

Rajasekar, V., Darne, P., Prabhune, A., Kao, R. Y.T., Solomon, A. P., Ramage, G., Samaranayake, L. and Neelakantan, P. (2021) A curcumin-sophorolipid nanocomplex inhibits *Candida albicans* filamentation and biofilm development. *Colloids and Surfaces B: Biointerfaces*, 200, 111617. (doi: [10.1016/j.colsurfb.2021.111617](https://doi.org/10.1016/j.colsurfb.2021.111617)).

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Deposited on: 10 February 2021

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A curcumin-sophorolipid nanocomplex inhibits *Candida albicans* filamentation and biofilm development

Abstract

Candida albicans is an opportunistic fungal pathogen that is highly resistant to contemporary antifungals, due to their biofilm lifestyle. The ability of *C. albicans* to invade human tissues is due to its filamentation. Therefore, inhibition of biofilms and filamentation of the yeast are high value targets to develop the next-generation antifungals. Curcumin (CU) is a natural polyphenol with excellent pharmacological attributes, but limitations such as poor solubility, acid, and enzyme tolerance have impeded its practical utility. Sophorolipids (SL) are biologically-derived surfactants that serve as efficient carriers of hydrophobic molecules such as curcumin into biofilms. Here, we synthesized a curcumin-sophorolipid nanocomplex (CU-SL), and comprehensively evaluated its effects on *C. albicans* biofilms and filamentation. Our results demonstrated that sub-inhibitory concentration of CU-SL (9.37 µg/mL) significantly inhibited fungal adhesion to substrates, and subsequent biofilm development, maturation, and filamentation. This effect was associated with significant downregulation of a select group of biofilm, adhesins, and hyphal regulatory genes. In conclusion, the curcumin-sophorolipid nanocomplex is a potent inhibitor of the two major virulence attributes of *C. albicans*, biofilm formation and filamentation, thus highlighting its promise as a putative anti-fungal agent with biofilm penetrative potential.

Keywords: Biofilm; Biosurfactant; *Candida albicans*; Curcumin; Filamentation; Hyphae; Sophorolipid.

1. Introduction

Candida albicans is an opportunistic fungal pathogen that causes superficial and invasive life threatening infections, particularly in immunocompromised patients, with fatality rates as high as 40% [1-3]. Patients undergoing cytotoxic chemotherapy, those on long-term antibiotics, diabetics, as well as elderly denture wearers are specifically at high risk for superficial mucosal fungal infections, that often invades the host tissues and serves as a reservoir to seed pathogens to distant sites [4]. The ability of this yeast to form extremely tenacious biofilms on abiotic and biotic surfaces, and its morphogenic transition from yeast to hyphal/filamentous phase, are well known as major reasons for the remarkably increased recalcitrance and tolerance of this yeast to existing antifungal agents [5].

The major drug groups currently available for the management of candidiasis are polyenes, azoles, and echinocandins [6]. However, the global emergence of multi-drug resistant yeast strains to even the most effective agents has seriously impeded their clinical use, and the novel antifungal pipeline is currently, virtually dry [7, 6]. This is compounded by the fact that, being a eukaryote, pathogen-specific drug targets for anti-candidal drugs are highly limited, leading to a lag in antifungal drug development in comparison to antibiotics. An alternative, innovative approach for drug design, specifically targeting the major virulence attributes of *C. albicans*, such as biofilm formation and filamentation, is therefore urgently warranted.

Natural compounds have gained immense popularity as safe alternatives to relatively toxic, synthetic drugs [8,9]. Curcumin (CU), a natural polyphenol extracted from the

perennial rhizome, *Curcuma longa* (Turmeric), has long been advocated as a health conferring phytochemical due to its wide ranging pharmacological attributes such as antibacterial, antifungal, antioxidant, antitumor, and anti-inflammatory action [10]. Curcumin (CU) is also active against *C. albicans*, due to the generation of fungal-toxic, reactive oxygen species (ROS) and suppression of hyphal development [11, 12]. Yet, factors such as its poor aqueous solubility and acid tolerance, low bioavailability, enzymatic degradation have impeded its clinical transition into a potent antimicrobial [13]. Hence, there is a pressing need to develop a potent drug delivery system that sustains the pharmacologic activity of curcumin, at the infective focus.

Sophorolipids (SL) are surfactants derived from non-pathogenic yeasts such as *Starmerella/Candida bombicola*, and hold great promise as vehicles for hydrophobic drug delivery [13-16]. These FDA-approved, biodegradable surfactants exist in two forms, lactonic and acidic, wherein the surfactant component, acidic sophorolipid (ASL), is a bola-amphiphile with two hydrophilic ends located on either end of a hydrophobic skeletal scaffold [16], while the lactonic sophorolipids may demonstrate antimicrobial effects [17]. As such, these glycolipid biosurfactants show excellent anti-adhesive property against various microorganisms. This inhibition of initial adhesion of microbes to biotic and abiotic surfaces prevents biofilm formation.

Recently, a curcumin-sophorolipid nanocomplex (CU-SL) theranostic platform was shown to tremendously improve the stability, photophysical property and availability of curcumin inside bacterial biofilms and bacterial cells without bactericidal effects. This complex was shown to inhibit biofilm formation and quorum sensing in bacteria [13, 18].

However, the antifungal effects of CU-SL remains to be investigated. Based on this background, we hypothesized that low, non-toxic concentrations of curcumin-sophorolipid nanocomplex (CU-SL) can selectively inhibit biofilm development as well as filamentation of *C. albicans* [18,19].

2. Materials and Methods

2.1.Synthesis and characterization of the curcumin-sophorolipid nanocomplex (CU-SL)

The synthesis and characterization have been described in detail elsewhere [13, 18, 19]. Briefly, 1 mg of CU was added to 40 mg SL in a beaker containing 10ml distilled water and probe sonicated (Branson Digital Sonifier 250) with constant pulse of 10 sec with 3 sec intervals. This cycle was repeated for 40 min. The solution obtained was clear yellow without precipitate indicating complete solubility of curcumin [18]. The CU-SL solution is a homogenous suspension of CU and SL. To characterize the particle size and its morphology, we performed scanning electron microscopic (SEM) analysis (FEI Quanta 200 3D) at voltage between 15-20 kV and transmission electron microscopic analysis (TEM) analysis FEI Technai G2 120 kV. About 10 µl of the CU-SL suspension was drop casted on a clean silicon wafer and dried overnight [13, 18].

2.3 Organism, media, chemicals and culture conditions

Candida albicans SC5314, the wild type reference strain that is most commonly used in antifungal studies, was obtained from the American Type Culture Collection (ATCC). The culture was maintained in Sabouraud Dextrose agar (SDA) at 37 °C in aerobic

conditions. The inoculum for each experiment was prepared by taking a loop full of a single colony from the SDA plates and suspending it in Yeast Peptone Dextrose broth (YPD). The broth culture was incubated overnight at 37 °C in aerobic conditions, centrifuged at 6000rpm for 10 min and washed twice with Phosphate Buffer Saline (PBS). A standard cell suspension of 1.5×10^7 yeast cells were prepared by optical density measurements. CU-SL was prepared as described previously [18]. All experiments were performed in triplicates on three independent occasions.

2.4 Susceptibility of planktonic cells

The effect of CU-SL on planktonic *C. albicans* was determined by measuring the OD₅₉₅. Initially, a stock solution was prepared by dissolving CU-SL in YPD broth. CU and SL were maintained as controls. A working concentration of 100 µg/ml of CU-SL, CU or SL was added to sterile 96-well polystyrene plates and serially diluted up to 0.585 µg/ml. The culture was prepared as mentioned earlier and 10 µl was added into each well. Untreated standard cell suspension with fresh YPD was considered as control. The plates were then incubated in aerobic conditions at 37 °C for 24 h to ascertain the minimum inhibitory concentration of the CU-SL combination. Later, the absorbance of each well was measured at OD₅₉₅ using a multimode detector and the inhibition percentages were calculated.

2.5 Evaluation of biofilm inhibition

A range of sub-inhibitory concentrations (sub-MIC) of the compounds (CU-SL, CU, SL) was determined based on the aforementioned planktonic cell studies. The inhibition of biofilm formation by the sub-MIC concentrations was determined by measuring the

biofilm biomass using the crystal violet (CV) assay [20]. Biofilm inhibition was investigated before and after the initial adhesion phase of *Candida albicans*, as described below [1]. Untreated standard cell suspension with fresh YPD was considered as control.

For treatment *before the initial adhesion* of the yeasts, 100 µl of varying concentrations of CU-SL, CU or SL were added to 10 µl of standard cell suspension. For treatment *after the initial adhesion* phase, 100 µl of the standard cell suspension was inoculated into the well plates and incubated for 90 min. Next, the loosely attached cells were washed with PBS and the adherent cells were exposed to varying concentrations of 100 µl of the compounds. Untreated cell suspensions with fresh media and untreated cell suspension with was maintained as control. The plates were then incubated under aerobic conditions at 37 °C for 24 h. Afterwards, the planktonic cells were removed and biofilms were washed twice with PBS and stained with 0.1% crystal violet for 15-20 min. The residual dye on the biofilm biomass was solubilized by adding 33% v/v acetic acid, transferred to another 96-well plate, and the absorbance was measured at OD₅₇₀ using a multimode detector.

2.6 Effect on filamentation

The effect of the compounds on filamentation was determined by allowing *C. albicans* to form hyphae on a solid substrate (glass coverslips in 6 well plates) under hyphal inducing conditions [21]. The inoculum was prepared by culturing *C. albicans* overnight at 30 °C in YPD + 10% FBS and the OD₅₂₀ was adjusted to yield a concentration of 1.5×10^7 CFU/mL cells. For hyphal development, 300µL of the inoculum was added to

each well along with 3 mL of varying concentrations of CU-SL and SL (9.37-0.585 µg/mL) prepared in fresh YPD + 10% FBS, and the plates were incubated at 37 °C for 24 h under aerobic conditions. The plates were fixed with 2.5% glutaraldehyde (Sigma Aldrich, USA) for 2 h and dehydrated in a series of ethanol solutions. Samples were then dried overnight, sputter-coated with gold and hyphal development was observed under a scanning electron microscope (SU1510, HITACHI, Minato-ku, Tokyo, Japan).

2.7 Confocal Laser Scanning Microscopic (CLSM) analysis

To characterize the biofilm inhibitory effects, CU-SL was added to chamber slides (µ 8-well plate chamber slides, ibidi GmbH, Gräfelfing, Germany) with 10µL of standard cell suspension. Untreated *C. albicans* biofilms were considered as controls. The plates were then incubated in aerobic conditions for 24 h. After the incubation period, the biofilms were washed with PBS and stained with 100 µL of Live/Dead™ stain (Molecular Probes, Life Technologies, Eugene, Oregon, USA). The slides were incubated in dark for 30 min, and the stained biofilms were visualized under CLSM (Olympus FV1000, Tokyo, Japan). Z-stack images were obtained from five randomly chosen regions and the attached cells/mm² were analysed using the CellC software [22, 23].

2.8 Effect of CU-SL on gene expression

To elucidate the potential mechanisms by which CU-SL inhibits *C. albicans* biofilms and filamentation, gene expression analysis was performed using qRT-PCR. Biofilms were developed with and without CU-SL on 6-well plates and incubated under aerobic conditions for 24 h at 37 °C. PBS-washed biofilms were then scraped and collected in 1.5 mL centrifuge tubes. The samples were centrifuged at 14000×g for 10 min and the

supernatant was discarded. The pellet was used to extract total RNA using the Promega SV Total RNA isolation system (Promega Corporation, Madison, Wisconsin, USA) following the manufacturer's guidelines. The purity and concentration of the extracted RNA were verified using Nanodrop (Thermo Scientific, USA). RNA was then converted into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) using the manufacturer recommended protocol. The gene expression was analyzed by qRT-PCR using 18S rRNA as the reference gene and the relative gene expression (fold change) was determined by the $2^{-\Delta\Delta CT}$ method [24]. The primer sequences used in this study is tabulated (Supplementary table 1).

2.9 Statistical analysis

Data were statistically analysed using GraphPad Prism 8.0.2. One-way ANOVA and Dunnett's T-test were performed to compare the significance between the control and treatment groups. The number of attached fungal cells/mm² in the CLSM analysis were compared using unpaired *t*-test. *P*<0.05 was considered to be statistically significant.

3. Results and Discussion

3.1 Electron Microscopic analysis of CU-SL

It has been reported that phospholipids, liposomes and bovine serum albumin enhance the stability of CU [25, 26]. The self-assembling property of a glycolipid biosurfactant makes it a potential candidate as a drug delivery vehicle. As mentioned earlier, "crude" sophorolipids contain both the lactonic and acidic components, wherein the former contributes to antimicrobial activity while the latter contributes to the drug delivery

application [27]. ASL micelles which confers the surfactant property to SL comprise three components, a hydrophilic outer shell, an aliphatic core layer and an intermediate palisade layer [13]. Therefore, it is plausible that the sophorolipid biosurfactant, which resembles the cell membrane by having a hydrophobic center attached to two hydrophilic tails at either end, enhances the stability and bioavailability of hydrophobic curcumin by forming micelles.

The SEM study revealed the presence of evenly formed particles of CU-SL in suspension in spherical to ellipsoidal morphology (Figure 1) indicating micelle formation as reported previously [18]. TEM analysis (Supplementary Figure 1) confirmed such spherical to ellipsoidal shape, with size ranging approximately 70-100 nm. The interfacial surface tension of the surfactant led to an even dispersion of the CU-SL complex. The curcumin powder used for the experiment was reduced to nanometer size due to physical force of sonication. Such a reduction in the size of curcumin to its nanoform has been shown to increase its bioavailability in wounds [28].

3.2 Susceptibility of planktonic and biofilm cells of *C. albicans* to CU-SL

CU-SL showed more than 70% growth inhibition at 100 µg/mL (Figure 2), while CU and SL showed 47% (9.37µg/mL) and no growth inhibition respectively. Therefore, concentrations <100 µg/mL (for CU-SL and SL) and ≤9.37µg/mL (for CU) were considered as sub-inhibitory (sub-MIC) concentrations. Hence, we evaluated the effect of these concentrations (9.37 to 0.59 µg/mL) of CU-SL, CU and SL on inhibition of biofilm development. The treatment was mechanistically divided into two phases of adhesion as before and after initial adhesion of *C. albicans* [1]. Within the range of sub-

inhibitory concentrations tested, only concentrations below 9.37 µg/mL of CU-SL were able to inhibit biofilms in both the pre- and post-adhesion phases (Figure 3). By contrast, sub-inhibitory concentrations of CU alone were unable to inhibit biofilms. Furthermore, the biofilm architecture of CU-SL (9.37 µg/mL) treated fungal biofilm was examined under CLSM which showed significant reduction in the number of attached cells/mm² compared to the control (Figure 4) ($p < 0.05$).

Our data clearly demonstrates that sub-MIC CU-SL nanocomplex (9.37 µg/mL) significantly suppressed both fungal adhesion and subsequent biofilm development, as compared to CU where no biofilm inhibition was observed at sub-MIC. It has been reported previously that CU needs a much higher concentration (>50 µg/mL) [29,12] to achieve these effects; however, this occurs at the expense of growth inhibition. SL on the other hand showed significant biofilm inhibition at 1.17 and 0.59 µg/mL after the initial adhesion phase. CU elicits its antifungal effects by damaging the cell wall integrity, ROS production and glycolipid biosurfactants in general has the tendency to disrupt the cell wall [31]. By contrast, our aim was to suppress the virulence properties of *C. albicans*, rather than fungicidal effects, which was achieved with sub-lethal concentrations of the CU-SL nanocomplex. The confocal micrographs also revealed that the fungal cells were not damaged and remained intact after CU-SL treatment (Figure 4). The SL present in the solution helped in the formation of micellar structures that encapsulated the finely formed CU within its core [18,13]. When the CU-SL complex is formed, the SL shell creates a hydrophobic surface on the CU core. The outer portion of SL is hydrophilic and thus facilitates its water solubility, and delivery of

CU into the biofilm cells by penetrating the extracellular polysaccharide matrix of the biofilm, though further investigations are required to test this hypothesis.

3.3 Biofilm inhibitory concentrations of CU-SL inhibit filamentation

To potentially develop this formulation for clinical applications, it is important that the concentrations which inhibit biofilm are able to inhibit the other key virulence phenotype of *C. albicans* i.e., filamentation. This phenotype is critical to confer an invasive nature of the yeast cells to cause disease. From the previous experiment, it was apparent that sub-MIC CU-SL inhibits biofilm, while sub-MIC CU alone is unable to inhibit biofilm development. Furthermore, SL showed significant biofilm inhibition after the initial adhesion phase. Therefore, only the sub-MIC of CU-SL and SL were investigated for their anti-filamentation effect. To confirm the anti-filamentation property of biofilm inhibitory concentrations of CU-SL and SL, standard cell suspensions in the presence of CU-SL and SL were grown under hyphal inducing conditions. It was evident that CU-SL abolished filamentation at 9.37 µg/mL (Figure 5). Notably, the same concentration also reduced biofilm formation in both the pre-and post-adhesion phases. whereas SL sub-MIC concentrations, which showed biofilm inhibition were unable to inhibit filamentation at all the sub-MIC concentrations tested. Taken together, the main advantage of CU-SL is that it inhibits both biofilm growth (at both *before* and after the initial adhesion phases) and filamentation at the same concentration.

The data shown above demonstrate that CU-SL significantly inhibits biofilm and hyphal formation in *C. albicans*. These effects were further investigated at the gene level by qRT-PCR, where four major transcriptional genes that govern biofilm formation, *ROB1*,

EFG 1, *BRG 1*, *NDT 80* were significantly downregulated ($p < 0.0001$) in biofilms exposed to CU-SL (Figure 6). Specifically, *ROB1* and *EFG1* were downregulated by more than 1.5 fold. *EFG1* positively regulates the formation of hyphae in *C. albicans* biofilms [32,33]. Our results revealed that CU-SL nanocomplex downregulated *EFG1*, resulting in inhibition of filamentation. CU-SL also significantly downregulated the adhesin genes *ALS1*, *SAP8* and *EAP1*, substantiating the confocal microscopic observations which showed inhibition of fungal cell adhesion to the substrate. Amongst these, *SAP8* was downregulated by more than 2 fold. Taken together, our studies indicate that CU-SL potentially inhibits fungal biofilms on abiotic substrates. Further studies are needed to test these effects on biotic substrates, since gene expression profiles and consequently the phenotypic effects are known to be substrate-dependent.

The global threat of antimicrobial resistance is true for antifungal drugs too, since fungi such as *C. albicans* have already developed resistance to the most recent antifungals. Currently available drugs such as azoles, show antifungal activity by binding to ergosterol in the fungal cell membrane. In particular, azole resistant strains of *C. albicans* are characterised by significant upregulation of genes involved in the ergosterol pathway such as *ERG11* [34]. Interestingly, one of the mechanism of antifungal activity of curcumin is by binding to the membrane ergosterol [35]. Our study revealed that CU-SL significantly downregulates the *ERG11* gene at sub-lethal concentrations. *ERG11*, which is involved in ergosterol biosynthesis pathway of lipid synthesis, has a highly polarized ergosterol-rich domain in the lipid raft of the membrane, and is responsible for filamentation. Furthermore, ergosterols are found to be associated in the septa of hypha [36]. *ERG11* is activated by the transcription factor

NDT80 which was also downregulated by CU-SL. Hyphal regulatory genes *SAP4*, *HWP1* and *HYR1* (Figure 6) were also downregulated by CU-SL explaining the microscopic observations on filamentation inhibition. Hence CU-SL exposure appears to have a wide ranging virulence suppressive effect on *C. albicans*. A noteworthy observation in our study was the downregulation of *RAS1*, a gene involved in hyphal formation under 30°C and at low nitrogen levels. This gene is known to be a master regulator of growth, stress response and morphogenesis of *C. albicans* [37]. Although it is tempting to attribute our observation to *RAS1* suppression, at least partially, an alternative explanation could also be offered, as Hsp90, which inhibits the RAS protein, and produced under the experimental we used (i.e. a temperature of 37°C) may have contributed to this finding. Therefore, the downregulation of *RAS1* in this study may be attributed to the culture conditions rather than a true effect of CU-SL treatment.

4. Conclusion

We report here for the first time, that a nanocomplex of the phytochemical curcumin and sophorolipid (CU-SL) is an effective anti-fungal against an ubiquitous human pathogen *Candida albicans*, that causes common mucosal and systemic diseases. Biofilm formation and hyphal development of the yeast appear to be the major virulence attributes that are suppressed by the curcumin-sophorolipid complex. Further studies are warranted to evaluate the biocompatibility to different cell lines and antifungal effects of CU-SL on biotic substrates including animal models.

5. Conflict of interests

The authors declare no conflicts of interest.

6. Acknowledgements

The authors sincerely thank Ms. Becky Cheung and Ms. Joyce Yau, Central Research Laboratories, Faculty of Dentistry, The University of Hong Kong and Dr. Yam Hill Cheung Bill, Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong for the technical expertise and support.

7. Authors' contributions

Prasanna Neelakantan, Adline Princy Solomon, Lakshman Samaranayake: Conceptualization; Vidhyashree Rajasekar, Priti Darne: Methodology, Data curation and Formal analysis; Prasanna Neelakantan, Richard Kao: Project administration; Prasanna Neelakantan, Richard Kao: Resources; Vidhyashree Rajasekar, Prasanna Neelakantan, Lakshman Samaranayake, Gordon Ramage: Writing - original draft; Vidhyashree Rajasekar, Asmita Prabhune, Adline Princy Solomon, Richard Kao, Lakshman Samaranayake, Gordon Ramage, Prasanna Neelakantan: Writing - review & editing.

8. Funding:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Legends to Figures

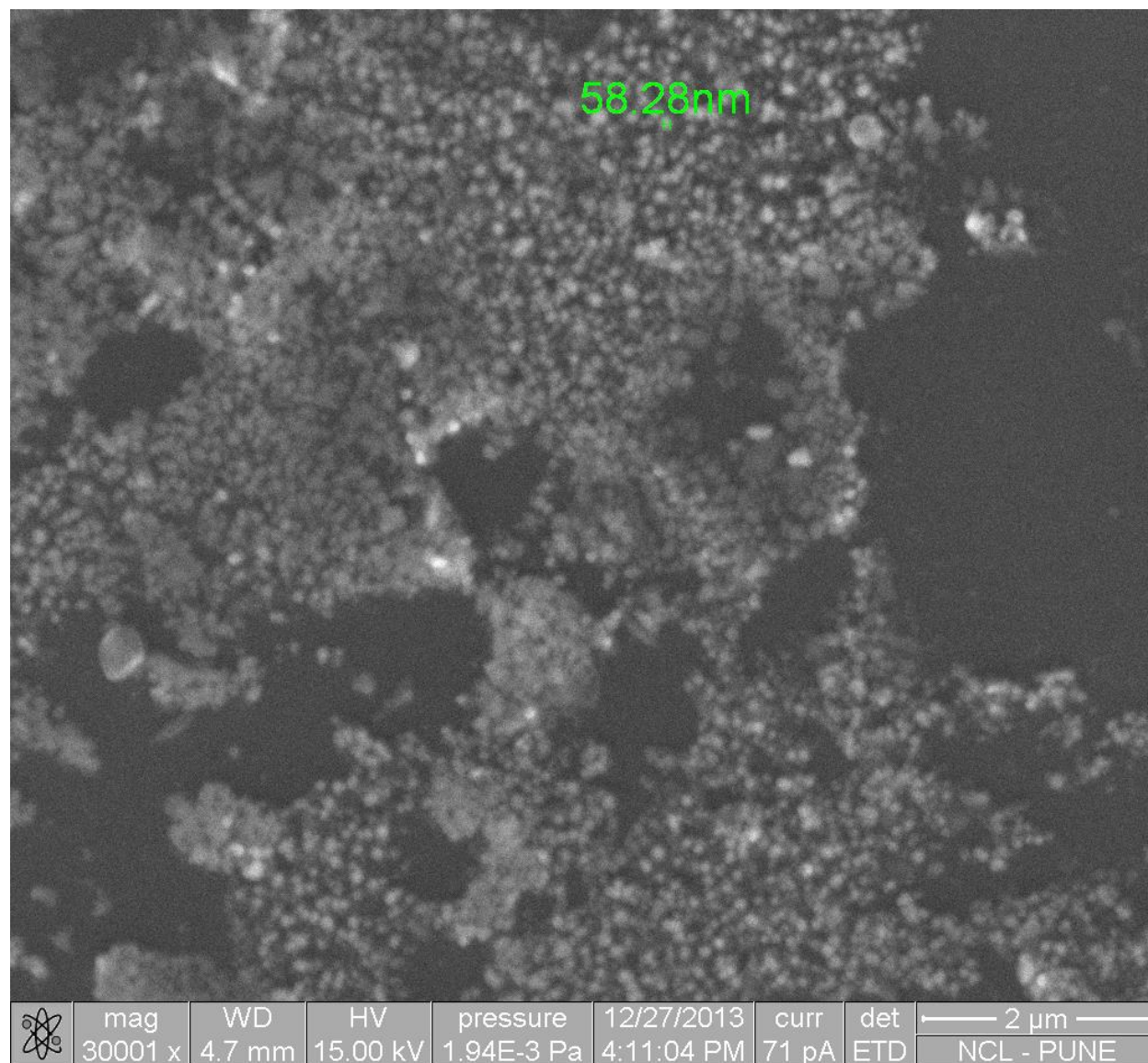


Figure 1. Scanning electron microscopic images (3000x) of the CU-SL nanocomplex. The SEM image revealed the presence of evenly formed particles of CU-SL in suspension with spherical to ellipsoidal morphology.

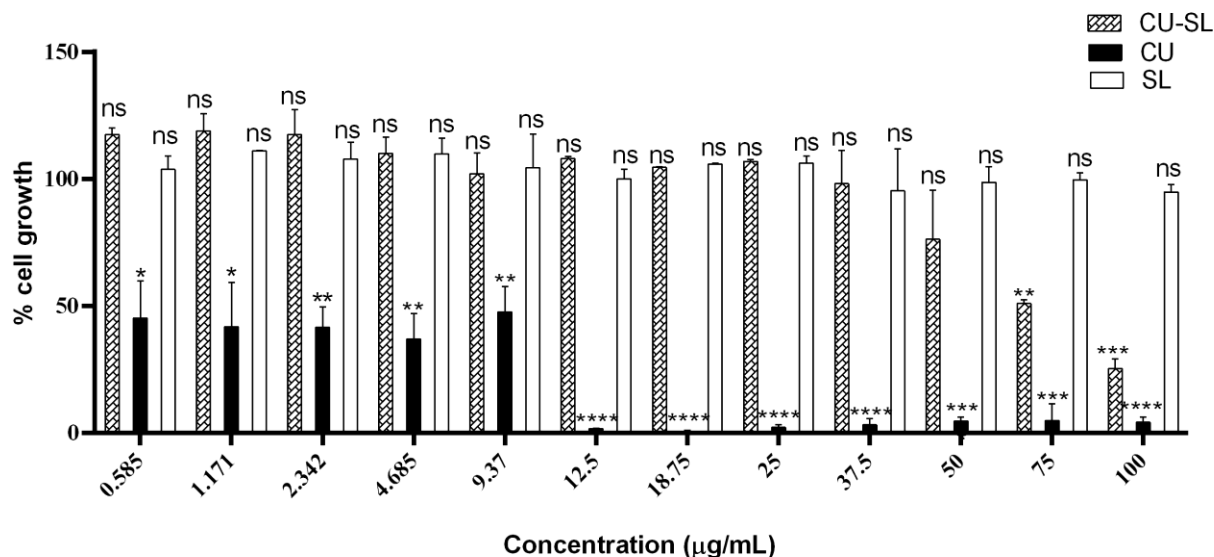


Figure 2. Effect of CU-SL on *C. albicans* growth. Planktonic *C. albicans* was treated with different concentrations of CU, SL and CU-SL. The cell growth was calculated by normalizing the inhibition data to 100%. Results represents the average of three independent experiments \pm SD. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$, **** $p \leq 0.0001$, ns-non-significant compared to the control

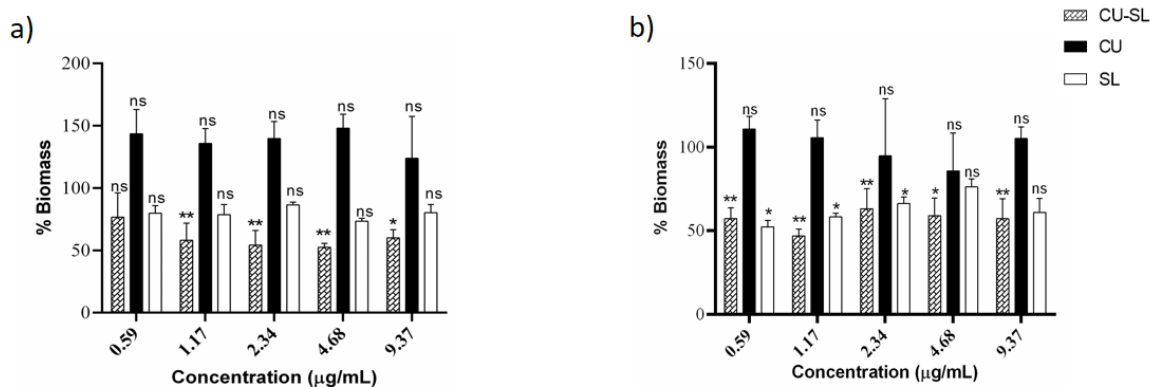


Figure 3. CU-SL is a potent inhibitor of *C. albicans* biofilms in sub-inhibitory concentrations. *C. albicans* biofilms were developed in the presence of different concentrations of CU-SL, CU and SL. (a) Biofilms treated at pre-adhesion phase, (b) Biofilms treated at post-adhesion phase. The biomass was calculated by normalizing inhibition data to 100%. Results represents the average

of three independent experiments \pm SD. * $p \leq 0.05$, ** $p \leq 0.005$, ns-not significant compared to the control.

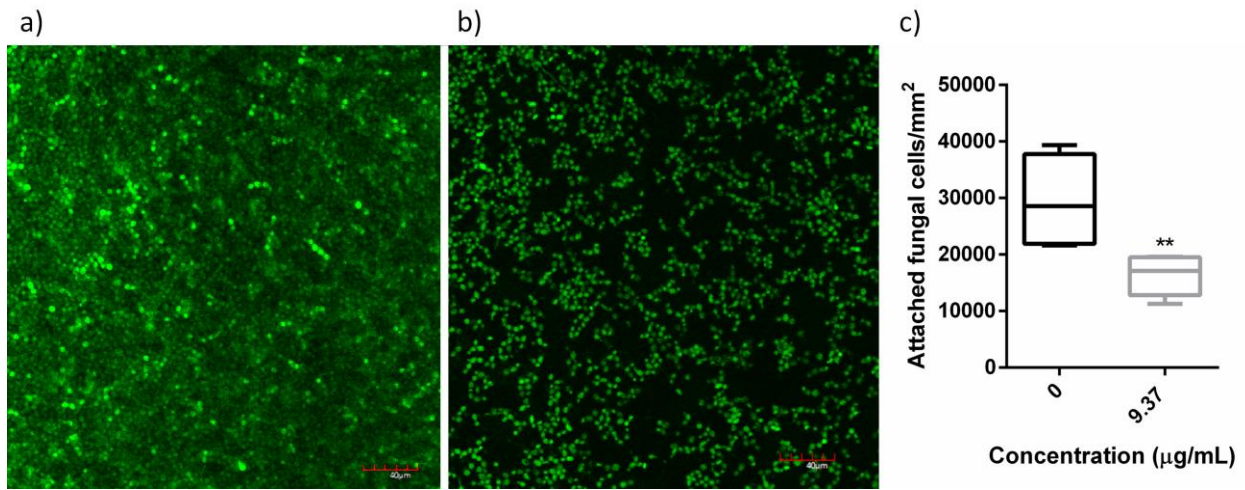


Figure 4: Confocal Laser Scanning Microscopic analysis (40x) of (a) control (untreated) and (b) CU-SL treated biofilms of *C. albicans*, (c) Bar graph representing the number of attached live cells on the biofilm. ** $p \leq 0.005$ compared to the control

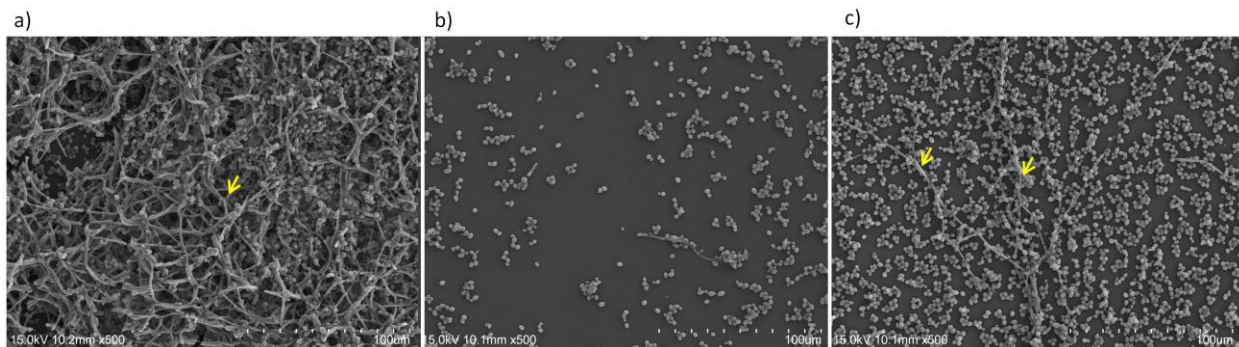


Figure 5: Scanning electron microscopic images (500X) of (a) untreated control, (b) CU-SL and (c) SL treated *C. albicans*. The untreated control shows dense interconnected hyphae (yellow arrows). CU-SL (9.37 μ g/mL) inhibited filamentation, and SL could not inhibit filamentation.

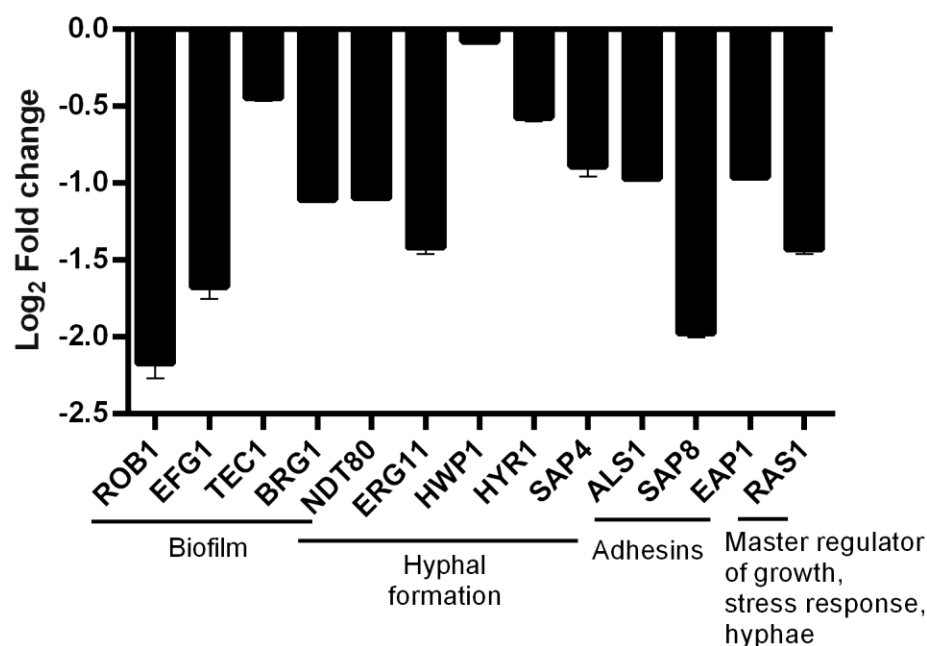
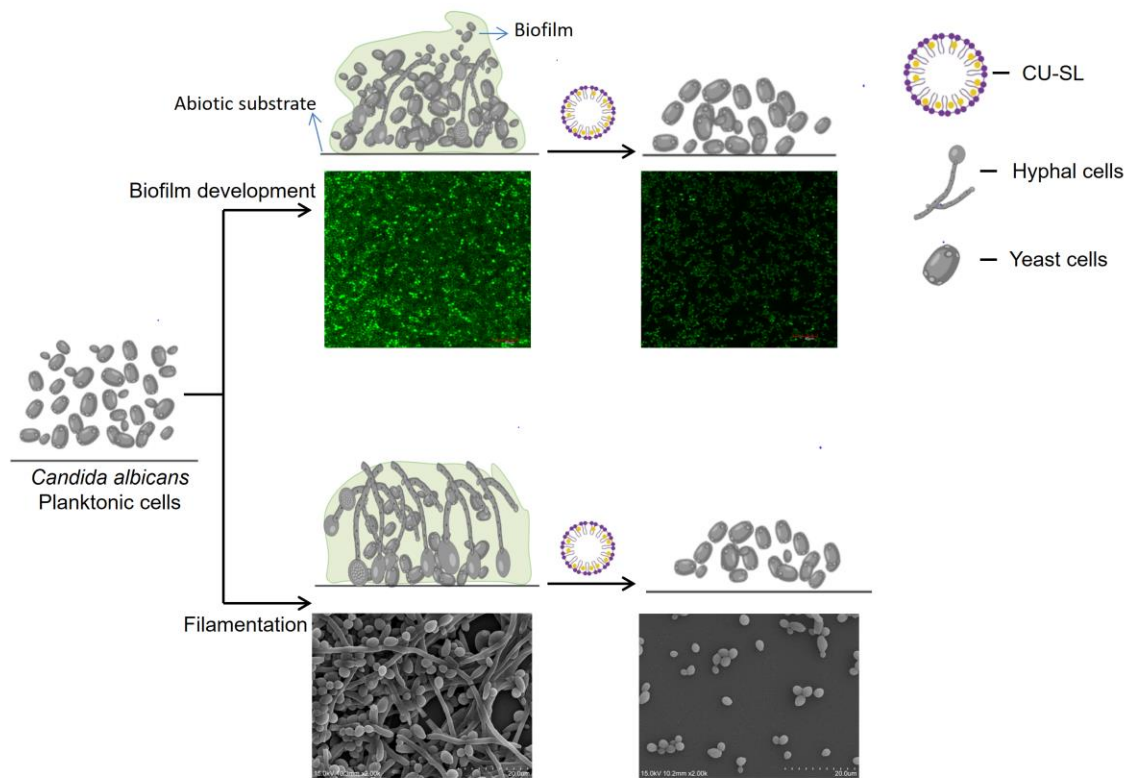


Figure 6: CU-SL downregulates multiple genes that regulate biofilm formation and virulence. Differential gene expression *C. albicans* biofilm following CU-SL treatment. Expression level of each gene is displayed after normalization with 18srRNA as housekeeping gene. The experiment was performed in duplicates. All comparisons were significantly different ($p < 0.05$) compared to the control. Error bars indicate the standard deviation.



Graphical Abstract