-Annexin V binding and showing PI uptake. The lower right quadrant represents the apoptotic cells, FITC-Annexin V positive and PI negative, demonstrating Annexin V binding and cytoplasmic membrane integrity.

The results shows that in comparison with the control sample there is no or less amount of $FITC^{+}/PI^{-}$, $FITC^{+}/PI^{+}$ and $FITC^{-}/PI^{+}$ cell populations. This data signifies that EO and TTO do not affect the viability of the C2C12 cell line.

References

1. Mansour James D., Schram James L., Schulte Thomas H. Fluorescence staining of intracellular and extracellular bacteria in blood. Journal of Clinical Microbiology, 19(4), 1984, 453-456.

2. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods, 184(1), 1995, 39-51.