Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Nature-Derived Gelatin-Based Antifungal Nanotherapeutics for combatting *Candida albicans* Biofilms †

*Ahmed Nabawy ‡, Jessa Marie Makabenta ‡, Jungmi Park ‡, Rui Huang ‡, Varun Nayar ‡, Robin Patel §, and Vincent M. Rotello ‡**

‡ Department of Chemistry, University of Massachusetts Amherst, 710 North Pleasant Street, Amherst, Massachusetts 01003, USA

§ Division of Clinical Microbiology, Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905

* Address correspondence to rotello@chem.umass.edu

KEYWORDS: Drug-resistant fungi, biofilms, nanomaterials, antimicrobials, *Candida albicans*, nanoemulsions, GRAS ingredients

† Dedicated to the memory of Joel Pedersen. An enthusiastic scientist, and a good person.

Experimental Methods

Materials and Methods. Riboflavin (98%, Cat #AC132355000), carvacrol (98%, Cat #C0026500G), Gelatin (Type B, Cat #AC612255000), Nile red (99%, Cat #AC415711000) were purchased from Fisher Chemical. The chemicals were used as received. All other reagents and solvents were purchased from Fisher Scientific and used as received. Tryptic soy broth (TSB) was purchase from Becton Dickinson. AlamarBlue™ cell viability reagent was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Gibco.

Fabrication of Nanoemulsions. Gelatin nanoemulsions (**GNE**) stock solutions were fabricated through emulsification of a suspension of riboflavin in carvacrol into an aqueous gelatin solution, followed by irradiation with 365 nm UV-light (Thermo Scientific™ 3UV Lamp (115V, 60Hz), Cat #95034). Briefly, suspension of riboflavin in carvacrol (3 µL, 0.1 wt%) was added to the gelatin aqueous solution (497 µL, 0.24 mg/ml). This solution was then emulsified for 50 seconds using an amalgamator (Zoneray HL-AH G8). The emulsion was then exposed to a UV lamp for 20 minutes.

Characterization. The hydrodynamic diameter of nanoemulsions (60 mg/L) was measured in triplicate using DLS (Malvern Zetasizer). TEM samples were prepared through drying a deposited drop of the nanoemulsions on 300 square mesh nickel grids with Formvar film (Electron Microscopy Sciences) and imaged using JEOL JEM-2000FX Transmission Electron Microscope.

Biofilm penetration study. Red fluorescent nanoemulsions were prepared through loading Nile Red, a hydrophobic red fluorescent dye, into the oil phase, carvacrol, of the nanoemulsions (1 mg/ 1000 µl). 3-day-old *Candida albicans* (IDRL-7033) biofilm stained with SYTO 9, a cell-permeable green fluorescent dye, to allow visualization of the biofilm. These biofilms were then treated with prepared red fluorescent nanoemulsions (60 mg/L) for 1 h. A Nikon A1 spectral detector confocal with FLIM module was used to monitor the penetration profile of the nanoemulsions. A penetration profile study was performed using Nikon A1 resonant scanning confocal with TIRF module. The images were processed using NIS-Elements.

Biofilm Formation. Fungi were cultured in tryptic soy broth (TSB) supplemented with 1% glucose at 30°C solution overnight. Fungi concentration was adjusted to an OD_{600} of 0.1 in TSB supplemented with 1% glucose (100 µL) were added to microplate wells. The microplates were then put under static conditions for 3 days at 30 °C. The resulting biofilms were washed with PBS solution to remove any adhering planktonic fungi. Different concentrations of **GNE** were prepared in TSB supplemented with 1% glucose and 100 µL of **GNE** solutions were added to the preformed biofilms at 37 °C. After 3 h of incubations with the nanoemulsions, Alamar Blue assay was used to determine the viability of the treated biofilms. After a wash step with PBS three times, cells were treated with 220 µL of 10% alamar blue in serum-containing media and incubated at 37 ºC under a humidified atmosphere of 5% CO_2 for 3 h. After incubation, 200 µL of solution from each wells was transferred in a 96-well black microplate. Red fluorescence, resulting from the reduction of Alamar blue solution, was quantified (excitation/emission: 560 nm/590 nm) on a SpectraMax M2 plate reader (Molecular Devices) to determine the cellular viability. Cells without any nanoemulsions were considered as 100% viable.

Biofilm-3T3 Fibroblast Cell Co-Culture. NIH 3T3 (ATCC CRL-1658) cells (20k/ well) were cultured in a 96 well plate overnight. Next, the 3T3 cells were washed using PBS solution followed

by addition of fungal solutions (10^8 CFU/mL). The cocultures were next incubated under static conditions at 37 °C for 24 h. The nanoemulsions were diluted using DMEM media to obtain the desired testing concentrations which were then added to the cocultures. After 3 h incubation, the mammalian cells in the cocultures were monitored utilizing LDH cytotoxicity assay. First, 50 µL of each sample medium was transferred to a 96-well flat-bottom plate. Then the working solution (50µL) was added to the plate for 30 min treatment without light at room temperature and then the stop solution (50µL) was added. SpectraMax M2 plate reader (Molecular Devices) was employed to measure the absorbance at 490 nm and 680 nm. Cells treated with PBS or lysis buffer were used as negative or positive control, respectively. Also, the biofilms in cocultures were dispersed through sonication and the remaining viable fungi were quantitatively determined using colony counting forming unit method.

Cytoplasmic Membrane Depolarization Assay of Gelatin Nanoemulsions. *C. albicans* were grown at 30 °C until reaching the mid-log phase (OD_{600} = 0.5). Next, the cells were centrifuged and washed with HEPES buffer (5 mM, 20 mM glucose, pH = 7.2) and suspended in HEPES buffer. The cells were then incubated with 50 nM DiSC3(5) followed by adding 10 mM KCl and the fluorescence emission (excitation λ=622 nm, emission λ=670 nm) was recorded (SpectraMax M2 plate reader, Molecular Devices). Once stable is obtained (~30 minutes), **GNE** and other controls were added to the cell suspension and the fluorescence signals were recorded continuously.

Selection of Resistance. *C. albicans* was inoculated in TSB media supplemented with 1% glucose-containing sub-lethal concentrations (1/3× MIC) of **GNE**. These cells were harvested, and their respective MICs were determined. *C. albicans* was cultured without **GNE** as well every time as a control for comparison of MICs. Each successive passage was prepared by exposing the fungi to 1/3× MIC of the previous therapeutic dosage.

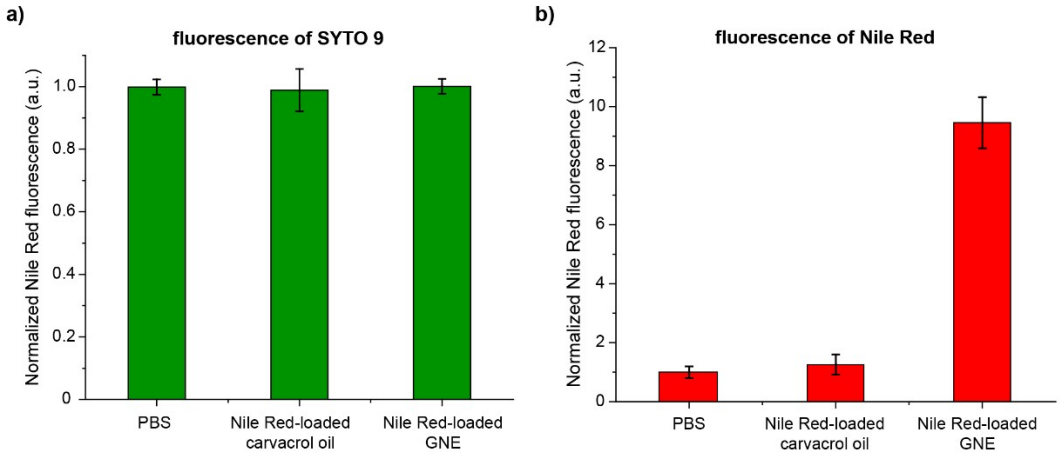

Figure S1. Integrated intensity of a) SYTO 9 and b) Nile Red in confocal study.

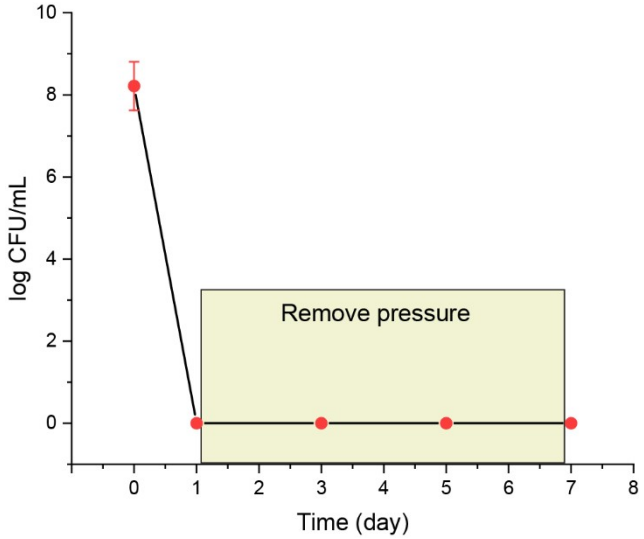

Figure S2. Carvacrol-loaded gelatin nanoemulsion eradicated *C. albicans* biofilm with no observed recurrence. Tracking of Viability of *C. albicans* (IDRL-7033) biofilms after 24 h treatment with carvacrol-loaded gelatin nanoemulsion (1920 mg/L). The tan box represents the absence of antimicrobial pressure.

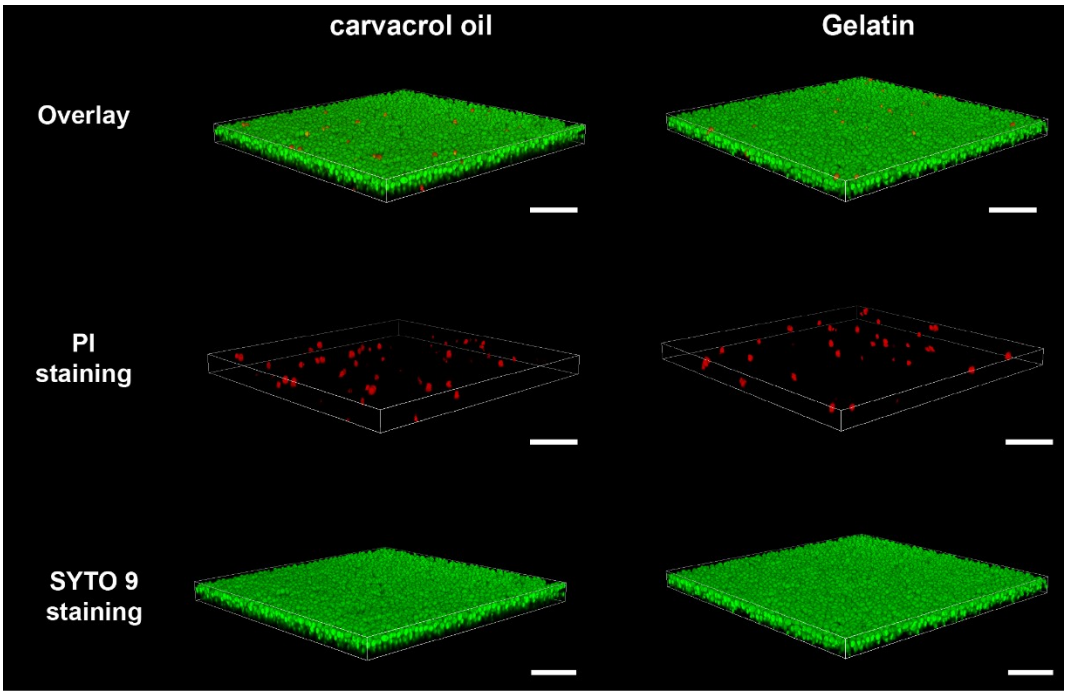

Figure S3. Representative 3D views of confocal images of *C. albicans* IDRL-7033 biofilm stained SYTO 9 (green fluorescence) and propidium iodide (PI, red fluorescence) after treatment with either carvacrol oil or gelatin only.

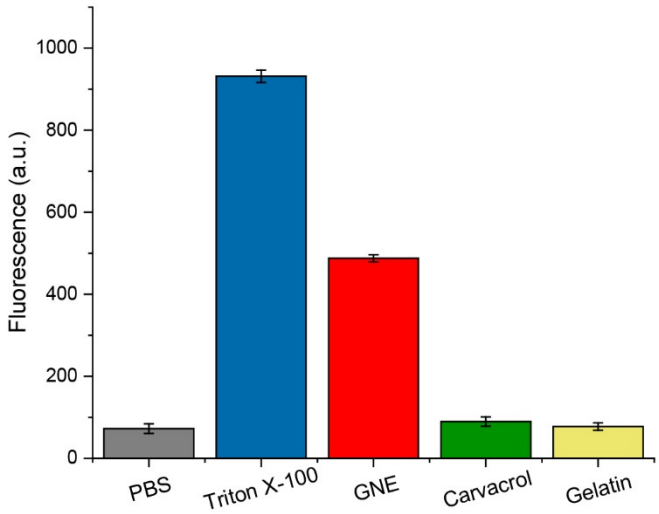

Figure S4. Cytoplasmic membrane depolarization of *C. albicans* IDRL-7033 treated with **GNE**, Carvacrol, or gelatin. Triton X-100 serves as a positive control.

pH	Zeta-Potential (mV)
7.4	-11 ± 4.5
5	12 ± 3.1

Table S1. Zeta potential of GNE at different pH.

Record Key	Genus	Species	Source
7033	Candida	albicans	Subcutaneous Abscess
7034	Candida	albicans	Pelvic Abscess
7116	Candida	albicans	Denture Stomatitis

Table S2. Sources of the *C. albicans* clinical isolates.