The evaluation of supramolecular self-associating amphiphiles as novel anti-biofilm compounds

by

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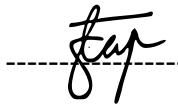

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Professional

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Conflict of interest

- The author declares no conflict of interest in this research project nor any in the writing of this thesis.

Contents

Chapter 1: Motivation and Literature Review

Motivation

The discovery of antibiotics has provided an arsenal of measures to combat infections and diseases (Hutchings et al. 2019). Furthermore, the evolution of antibacterial compounds has produced various antifungal compounds with the ability to treat an array of fungal infections (Espinel-Ingroff 1997). The incorporation of antimicrobial compounds into industrialised sectors has contributed to the global health sector, agricultural practices and developing countries (Goel 2015; Lamichhane et al. 2018; Hutchings et al. 2019). However, the establishment of antimicrobial resistance (AMR) has proved a formidable opponent to currently available and established antimicrobial substances. A review published in The Lancet (2022) reported that 4.95 million deaths were associated with antimicrobial-resistant bacterial infections and 1.27 million deaths were directly attributed to AMR. Interestingly, lower respiratory infections (LRIs) accounted for more than 1.5 million deaths related to AMR. From their review, six pathogens were identified as the leading causative agents responsible for 3.57 million AMR-associated deaths. These six pathogens have been identified as *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Antimicrobial Resistance Collaborators 2022).

Since the discovery of antimicrobials, there has been a rapid decline in antimicrobial production, with newly discovered substances being reserved for last-resort prescription and use (Prescott 2014). This decline has been exacerbated by a lack of financial return. When considering that the purpose of The Global Fund is to invest in programmes that work toward ending infectious diseases that pose a threat to humankind, it is evident that investment is urgently required (The Lancet 2022). Antimicrobial resistance develops through various mechanisms (Mcdermott et al. 2003) and is prevalent in a wide range of settings (Prescott 2014). Settings such as hospitals have been identified as particularly susceptible to contamination by opportunistic pathogens such as *Pseudomonas aeruginosa* and *Candida albicans* (Smith and Hunter 2008; Fattouh et al. 2021). *Pseudomonas aeruginosa* is a ubiquitous bacterium frequently isolated from indwelling medical apparatus, such as catheters (Azevedo et al. 2017). Similarly, *Candida albicans* is a commensal yeast frequently isolated from indwelling devices (Lynch and Robertson 2008). These pathogens display various virulence factors, ranging from metabolic plasticity to biofilm formation (Strateva and Mitov 2011; Mayer et al. 2013).

In addition to the review published by The Lancet, the World Health Organization (WHO) has published a list of critically important pathogens that require immediate research and development of novel antimicrobial substances. To complement the list, a review by Rice (2008) identified six bacterial pathogens of immediate interest which displayed antimicrobial resistance properties to various classes of antibiotics. These six pathogens are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. These pathogens form the coined term ESKAPE pathogens (Rice 2008). Notably, some of the pathogens described in 2008 are mentioned in the review published by The Lancet in 2022. This is indicative of their pathogenic fitness and ability to circumvent antimicrobial action. Additionally, the WHO recently published a list of critically important fungal pathogens that require immediate research. The yeast *Candida albicans* was noted as one of the four pathogens in the critical priority group (2022).

The ability of pathogens such as *Pseudomonas aeruginosa* and *Candida albicans* to produce biofilms not only contributes to their virulence (Botto et al. 1998; Jabra-Rizk et al. 2004) but has also been identified as a noteworthy antimicrobial resistance mechanism contributing to consistent and recurring infections (Jabra-Rizk et al. 2004). Biofilms also display enhanced resistance to various host defence mechanisms, including pH changes and immune responses (Wilkins et al. 2014). This biofilm-related resistance is facilitated via slow substance penetration, differential chemical microenvironments, adaptive stress response pathways and the establishment of tolerant persister cells that allow for further propagation and dispersal (van Acker et al. 2014). The resistance mechanisms displayed by biofilms are exacerbated in the case of a polymicrobial biofilm (Patel 2005; Desai et al. 2014).

The characteristics displayed in mixed-species biofilms are a result of cell-to-cell contact and the production and secretion of metabolites and intermediates (Desai et al. 2014). Metabolites produced in mixed-species biofilms include quorum-sensing molecules (QSM). For example, *P. aeruginosa* produces homoserine lactone (HSL) which inhibits *C. albicans* hyphal formation through the inhibition of the Ras-adenylate cyclase pathway (Davis-Hanna et al. 2008; Hall et al. 2011; Desai et al. 2014). Similarly, *C. albicans* produces a quorum-sensing molecule, farnesol, which inhibits *P. aeruginosa* virulence factors by interacting with *P. aeruginosa* quorum sensing (Cugini et al. 2007; Desai et al. 2014). The interaction and association between these pathogens is highlighted by their characteristic antagonistic and bidirectional relationship as observed in cystic fibrosis patients (Reece et al. 2021). *Pseudomonas aeruginosa* inhibits *C. albicans* growth, hyphal development, as well as biofilm formation. *Candida albicans* inhibits *P. aeruginosa* virulence-metabolites and proteins such as pyocyanin and haemolysin (Brand et al. 2008; Reece et al. 2021).

The interactions between these pathogens further illustrate the importance of treating infections in a polymicrobial manner and considering novel antimicrobial therapies. Approaches to novel antimicrobial therapies include redesigning antimicrobial compounds and revising currently available substances and established mechanisms of action to address a polymicrobial infection (Dharmaprakash et al. 2015). Other avenues of interest include searching for new target genes and considering rational drug design to develop novel antimicrobial compounds (Nicola et al. 2019).

The use of amphiphilic macromolecules provides an opportunity to develop novel excipients with the potential for classes of antimicrobial compounds (Lombardo et al. 2015). Amphiphiles consist of a hydrophilic and lipophilic component enabling self-assembly into a wide variety of structures that can mimic biological systems. Hiscock et al. (2016a) have developed a novel tetrabutylammonium (TBA) sulfonate-urea salt capable of producing hydrogen-bonded nanostructures. This class of novel compounds, deemed supramolecular self-associating amphiphiles (Figure 1), has shown antimicrobial efficacy against planktonic cells of clinically relevant strains of antimicrobial-resistant pathogens, such as the Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative *Escherichia coli* (Tyuleva et al. 2019; White et al. 2020b).

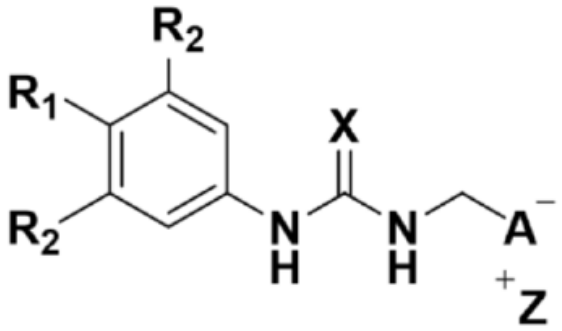

Figure 1. Supramolecular self-associating amphiphile (SSA) backbone structure. The structure of the relevant countercation has been omitted for clarity (Blackholly et al. 2016; Hiscock et al. 2016a)

It is thus important to consider and evaluate the efficacy of this potential class of compounds against other relevant pathogens, such as *P. aeruginosa* and *C. albicans*, as this could give invaluable insight into the development of novel antimicrobial compounds. In addition, given the enhanced drug resistance of microbial biofilms, the ability of supramolecular self-associating amphiphiles to affect the biofilms of these pathogens is also important to consider.

Introduction

The ability of naturally occurring substances to inhibit bacterial growth has proven to be one of mankind's cornerstone discoveries (Fleming 1929; Whitehead 1933). The potential of these substances was highlighted when Alexander Fleming noticed the inhibition and lysis of *Staphylococcus* colonies when cultured in the presence of ascomycetous fungus of the genus *Penicillium* (Fleming 1929). The ability of *Penicillium* to inhibit bacterial growth was attributed to the production of a substance termed penicillin (Fleming 1929). A characteristic property of antibacterial substances is their selective action; some substances are effective against either Gram-negative or Gram-positive bacteria and in some instances, both (Waksman 1944).

When considering that these substances differ in chemical composition, it is evident that the selective action of antibacterial substances directly relates to their mechanism of action. Additionally, the qualitative characteristic of varying concentrations directly influences the substance's efficacy. It is established that higher concentrations of antibacterial substances result in exaggerated inhibition (Waksman 1944). From these findings, the use and application of antibiotic substances have enabled the treatment of various bacterial infections and revolutionised the healthcare industry (Espinel-Ingroff 2003; Tomson and Vlad 2014).

The discovery and application of antibacterial substances promoted the search for antifungal substances (Espinel-Ingroff 1997). The efforts of Elizabeth Hazen and Rachel Brown lead to the discovery of an antifungal substance produced by soil actinomycetes. The substance termed fungicidin or nystatin indicated fungistatic and fungicidal activity (Hazen and Brown 1951). From these findings, other cultures were screened for antifungal substances and inhibitory potential. In 1953, Steinberg and co-workers identified and isolated the antifungal substances amphotericin A and B from a Streptomycete culture (Steinberg et al. 1953, Dutcher 1968). A report by Milton Sloane supported the

efficacy of antifungal substances when used in the treatment of candidiasis; proving the use of nystatin to be effective (Sloane 1955).

The use of these substances greatly contribute to the management of fungal infections arising from antibacterial treatment (Hazen and Brown 1951; Kull et al. 1961; Scardavi 1966; Hartshorn 1969). Since the discovery and use of antimicrobial substances, organisms have adapted to their inhibitory effects (Kasuya 1964). Antimicrobial resistance may be innate, acquired, or induced by adaptive mutations (Gopal Rao 1998). Innate antimicrobial resistance mechanisms are based on the biochemical composition of organisms. The most common innate resistance mechanisms include the reduced permeability of the outer membrane and efflux pumps that actively remove antimicrobial substances from the intercellular space (Rouveix 2007; Reygaert 2018). Innate resistance can also be described as the inability of antimicrobial substances to bind to host cells; either through lack of enzymatic function or absence of the appropriate binding site (Hancock and Speert 2000; Mcdermott et al. 2003; Hollenbeck and Rice 2012).

In addition to intrinsic resistance mechanisms, microbes can acquire resistance through genetic material (Marsit et al. 2015; Alexander et al. 2016) by horizontal evolution (Tenover 2006; Fitzpatrick 2012; Reygaert 2018). The exchange of genetic material occurs through conjugation, transduction and transformation (Murray et al. 2003; Tenover 2006). In addition, genetic material can be adopted through the use of resistance plasmids, transposons and integrons (Kasuya 1964; van der Bliek and Borst 1989; Kruse and Sorum 1994; Mølbak 2004). Adaptive resistance mechanisms are implemented either transiently or permanently by microbes in response to extracellular environmental signals and conditions (Arzanlou et al. 2017). Transient adaptive responses include the overexpression of efflux pumps, and porin channel- and biofilm formation (Patel 2005; Christaki et al. 2020). In comparison, permanent adaptive resistance is attributed to genetic expression modulations and these modulations facilitate hypermutability (Hamad et al. 2022).

In addition to extracellular environmental signals and conditions, prolonged exposure to antimicrobial substances also exerts selective pressure and leads to the survival of resistant organisms. Furthermore, selective pressure may also confer cross-resistance to a range of other antimicrobial compounds (van der Bliek and Borst 1989; Pastan and Gottesman 1991; Gopal Rao 1998). Therefore, the establishment of resistant organisms has significantly impacted the success of treating infections and diseases (Cars and Nordberg 2005; Okeke et al. 2005).

Antimicrobial substances

Antimicrobial substances are classified based on their chemical composition and mechanisms of action (Hartshorn 1969). A mechanism of action utilised by antibiotic substances includes the inhibition of protein synthesis (Hashmi 2020). Aminoglycosides, such as streptomycin (Figure 2), contain an amino-sugar substructure which facilitates high affinity binding to the A-site on the 16S ribosomal RNA of the 30S ribosome (Kotra et al. 2000). The binding interaction induces a conformation change in the A-site and results in codon mistranslation. This results in incorrect polypeptide synthesis, ultimately damaging the cell membrane (Krause et al. 2016).

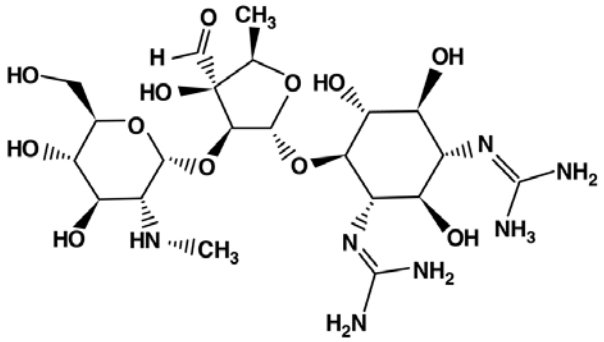

Figure 2. Structure of streptomycin

Another mechanism of action utilised by antibiotic substances includes the inhibition of cell wall synthesis. β-lactam antibiotics, such as penicillin (Figure 3), contain a core β-lactam ring which binds to penicillin-binding proteins. The binding prevents the transpeptidation of peptidoglycan strands (Hashmi 2020). The inefficient transpeptidation of peptidoglycan strands results in incomplete cell wall synthesis and, therefore, constitutes the bactericidal activity of β-lactams (Zhanel et al. 2005).

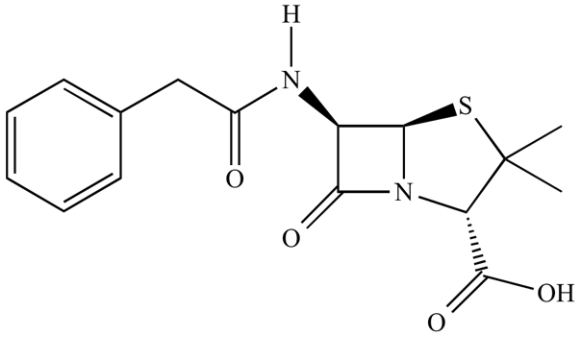

Figure 3. Structure of penicillin

Quinolones, such as ciprofloxacin (Figure 4), can inhibit bacterial DNA synthesis (Jia and Zhao 2021) by targeting the bacterial topoisomerase II, IV and gyrase enzymes (Aldred et al. 2014). The mechanism of action is based on the binding ability of the compound to the DNA-enzyme complex, which prevents successful DNA ligation. As a result, permanent chromosomal breaks are induced and stress response mechanisms, such as DNA repair and SOS signals, are triggered. The inability of the organism to repair these chromosomal breaks and the cumulative effect of the enzymatic-activity loss facilitates cell death (Aldred et al. 2014; Hashmi 2020). Similar to DNA synthesis inhibition, the ability of antibacterial substances to hinder RNA synthesis is an important antibacterial mechanism (McClure and Cech 1978).

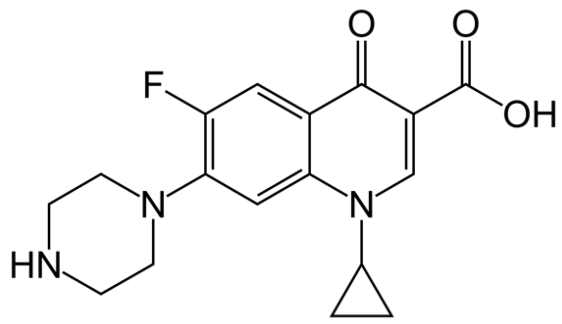

Figure 4. Structure of ciprofloxacin

The rifamycin class contains the antibacterial substance rifampicin (Figure 5) (Goldstein 2014). The mechanism of action of this class is based on the high binding affinity of the compound to the DNA-dependent RNA polymerase enzyme (Campbell et al. 2001). Rifampicin causes steric hindrance by binding to the β-subunit and effectively inhibits elongation and transcription, which ultimately disrupts and prevents bacterial RNA synthesis (McClure and Cech 1978; Campbell et al. 2001).

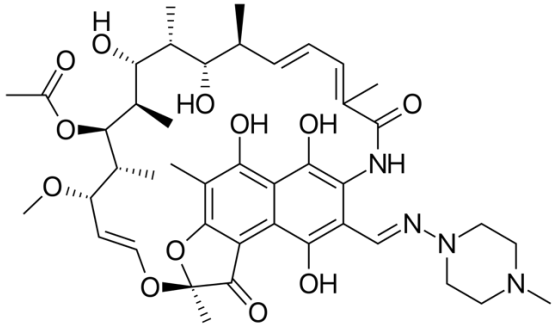

Figure 5. Structure of rifampicin

Similar to antibacterial substances, antifungals are also classified based on their mechanism of action. Two established antifungal classes have a mechanism of action directed toward the fungal cell membrane (Ghannoum and Rice 1999). The first of these classes is the azole class, which contains drugs such as fluconazole (Figure 6) and ketoconazole (Shafiei et al. 2020). The general structure of azoles is a five-membered aromatic ring with two heteroatoms, of which one is always nitrogen (Shafiei et al. 2020). Azoles affect the fungal cell membrane by inhibiting the fungal cytochrome P450 14α-demethylase; an enzyme responsible for the demethylation of methyl sterol intermediates, to produce ergosterol (Ghannoum and Rice 1999; Chapman et al. 2008).

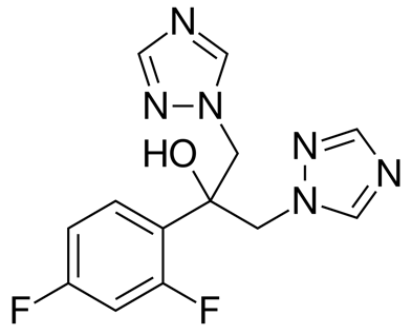

Figure 6. Structure of fluconazole

The second antifungal class, the polyenes, which also targets the fungal cell membrane (Ghannoum and Rice 1999), consists of a hydrophilic head and hydrophobic tail. The characteristic polyene hydrophilic head contains a mycosamine group and polyol chain that accommodates various hydroxyl groups (Carolus et al. 2020). Polyenes can selectively bind to the fungal membrane (Chapman et al. 2008). The selective interaction has several methods of action, however, the best studied is the ability of polyenes to bind to ergosterol, resulting in pore formation and generation of reactive oxygen species (ROS) (Ruiz-Baca et al. 2021). An example of a drug from this class is the well-known amphotericin B (Figure 7) (Carolus et al. 2020; Ruiz-Baca et al. 2021).

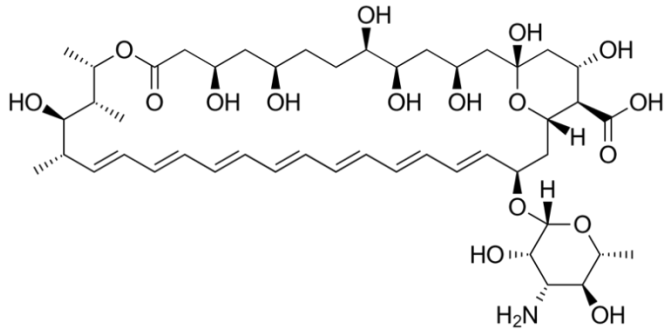

Figure 7. Structure of amphotericin B

A novel antifungal class that targets a fungal component not found in the host, is the echinocandins, which target fungal cell wall biosynthesis. Caspofungin (Figure 8) is an example of this class of antifungal (Houšť et al. 2020). Echinocandins are natural or synthetic amphiphilic cyclic lipopeptides that contain an *N*-linked acyl side-chain, which is required to anchor the compound to the membrane (Denning 2003; Szymański et al. 2022). Echinocandins affect the fungal cell wall by acting as inhibitors to the synthesis of α-1,3-β-glucan, a polysaccharide and important component of the fungal cell wall (Nivoix et al. 2020).

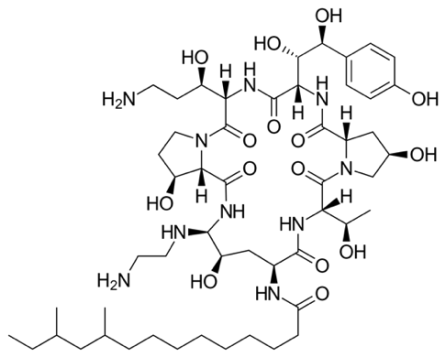

Figure 8. Structure of caspofungin

Antifungal pyrimidine analogues have a characteristic pyrimidine-related structural backbone (Ghannoum and Rice 1999; Sanglard 2016). This class of synthetic antifungals contain the substance flucytosine (5-FC) (Figure 9) (Viviani 1995). Members of this class do not indicate any intrinsic antifungal capabilities. When the antifungal is assimilated by susceptible cells (by cytosine permease) and converted into 5-fluorouracil (by cytosine deaminase) the substance can affect fungal RNA and DNA synthesis (Ghannoum and Rice 1999).

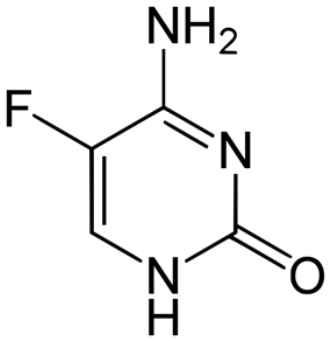

Figure 9. Structure of flucytosine

There are two distinct mechanisms of action of flucytosine. The first mechanism is the conversion of 5-fluorouracil (5-FU) from 5-fluorouridine monophosphate (FUMP) and 5-fluorouridine diphosphate (FUDP) into 5-fluorouridine triphosphate (FUTP) by the cytosine deaminase (Vermes et al. 2000). The incorporation of FUTP into fungal RNA replaces uridylic acid. This substitution alters the relevant amino acid pool and subsequent aminoacylation of tRNA, thereby affecting RNA synthesis and inhibiting protein synthesis (Waldorf and Polak 1983; Vermes et al. 2000; Loyse et al. 2013).

The second mechanism is the conversion of 5-fluorouracil to 5-fluorodeoxyuridine monophosphate (FdUMP) by the uridine monophosphate pyrophosphorylase (Vermes et al. 2000). FdUMP acts as an inhibitor of thymidylate synthetase (a crucial source of the nucleoside thymidine) which results in the inhibition of DNA synthesis (Vermes et al. 2000) (Figure 10). Although the substance cannot effectively inhibit fungal pathogens, when used in combination with other classes of antifungals, increased inhibitory potential is observed (Sanglard 2016).

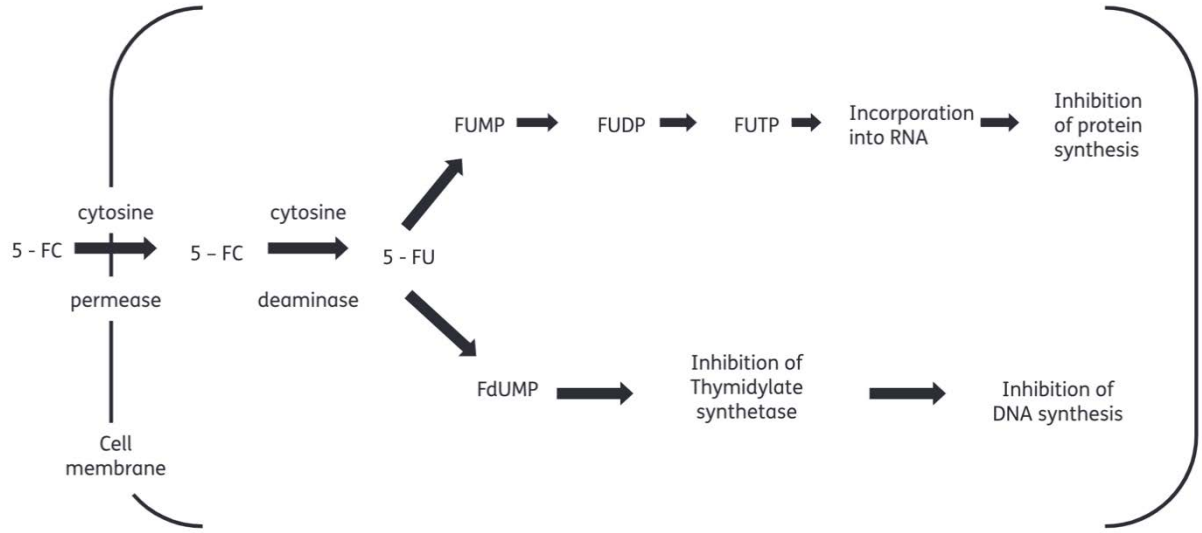

Figure 10. The intracellular pathways and mechanisms of action of flucytosine. Flucytosine is assimilated into the fungal cell and converted into 5-fluorouracil. 5-fluorouracil is converted into 5-fluorouridine triphosphate and alters the aminoacylation of tRNA. This results in the inhibition of protein synthesis. Alternatively, 5-fluorouracil is converted into 5-fluorodeoxyuridine monophosphate. The 5-fluorodeoxyuridine monophosphate is an inhibitor of thymidylate synthetase which prevents DNA synthesis (Waldorf and Polak 1983; Loyse et al. 2013)

Antimicrobial resistance and *Pseudomonas aeruginosa*

From these findings, it is logical to consider the application of antibacterial and antifungal substances in other sectors. As the use of antimicrobial substances became more apparent and the efficacy thereof improved, these substances have been introduced to different industrialised sectors such as agricultural and veterinary practices (Gopal Rao 1998). As a result, antimicrobials are used to prevent, control and treat various diseases in crops and livestock (Anderson et al. 2003).

Mass-medication events are used instead of individual treatment procedures or natural preventative protocols, to ensure the feasibility of the antimicrobial application. However, most uses are nontherapeutic promoters of enhanced growth and improved feed efficiency as producers administer antimicrobials to promote commodity production. The antimicrobials used in these practices are identical or similar to those administered to humans and when considering the resistant properties of organisms, it is obvious that nontherapeutic uses are controversial and irresponsible (Anderson et al. 2003). The extensive application and misuse of antimicrobial substances in different industrialised sectors have established antimicrobial-resistant organisms in the food chain (Vidaver 2002; Anderson et al. 2003).

Moreover, in clinical settings, such as hospitals where patients are treated for severe diseases, the selective pressure exerted on pathogenic organisms is intensified (Wenzel et al. 1976). The resultant cross-resistance in pathogenic organisms has facilitated the establishment of organisms resistant to more than one class of antimicrobial compound (van der Bliek and Borst 1989). The prevalence of multi-drug resistant pathogens has significantly contributed to global mortalities, decreased therapeutic success, prolonged illness and hospitalisation, and related medical costs (Tanwar et al. 2014).

A group of bacteria coined the ESKAPE pathogens are of immediate concern as they are responsible for the largest percentage of nosocomial infections. Furthermore, the ESKAPE pathogens represent

the most noticeable and extensive multi-drug resistant characteristics (Alekshun and Levy 2007; Rice 2008; Boucher et al. 2009; Rice 2010). The ability of ESKAPE pathogens to readily colonise immunocompromised individuals is of clinical importance, and this is presented in the example of *Pseudomonas aeruginosa* (Bodro et al. 2013; Pendleton et al. 2013). *Pseudomonas aeruginosa* is a Gram-negative bacterium associated with nosocomial infections, bacteraemia, and diseases such as cystic fibrosis (Hancock 1998; Hancock and Speert 2000). *Pseudomonas aeruginosa* virulence factors include surface structures, secreted substances and cell-to-cell interactions (Liao et al. 2022). Surface structures contributing to *P. aeruginosa* pathogenicity include pili, flagella and outer membrane components and are used for bacterial adhesion, motility and biofilm formation (de Kievit et al. 2001; Liao et al. 2022). Secreted substances such as exopolysaccharides, siderophores and toxins further contribute to the organisms' pathogenicity. Exopolysaccharides are used in biofilm formation, whereas siderophores and toxins act as chelating agents and virulence factors, respectively.

Cell-to-cell interactions include biofilms which further promote and contribute to the efficacy of the pathogen (Soong et al. 2008; Hall et al. 2016; Rocha et al. 2019). To complement the virulence factors mentioned, *P. aeruginosa* displays various forms of intrinsic, acquired and genetic resistance to antimicrobial substances (Pai et al. 2001). Similar to the other ESKAPE pathogens, *P. aeruginosa* utilizes structural modifications, the upregulation of efflux pumps, and biofilm formation to circumvent antimicrobial action (Moore and Flaws 2011). Biofilms produced by *P. aeruginosa* facilitates the capacity to interact and coexist with other organisms in complex communities, which is normally detrimental to the host (Cendra and Torrents 2021).

Antifungal resistance and *Candida albicans*

In addition to the ESKAPE pathogens, other organisms, such as the yeast *Candida albicans,* are able to successfully colonise immunocompromised individuals (Uppuluri et al. 2009b; Kim and Sudbery 2011; Shor and Perlin 2015). In a recent report published by the World Health Organisation (WHO, 2022), *C. albicans* has been identified as one of the top four fungal pathogens in the critical priority grouping. Infections caused by *C. albicans* range from superficial conditions of thrush (McCullough et al. 1996) to systemic and life-threatening conditions of fungemia (Rex et al. 1995). Okoye et al. (2022) reported that *C. albicans* was the most frequently isolated yeast contributing 32.6% to candidemia in Africa.

Various virulence factors contribute to the success of *C. albicans* in colonising different environments. *Candida albicans* is a polymorphic yeast, meaning the yeast can switch between various morphological forms in response to environmental conditions, that also display different phenotypes (Timpel et al. 2000; Lan et al. 2002; Yang 2003; Mayer et al. 2013; Soll 2014). The ability of *C. albicans* to transition between yeast and hyphal forms greatly contributes to its' virulence. The morphological transitions of *C. albicans* are induced by host environmental signals. These signals are interpreted through signalling cascades such as the cAMP-dependent protein kinase A pathway, the *CEK1* mitogen-activated protein kinase pathway, and the *RIM101*-dependent pathway (Csank et al. 1998; Klengel et al. 2005; Hall et al. 2011).

Other genomic virulence factors that contribute to the pathogenesis of *C. albicans* include alterations to the fungal cell membrane. One such example is the GH72 family of enzymes involved in the formation of the fungal cell membrane. The GH72 enzymes contribute to the formation of β-1,3-glucan, a major constituent of the fungal cell membrane. The *PHR* gene family is responsible for encoding the GH72 enzymes (Calderon et al. 2010; Popolo et al. 2017) and *C. albicans* utilizes the expression of *PHR1* to facilitate the adaption to alkaline environments by modifying cell morphology (Saporito-Irwin et al. 1995; Yang 2003). Similarly, the expression of *PHR2* facilitates adaptation to acidic environments through morphogenesis (Mühlschlegel and Fonzi 1997; Yang 2003). To complement membrane adaptability, *C. albicans* mutates drug target-site genes to reduce the antimicrobial binding affinity. In addition, *C. albicans* overexpresses the gene responsible for encoding 14-α-demethylase, *ERG11* (Song et al. 2004). The expression of this gene contributes towards ergosterol synthesis and overexpression results in increased drug target sites, which requires a higher azole concentration to facilitate inhibition (Ruiz-Baca et al. 2021).

When considering the genomic plasticity of *C. albicans* it is no surprise that antimicrobial resistance has been established in this opportunistic pathogen (Hawser and Douglas 1995). *Candida albicans* also displays acquired antimicrobial resistance through the overexpression of membrane proteins (Odds 2004; Bhattacharya et al. 2020). Membrane proteins facilitate processes such as regulating drug- efflux, modification and detoxification (Bhattacharya et al. 2020). There are two main efflux protein families, namely, adenosine triphosphate binding cassette (ABC) and major facilitator superfamily (MFS) transporters (Marger and Saier 1993; Cannon et al. 2009). *Candida* drug resistance 1 and 2 (*CDR1* and *CDR2*) and *MDR1* genes encode for the respective transporter proteins (Jha and Kumar 2019). The upregulation of these three genes significantly contribute to *C. albicans* pathogenesis (Revie et al. 2018).

Further acquired modifications include ergosterol biosynthetic pathways. The *ERG3* gene encodes for a Δ-5,6-desaturase and confers azole and polyene resistance by facilitating the accumulation of alternative sterols to compensate for the depletion of ergosterol (Revie et al. 2018). Additionally, *C. albicans* is known to produce biofilms which act as both a virulence factor and mechanism of resistance to antimicrobial substances (Baillie and Douglas 1999). The complex heterogenous structure produced by *C. albicans* acts as a virulence factor by establishing a niche environment for fungal propagation (Jabra-Rizk et al. 2004). When considering the nature of a biofilm, it is evident that resistance to antimicrobial substances is facilitated through slow penetration and permeation events (Ramage et al. 2005).

Microbial biofilms

Pathogenic planktonic cells differ from biofilm cells in various ways (Bester et al. 2005; Brown et al. 2016). Planktonic cells are exposed to diverse environmental conditions and rely on intrinsic resistance mechanisms to successfully propagate and colonise an environment (Hawser and Douglas 1995). A resistance mechanism of interest is the ability of pathogens to establish and maintain biofilms (la Tourette Prosser et al. 1987). A biofilm is an interactive microcolony of sessile cells encapsulated in a polymeric substance on biotic or abiotic surfaces (Hoyle and Costerton 1991). Biofilm formation can

be summarised as a four-step process (Figure 11). Briefly, planktonic cells actively adhere to a surface through motility, chemotaxis or passive diffusion (Hoyle and Costerton 1991). Thereafter, cells reproduce and are dispersed into the surrounding environment. The dispersal of cells allows for further colonisation which results in subsequent and chronic secondary infections (Hoyle and Costerton 1991; Stewart and Costerton 2001).

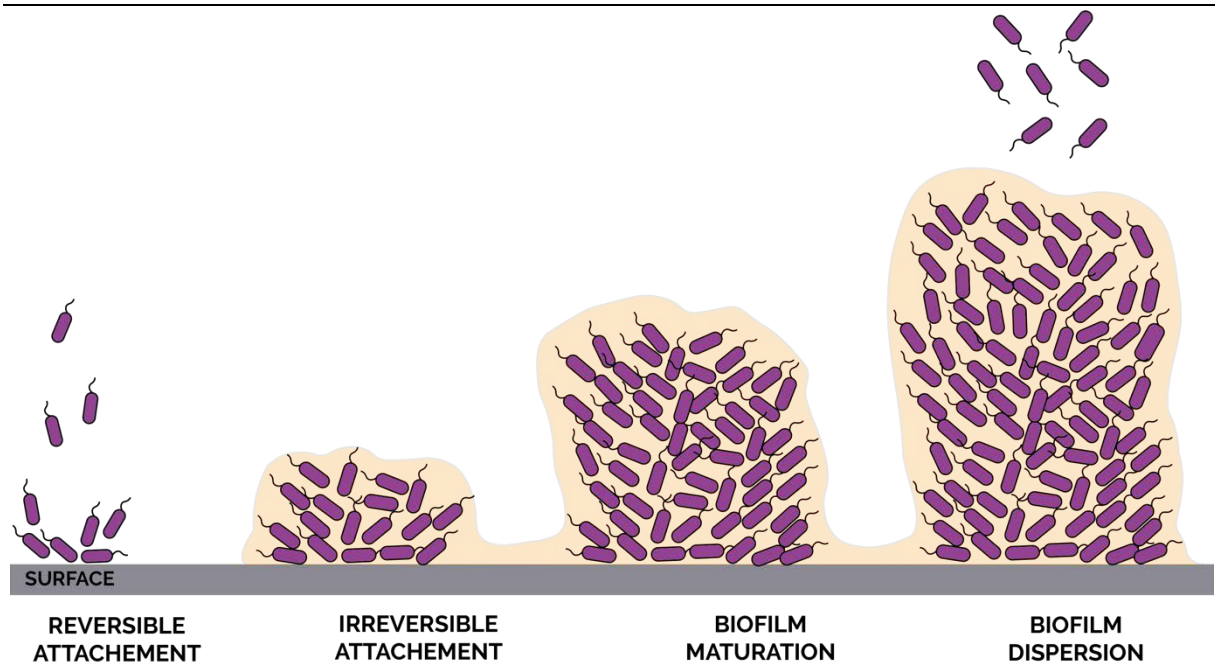

Figure 11. Illustration of the process of biofilm formation. The first step in biofilm formation is the adhesion of motile planktonic cells to a surface of interest. The cells adapt to regulate the production of adhesion proteins and cell-surface structures to ensure irreversible attachment. Once successfully attached, cells produce an exopolymeric-rich extracellular matrix. The cellular community matures into a complex and population-dense structure. Finally, motile cells are released from the tertiary structure in response to inducing conditions and allowed to colonise distal sites (Maunders and Welch 2017)

Sessile cells produce an extracellular biofilm matrix (EBM) which, depending on the environment and organism (Jain et al. 2007; de Cremer et al. 2015), consists mainly of polysaccharides, proteins, and extracellular DNA (eDNA) (la Tourette Prosser et al. 1987). eDNA is produced by the microbe (or acquired from the host) and acts as a scaffold-support component that facilitates and maintains cellular attachment and intercellular interactions (Goodman and Bakaletz 2022). Furthermore, eDNA is used to store important atoms such as carbon and fixed nitrogen (Guo et al. 2021). Polysaccharides contribute to the structural integrity of biofilms by providing protection against unfavourable environmental conditions and facilitating antimicrobial resistance. Furthermore, polysaccharides also play an important role in cell-to-cell binding (Karygianni et al. 2020). Similar to polysaccharides, proteins in the EBM contribute to adhesion, mechanosensing and host immunity evasion (Karygianni et al. 2020; Tan et al. 2020). The microenvironments established within the EBM result in gradients of nutrients, oxygen, secreted factors and even waste products (Xu et al. 1998). This may benefit biofilm cells by concentrating nutrients and shielding cells from antimicrobial substances (Costerton et al. 1981; la Tourette Prosser et al. 1987; Goodman and Bakaletz 2022). Therefore, when considering the extensive

and impressive characteristics of biofilms, it is evident that the EBM is a key component to its success (Davies et al. 1998).

When comparing the phenotypes of planktonic and biofilm cells, it is evident that differential profiles are observed concerning protein expression and growth rates (Aaron et al. 2002; Bester et al. 2005). A study by Seyer and co-workers evaluated the outer membrane protein (OMP) patterns of planktonic and sessile *P. aeruginosa* cells and noted variable OMP patterns in adherent cells (Seyer et al. 2005). Furthermore, Mikkelsen and co-workers showed that the protein profiles of exponentially growing planktonic cells closely resembled those of biofilm cells (Mikkelsen et al. 2007). When referring to the growth kinetics of planktonic and sessile cells, it is established that there is an apparent difference between the two types of cells. Planktonic cells display a higher growth rate compared to sessile cells, whereas sessile cells display a delayed lag phase (Rollet et al. 2009). The up-regulation of genes involved in various cellular processes such as metabolism, signalling and adherence also exhibited apparent differences between the cell types (Planchon et al. 2009; Booth et al. 2011).

Biofilm-production by *Pseudomonas aeruginosa*

The ESKAPE pathogen, *Pseudomonas aeruginosa,* is frequently isolated from hospitalised patients diagnosed with recalcitrant infections (Sherrard et al. 2014). The nutritional versatility of *P. aeruginosa* allows this opportunistic pathogen to comfortably colonise clinical environments (Frimmersdorf et al. 2010; Perez et al. 2011). *Pseudomonas aeruginosa* displays various virulence factors, controlled by multiple mechanisms, which contribute to its pathogenicity (Hancock and Speert 2000). The virulence factor relevant to this study is its formidable ability to form biofilms (Figure 12) (Jesaitis et al. 2003; Arai 2011).

Pseudomonas aeruginosa biofilms adhere to the generalised process of formation. Planktonic *P. aeruginosa* cells propel to an appropriate surface using flagella, followed by irreversible attachment (Ozer et al. 2021). Once attachment is established, *P. aeruginosa* secretes exopolymeric substances to function as a structural scaffold and to promote colony formation. The formation of mature colonies results in planktonic dispersal for further propagation (Ma et al. 2009). It is noteworthy to mention that several reports speculated the importance of flagella in establishing and supporting biofilm formation (Barken et al. 2008; Belas 2014). Ozer and co-workers indicated that flagella are present throughout the entire life cycle of a biofilm. Moreover, those flagellated cells are continuously dispersed from a biofilm; supporting the notion that dispersal facilitates secondary and recalcitrant infections. Interestingly, it was proven that only the middle and lower regions of biofilm cells continuously synthesized flagella.

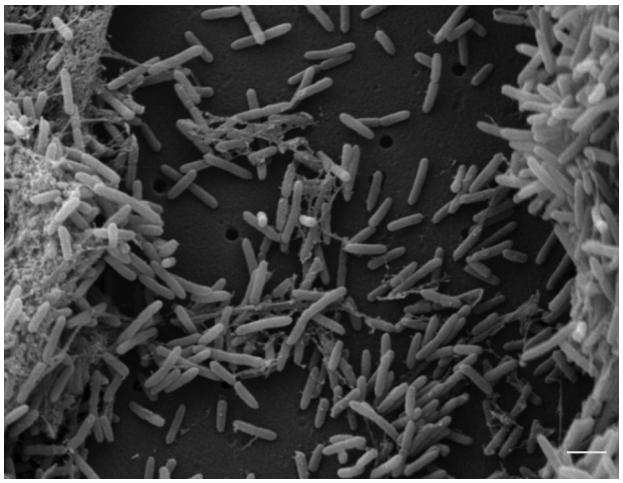

Figure 12. Scanning electron micrograph of *Pseudomonas aeruginosa* PAO1 biofilm. The scale bar represents 5 µm (Woodworth et al. 2008)

The process of biofilm formation in *P. aeruginosa* is under the regulation of cell-density-dependent autoinducers (Ais). *P. aeruginosa* uses quorum sensing in dense populations to regulate gene expression and colony behaviour (Qin et al. 2022). There are two types of systems used by *P. aeruginosa,* namely, *N*-acyl homoserine lactone (AHL) signal molecules (Figure 13) and 4-quinolones (4Qs) (Figure 14) (Bjarnsholt and Givskov 2007). As the microbial population increases, Ais accumulate until an intracellular threshold concentration is reached, resulting in the activation of transcriptional regulators (Smith and Iglewski 2003). There are two interrelated AHL-dependent systems used by *P. aeruginosa*, the *las* and *rhl* systems (Smith and Iglewski 2003). The *las* system consists of a transcriptional activator (LasR) and AI synthase (LasI) directing the synthesis of the *N*-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL) signalling molecule. Similarly, the *rhl* system consists of a transcriptional factor (Rh1R) and AI synthase (Rh1L) directing the synthesis of the *N*-butyryl homoserine lactone (C_4-HSL) signalling molecule (Kievit et al. 2002). These signalling systems are used to regulate various functions such as bioluminescence, the production of virulence factors, the production of exopolysaccharides and biofilm development (Pearson et al. 1997; Boyer and Wisniewski-Dyé 2009).

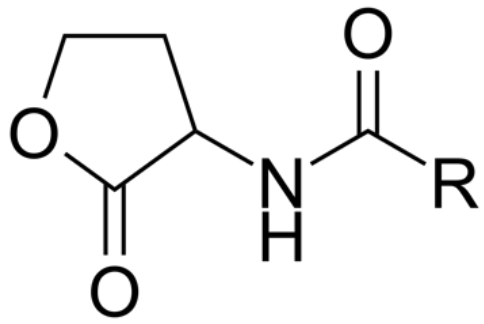

Figure 13. Structure of *N*-acyl homoserine lactone

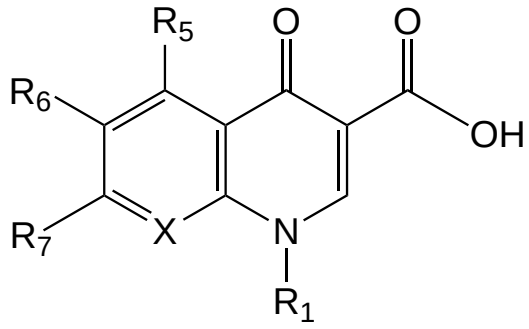

Figure 14. Structure of 4-quinolone

The EBM of *P. aeruginosa* consists mainly of three different exopolysaccharides; Pel, Psl and alginate (Ma et al. 2009). The Pel (encoded by the *pel* locus) and Psl (encoded by the polysaccharide synthesis locus) exopolysaccharides are composed of various substrates and include galactose, glucose, mannose, rhamnose and trace amounts of xylose (Colvin et al. 2012; Jennings et al. 2015). Both Pel and Psl contribute to bacterial adhesion and maintenance of the biofilm tertiary structure, however, the extent of synthesis and function of each is strain-specific. For example, *P. aeruginosa* PAO1 relies primarily on Psl for cell adhesion and biofilm structure maintenance (Colvin et al. 2012). Alginate is a capsular polysaccharide that contributes to biofilm development and architecture (Stapper et al. 2004). Although alginate is present in the EBM, studies have shown that it does not specifically contribute to antimicrobial resistance (Mathee et al. 1999; Wozniak et al. 2003; Matsukawa and Greenberg 2004).

eDNA released by *P. aeruginosa* has various roles such as providing nutrients to surrounding cells, disseminating genes to competent cells, and contributing to biofilm matrix architecture (Jakubovics et al. 2013). The characteristic high molecular weight and viscous properties of DNA provide protection and support to biofilm cells, and can further promote adhesion to hydrophobic substrates (Jakubovics et al. 2013; Devaraj et al. 2019; Goodman and Bakaletz 2022). The mentioned constituents found in *P. aeruginosa* biofilms contribute to the success of persistent and chronic infections arising from colonisation (Hancock 1998; Mah et al. 2003; Ma et al. 2009).

Biofilm-production by *Candida albicans*

It has been established that similar to bacteria, fungal pathogens are known to establish daunting biofilms (Douglas 2003). The ability of fungal pathogens to infect and colonise clinically-related environments such as hospitals, and more importantly, indwelling medical devices are a great concern to the global public health sector (Karygianni et al. 2020). *Candida albicans* has proven to utilise biofilm formation (Figure 15) as a formidable virulence factor and mechanism of antimicrobial resistance (Hawser and Douglas 1995). Similar to other pathogens, *C. albicans*' biofilm formation progresses through various stages, with adhesion and filamentation playing essential roles in the success of the biofilm (Uppuluri et al. 2010). *Candida albicans* follows the generalised scheme of biofilm formation; cells adhere to a suitable surface, followed by cell proliferation, hyphal development, and the production of an exopolymeric substance to facilitate the completion of the tertiary complex structure (Chandra et al. 2001).

Notably, *C. albicans* utilizes various cellular forms in a biofilm, including oval yeast cells, pseudohyphae, and tubular hyphal forms (Desai and Mitchell 2015; Pierce et al. 2017). As with any other biofilm life cycle, dispersal is essential to further propagation. For *C. albicans*, the hyphal layer in the biofilm produces yeast cells which are continuously dispersed to colonise distal sites and restart the entire process (Uppuluri et al. 2009).

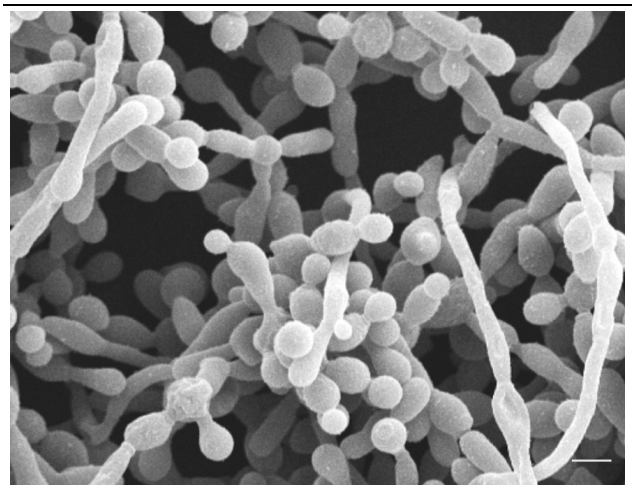

Figure 15. Scanning electron micrograph of *Candida albicans* biofilm (Lopez-Ribot 2005). The scale bar represents 10 µm

Several determinants are involved in the orchestration of biofilm formation and development. Various quorum-sensing molecules have been identified to play a role in *C. albicans* biofilms. These molecules include, tyrosine, farnesol (Figure 16), and their derivatives such as tryptophol, tyrosol (Figure 17), and farnesoic acid, respectively (Lingappa et al. 1969; Oh et al. 2001; Hornby et al. 2001; Chen et al. 2004). The auto-signalling tyrosine-derivative molecule produced by *C. albicans*, tyrosol, is released by *C. albicans* cells to diminish the lag phase normally seen in culture conditions. Furthermore, tyrosol accelerates and promotes germ tube- and hyphal formation thereby contributing to biofilm establishment (Chen et al. 2004; Alem et al. 2006). In comparison, another derivative, tryptophol, inhibits filamentation and promotes yeast cell dispersion (Ramage et al. 2002; Wongsuk et al. 2016).

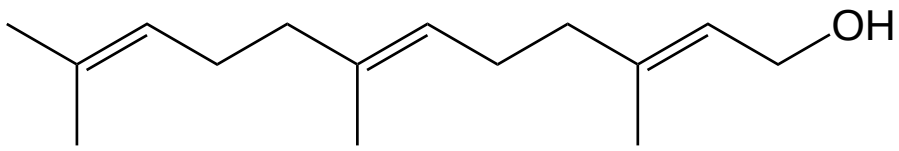

Figure 16. Structure of farnesol

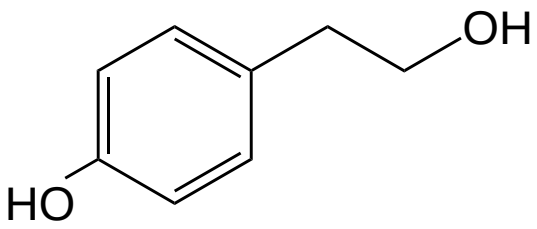

Figure 17. Structure of tyrosol

Candida albicans also utilizes farnesol as a QSM to facilitate biofilm formation and establishment. Farnesol successively blocks the morphological transition of *C. albicans* from yeasts to hyphae at high cell density (Alem et al. 2006). Furthermore, farnesol and its derivative farnesoic acid facilitate the dispersal of yeast cells by inhibiting germ tube and hyphae formation (Navarathna et al. 2005; Lindsay et al. 2012; Dixon and Hall 2015). The regulation of *C. albicans* morphology in biofilms contributes to population diversification and structure formation within the community (Davis-Hanna et al. 2008). In addition, a complex molecular pathway controls various stages of the biofilm lifecycle (Sebaa et al. 2019).

The regulation of adhesins, produced by hyphal layers, are regulated by the TEC1 cascade. TEC1 is a transcription factor responsible for the expression of the biofilm and cell wall regulator 1 (*BCR1*) gene, which produces the Bcr1p protein (Staib et al. 2004; Nobile and Mitchell 2005). The Bcr1p C_2H_2 zinc finger protein is not only required for cell wall protein expression but acts as a master regulator for other adherence proteins (Nobile et al. 2006). Adherence proteins are dependent and include the agglutinin-like sequence (ALS) gene family (Naglik et al. 2006; Nobile et al. 2006). ALS proteins facilitate cellular adhesion and biofilm formation through the complementary action of Als1, Als3 and hyphal wall protein 1 (Hwp1) (Hoyer 2001; Nobile and Mitchell 2005; Nobile et al. 2008).

It is established that quorum-sensing molecules used by *C. albicans* directly influence various metabolic pathways involved in successful biofilm formation. For example, farnesol regulates hyphal growth by inhibiting the Ras-cyclic AMP (cAMP)-protein kinase A (PKA) cascade (Padder et al. 2018). Briefly, the Ras1-dependent activation of cAMP production, by adenylate cyclase (Cyr1), and the resultant activation of PKA is to facilitate hyphal growth (Lindsay et al. 2012). Hyphal growth is facilitated by the activation of transcription factors (by PKA) and the decrease of transcriptional repressors (such as Nrg1 and Tup1) (Cao et al. 2005; Kebaara et al. 2008; Lindsay et al. 2012).

In addition to the role of QSMs and their related molecular pathways, *C. albicans* produces an extracellular matrix (EXM) which significantly contributes to the success of biofilms (Roemer et al. 2003; Lopez-Ribot 2014). *Candida albicans*' EXM consists mainly of proteins, polysaccharides, lipids, and nucleic acids (Nobile and Mitchell 2006; Desai and Mitchell 2015). Proteins in the EXM of *C. albicans* ,include ALS and Hwp1, facilitate cell-to-cell binding and fungal-bacterial interactions. Furthermore, functionally classified enzymes contribute to metabolic activity, translation processes, protein folding, replication, and the repair of proteins related to the biofilm (Karygianni et al. 2020). Polysaccharides utilised in *C. albicans* biofilms include α-mannans and β-glucans, specifically, α-1,6 mannan, β-1,3 glucan, and β-1,6 glucan (Karygianni et al. 2020). Both groups of polysaccharides contribute to the

scaffolding and integrity of the biofilm structure, cellular protection, and antifungal resistance (Nett and Andes 2020). Furthermore, these polysaccharides exist in a multicomponent interaction referred to as the mannan-glucan complex (MGCx), which contributes to biofilm matrix biogenesis and pathogenicity (Nobile et al. 2009; Mitchell et al. 2015).

Other macromolecules identified in the *C. albicans* biofilm EXM are lipids and nucleic acids. The lipids of interest to *C. albicans* are eicosanoids, neutral and polar glycerolipids and sphingolipids (Karygianni et al. 2020). Notably, the role of these lipids remains largely unexplored (Martins et al. 2010; Lattif et al. 2011; Zarnowski et al. 2014). Lastly, the nucleic acid constituent of *C. albicans* biofilms appears to be comprised of non-coding DNA (Kasai et al. 2006) plays a dominant role in biofilm integrity and maintenance (Martins et al. 2010; Mathé and van Dijck 2013).

Polymicrobial biofilm interactions

There is a high level of intra– and interspecies interactions coinciding with opportunistic infections (Jabra-Rizk 2011). Notably, infections are rarely homogeneous but are rather presented as mixed kingdom, with polymicrobial biofilms (Figure 18) consisting of bacteria, fungi, and viruses (Mazaheritehrani et al. 2014; van Dyck et al. 2021). The formation and complexity of polymicrobial biofilm communities occur through a sequential attachment process known as coaggregation (Peters et al. 2012). Coaggregation occurs in two different ways. Several organisms can aggregate, and induce phenotypic changes, which promotes further coaggregation and biofilm formation through intercellular communication. Alternately, secondary colonisers can bind to specific molecules in the mature biofilm structure and induce another coaggregation cascade (Flemming and Wingender 2010; Peters et al. 2012). These complex microbial environments may be synergistic, through the colonisation of one organism which promotes and enables a secondary infection by a different pathogen. Similarly, these interactions may be antagonistic in which the colonisation of a pathogen inhibits the growth of another (Nadell et al. 2009). Commensal interactions, where one organism benefits from an interactive association without affecting another, may be observed when antibiotic-resistant pathogens protect antibiotic-sensitive organisms from inhibitory action (O'Connell et al. 2006).

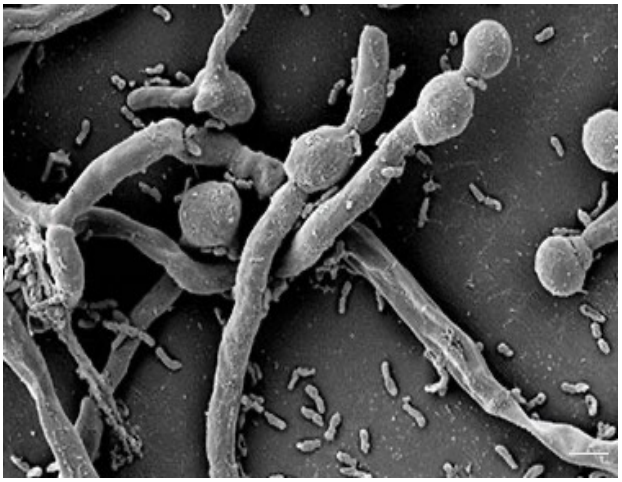

Figure 16. Scanning electron micrograph of *Pseudomonas aeruginosa* PAO1 and *Candida albicans* SC5314 polymicrobial biofilm (Fourie et al. 2021). The scale bar represents 10 µm

When considering the metabolic and genomic variances presented in a polymicrobial biofilm, it is evident that the relationship between constituents is far more complex than what is understood. In the case of *P. aeruginosa* and *C. albicans* being co-cultivated in a polymicrobial setting, both pathogens display ambiguous characteristics depending on cultivation and environmental conditions (Peters et al. 2012; O'Brien et al. 2022). To elaborate on these ambiguous characteristics, patients diagnosed with cystic fibrosis are used as an example.

The autosomal disease, cystic fibrosis, is caused by mutations in the gene encoding for cystic fibrosis transmembrane conductance regulators. The disease is characterised by bacterial colonisation and infection which ultimately results in respiratory failure (Mathee et al. 1999). The bacterial pathogen responsible for most morbidities and mortalities is *P. aeruginosa* (Koch and Høiby 1993; Mathee et al. 1999). Although *C. albicans* is a commensal organism isolated from the human gastrointestinal tract, it is not often associated with pulmonary colonisation and isolation (Brook et al. 1992; Burns et al. 1999; Harriott and Noverr 2011). However, several reports have established the colonisation and infection of *C. albicans* in patients diagnosed with cystic fibrosis (Hughes and Kim 1973; Welch et al. 1987; Rodrigues et al. 2017; Reece et al. 2021). A study by Hogan and Kolter (2002) showed that *P. aeruginosa* cells adhered to *C. albicans* filaments and rarely adhered to *C. albicans* yeast cells. The study revealed that *P. aeruginosa* lysed and killed the filaments it was attached to, indicating an antagonistic relationship between the pathogens when co-inhabited in a polymicrobial biofilm.

Pseudomonas aeruginosa interacts with *C. albicans* by attaching to the hyphal form of the yeast through type IV pili and bacterial lipopolysaccharides (Hogan et al. 2004; Bandara et al. 2010; Bandara et al. 2013). Once attached, *P. aeruginosa* produces nitrogen-containing heterocyclic compounds known as phenazines (Figure 19). Phenazines serve as signals to regulate gene expression, contribute to biofilm formation, and enhance pathogenic fitness (Pierson and Pierson 2010; Harriott and Noverr 2011). Phenazines produced by *P. aeruginosa* include phenazine-1-carboxamide, phenazine-1-carboxylate, pyocyanin, and 5-methyl-phenazinium-1-carboxylate (Kerr 1994; Kerr et al. 1999; Pierson and Pierson 2010; Morales et al. 2010).

These bacterially produced molecules display antifungal characteristics by inhibiting yeast to hyphal transitions, inducing hyphal cell lysis, and generating reactive oxygen species (Kerr et al. 1999; Brand et al. 2008; Morales et al. 2010). Interestingly, the co-cultivation of *P. aeruginosa* and *C. albicans* results in a positive feedback loop. Ethanol produced by the yeast promotes *P. aeruginosa* biofilm production, which results in the production of phenazines, which results in the production of more ethanol (Fourie and Pohl 2019).

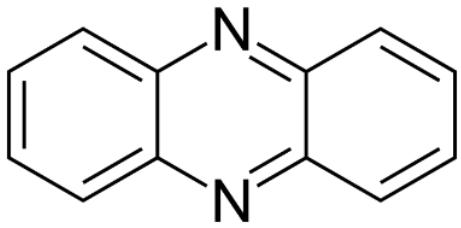

Figure 17. Structure of phenazine

Other interactions of interest to this antagonistic relationship include the production, secretion, and exchange of quorum-sensing molecules. The production of 3-oxododecanoyl-L-homoserine lactone (3-oxo-HSL) by *P. aeruginosa* is required for adhesion to *C. albicans* hyphae, and not only inhibits the morphological transition of yeast cells to hyphal cells, but ,reverts hyphal cells to yeast form (Hogan et al. 2004; Ovchinnikova et al. 2012; Fourie and Pohl 2019). In addition to HSL molecules, *P. aeruginosa* also produces quinolone molecules which influence *C. albicans* fitness (Lépine et al. 2003; Reen et al. 2011). The molecule 2-heptyl-3-hydroxyl-4-quinolone modulates bacterial swarming and contributes to the production of phenazines (Pesci et al. 1999). Furthermore, this quinolone molecule inhibits *C. albicans* biofilm formation (McAlester et al. 2008; Reen et al. 2011).

The interaction between *P. aeruginosa* and *C. albicans* has been described as bidirectional; as *C. albicans* produces QSM in response to the presence of the bacteria (Ramage et al. 2005; Purschke et al. 2012). Similar to the bacterial quorum sensing molecule HSL, *C. albicans* produces farnesol to control fungal growth. Farnesol controls fungal growth by inhibiting the germination of blastospores, which results in fungal dispersal, and the propagation of new communities (Hornby et al. 2001; Ramage et al. 2002; Lindsay et al. 2012). Farnesol is also a fungal virulence factor and inhibits the production of bacterial phenazines and quinolones (Cugini et al. 2007; Abdel-Rhman et al. 2015; Rodrigues and Černáková 2020; Reece et al. 2021). When considering the influence of these QSM, it is evident that the interaction between these pathogens is bidirectional and directly influences their related virulence mechanisms (Reece et al. 2021).

The intricate interactions and microenvironments of a polymicrobial biofilm present various advantages to establishing and promoting antimicrobial resistance. These advantages are enabled through an enlarged gene pool, passive resistance mechanisms, complimentary metabolic capabilities, and quorum sensing advances (Wolcott et al. 2013; Costa-Orlandi et al. 2017). A formidable resistance mechanism presented in a polymicrobial biofilm is an enlarged gene pool (Heidari et al. 2022; Bridier and Briandet 2022; Thaarup et al. 2022). Pathogens in a biofilm respond genetically to their environment, resulting in differential phenotypes (Rodrigues et al. 2019; Nikolaev et al. 2022).

Biofilms present the opportunity for microorganisms to access complimentary metabolic opportunities. The proximity of members in a biofilm allows a combination of resources to support the growth of all constituents (Dixon and Hall 2015). The acquisition of substrates for metabolic processes and the dispersion of relevant end products contribute to the success of a polymicrobial biofilm and its antimicrobial resistance properties (Fischbach and Sonnenburg 2011).

In addition to metabolic advances, the presence of quorum-sensing metabolites in a biofilm further contributes to the success of the complex community (Tait et al. 2009). Quorum-sensing molecules such as alcohol derivates and peptides contribute to the success and antimicrobial resistance of a biofilm by inducing resistance to oxidative stress and promoting the production of virulence factors (Lee et al. 2007; Hall et al. 2011; Albuquerque et al. 2013). The intricate relationship observed between consortium members is also indicative of differential gene expression and cellular response systems (Tuttle et al. 2011; Elias and Banin 2012). A study by Purschke et al. (2012) evaluated the secretome of mono – and polymicrobial biofilms of *P. aeruginosa* and *C. albicans*, respectively. Their study isolated 131 individual proteins in a polymicrobial biofilm, only 39 were assigned to the yeast, whereas 92 proteins belonged to *P. aeruginosa*. Both organisms indicated a larger diversity of proteins; with 73 proteins being assigned to the yeast, and 154 proteins being assigned to the bacteria.

Furthermore, studies by Bandara et al. (2020) proved that the production of the bacterial QSM 3-oxo-HSL induces fungal resistance to the antimicrobial compound fluconazole. Antifungal resistance is achieved by the QSM preventing changes to the ergosterol biosynthesis pathway, promoting drug efflux activity, and maintaining fungal cell membrane activity (Bandara et al. 2020). When considering the complex interactions between these pathogens, it may be suggested that the relationship between them is not unilaterally antagonistic, and recalcitrant properties may be promoted to benefit the consortium (Peters et al. 2012; Orazi and O'Toole 2020; Bisht et al. 2020).

Antimicrobial alternatives

As indicated above, microorganisms naturally live in dynamic structured communities, known as biofilms, which provide various advantageous strategies and opportunities for microbial propagation and growth (Neglo et al. 2022). It is established that mono – and polymicrobial biofilms pose a significant threat to the global public health and is of immediate clinical importance. Although many antimicrobial agents are being used to treat biofilms and their related infections, the inappropriate application of conventional therapeutic strategies may contribute to pathogenic and biofilm resistance mechanisms (Rumbaugh 2014).

A standard approach used to treat biofilms, and biofilm-related infections is the combination of two or more currently available antimicrobials (Brook 2002). This approach is referred to as combination therapy (Aaron et al. 2002). However, the establishment of multidrug – and extensively drug-resistant pathogens threaten the efficacy of this approach. Moreover, the risk of selecting resistant characteristics in opportunistic pathogens poses a different problem altogether (Dharmaprakash et al. 2015). Pre- and probiotics have been suggested to circumvent the challenges presented by combination therapy (Dobrogosz et al. 2010; Hancock et al. 2010). Interestingly, Wang and co-workers have assessed probiotic substances using whole genome sequencing (WGS) and have urged caution. The commercial production of these substances poses various risk factors, such as incorrect and inconsistent strain information, which may contribute to the fitness of opportunistic pathogens through downstream metabolic interactions (Wang et al. 2021).

When referring to the biofilms formed by *P. aeruginosa* and *C. albicans*, a possible treatment to address indwelling medical apparatus-related biofilm infections is the antimicrobial lock therapy (ALT). The principle of this approach is that the relevant medical device is internally and externally coated with doses of 100 – to 1000-fold the minimum inhibitory concentration (MIC) of an appropriate antimicrobial substance. This 'locks' the device for a certain time to retard biofilm formation (Carratalà 2002). This approach has promising results. This treatment is pathogen-specific and complete biofilm inhibition has not yet been achieved. Furthermore, this treatment is not sensitive enough to address polymicrobial biofilms or infections (Tournu and van Dijck 2012).

When considering the phenotypic plasticity and fluidity of a biofilm, it is evident that various mechanisms interact and work together (Smith 2005). Additionally, it is important to consider the nature of antimicrobial action when attempting to eradicate biofilms and their related infections. Approaching infections in the same manner as growing cultures of individual cells has proved inadequate (Masterton 2005; Leite et al. 2014). Furthermore, this approach contributes to antimicrobial resistance and the recalcitrant nature of opportunistic pathogens (Gilbert et al. 2002; Hall-Stoodley and Stoodley 2009). Current antibiofilm approaches have focused on biofilm formation, the biofilm-related EXM, and relevant virulence factors (Roy et al. 2018; Paluch et al. 2020; Jiang et al. 2020).

The intimate and complex nature of biofilm formation alludes to a possible antibiofilm strategy (Tan et al. 2020). Biofilm formation relies on coaggregation and the integrity of the resultant tertiary structure. Therefore, a potential strategy may be to compromise the multicellular structure of a biofilm to avail target sites and susceptible cells to antimicrobial action (Goodman and Bakaletz 2022). Possible therapies to achieve this include enzyme-substrate analogues to interfere with intra– and intercellular communication, enzymatic reactions to prevent biofilm matrix formation, and enzymatic degradation of the biofilm matrix (Yasuda et al. 1993; Parsek and Greenberg 2000; Stewart and Costerton 2001; Peng et al. 2016). For example, antibiofilm therapies may target stages of biofilm growth such as the initial stage of biofilm formation attachment. The attachment of sessile cells may be addressed by targeting cell surface proteins such as adhesins to disrupt the interactions between the host and pathogens (Jiang et al. 2020). However, many organisms display the ability to utilise multiple receptor-ligand interactions to facilitate host binding (Karygianni et al. 2020).

A sequential approach would suggest that the next target be the production of the EXM. However, the process of EXM production and secretion is multifaceted and relies on both signalling – and non-signalling mechanisms (Peng et al. 2016; Jiang et al. 2020). Therefore, it is suggested that targeting the components of the EXM, and their related synthetic pathways may provide better results (Mitchell et al. 2016; Dragoš and Kovács 2017). Whitchurch et al. (2002) established that deoxyribonucleases (DNases) are effective in inhibiting biofilm formation by degrading the eDNA component of the biofilm matrix. Interestingly, DNases were mainly effective against immature biofilms of up to 24 hours (Whitchurch et al. 2002; Tetz et al. 2009).

At this juncture, it is important to consider antimicrobial substances in terms of polymicrobial biofilms as even a single species biofilm consists of a heterogeneous population. Microorganisms in a biofilm exist

in different phenotypic states due to various gradients found in the biofilm environment. Additionally, genes responsible for related stress response mechanisms are differentially regulated in these gradients. Therefore, the genes expressed in these phenotypes may not directly contribute to biofilm fitness but are expressed in response to a gradient environment (Dharmaprakash et al. 2015). As a result, elements and regulatory pathways persistently involved in both planktonic and biofilm cells might be worth pursuing as a potential antibiofilm target (Jiang et al. 2020). Elements and regulatory pathways of interest include quorum sensing systems and regulatory pathways and natural by-products (Tournu and van Dijck 2012; Sambanthamoorthy et al. 2014; Dharmaprakash et al. 2015). It is noteworthy to mention that the exact role of QSMs in polymicrobial biofilms and their related resistance is unclear, however, the use of quorum sensing inhibitors (QSI) has been investigated as a potential antibiofilm strategy. Quorum-sensing can be addressed by targeting ligand-receptor pathways, inhibiting receptor synthesis and inhibiting ligand-receptor binding (Raffa et al. 2005; Rumbaugh 2014).

Natural by-products produced by constituents of a polymicrobial biofilm include biosurfactants (BS) which are biological compounds with the ability to reduce surface tension in liquids. These complex biological compounds include lipopeptides, phospholipids and polysaccharide-proteins (Sambanthamoorthy et al. 2014). These biological compounds can interfere with biofilm formation by modifying adhesive properties and interfering with cell-to-cell communication (Rumbaugh 2014; Sambanthamoorthy et al. 2014). Furthermore, a characteristic of surfactants is the ability to self-assemble into aggregates to achieve the separation of hydrophobic moieties from water. This self-assembly occurs above a concentration known as the critical micelle concentration (CMC) (Blanco et al. 2005).

Naturally derived antimicrobial peptides (AMPs) are a diverse group of protein-antimicrobials forming part of the innate immune system (Boman 1995). AMPs demonstrate antimicrobial activity by targeting microbial membranes and are promising antimicrobial candidates (Hancock and Sahl 2006). AMPs demonstrate the ability to bind surface active agents (surfactants), which results in the formation of a protein-surfactant complex. In this complex, the hydrophobic moieties of the relevant surfactant react with the nonpolar amino acids of the protein and prevent unwanted solvent contact (Faustino et al. 2009a). Chemical recombination techniques may be used to modify these proteins and act as a starting point for designing novel antimicrobial substances or classes (Li 2011; Rai et al. 2016).

The use of molecules containing both a lipophilic and hydrophilic group (amphiphiles) provides a unique opportunity to design novel materials for advanced antimicrobial nanotechnology applications. Conventional amphiphiles consist of a long saturated or unsaturated hydrocarbon chain as the lipophilic component. Whereas the hydrophilic component is either ionic or non-ionic, the zwitterion amphiphiles have a headgroup with both a positive and negative charge. Amphiphiles can self-assemble into highly ordered structures and can mimic biological systems and, therefore, presents a desirable avenue for novel antimicrobial design (Lombardo et al. 2015; Blackholly et al. 2016).

Brahmachari and co-workers (2010) developed antimicrobial amphiphiles to elaborate on the potential application of these supramolecular materials. The design of hydrogelators containing a hydrophobic alkyl chain coupled to a quaternary pyridinium molecule facilitated the formation of complex amphiphilic supramolecular aggregates with inherent antibacterial activity. The hydrogelators produced indicated promising antibacterial activity against Gram-positive and negative bacteria (Haldar et al. 2005; Brahmachari et al. 2010). Additionally, Debnath and co-workers reported a novel class of antibacterial hydrogelators based on *N*-fluorenyl-9-methoxycarbonyl (Fmoc) amino acid functionalised cationic amphiphiles. This novel class of positively charged hydrogelators was designed by the incorporation of a pyridinium moiety, known for its membrane permeation and antibacterial properties. Similar to the hydrogelator molecules produced by Brahmachari and co-workers, this novel class also indicated antibacterial activity against Gram-positive and negative bacteria (Haldar et al. 2005; Debnath et al. 2010).

Faustino and co-workers designed a novel molecule based on the formation of hydrogen bond donor (HBD):anion complexes to act as surfactants (Faustino et al. 2009a). The modification of these complexes results in the formation of anion-spacer-urea-based molecules with both a hydrophobic and hydrophilic group. It was concluded that these complexes did not indicate the potential to be developed as antimicrobial agents. To complement their work, Pittelkow et al. (2004) evaluated the characteristics of similar urea-based molecules in a host-guest system and alluded to a potential novel antimicrobial therapy (Faustino et al. 2009b). Furthermore, Hiscock and co-workers developed a novel tetrabutylammonium (TBA) sulfonate-urea salt (Figure 20) with the ability to produce hydrogen-bonded nanostructures (Blackholly et al. 2016; Hiscock et al. 2016a; Hiscock et al. 2016b). The molecular self-assembly and resultant nanostructure formation rely on non-covalent intermolecular interactions such as hydrogen bonding. The integration of these self-association events and supramolecular complex formations has established novel programmable supramolecular frameworks and nanostructures (White et al. 2017).

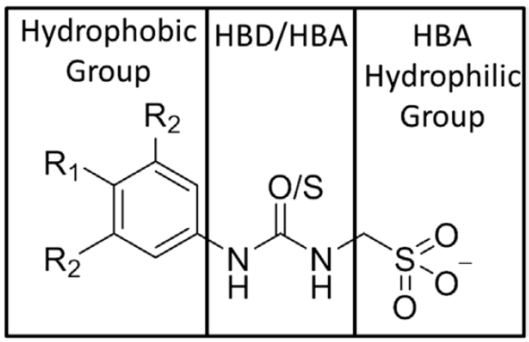

Figure 18. Schematic of the general structure of sulfonate-urea amphiphilic salts. The hydrophobic group of the amphiphile, represented by the phenyl ring, presents three different binding sites for moiety modifications. The urea (or thiourea in the case of a sulphur substitution) moiety represents the hydrogen bond donor or acceptor group. The sulfonate moiety also presents an opportunity for structure modification and acts as the hydrophilic group in the amphiphilic compound. The structure of the relevant countercation has been omitted for clarity (Blackholly et al. 2016; Hiscock et al. 2016a)

From the work done by Hiscock et al. (2016), a novel class of antimicrobial compounds has been established by modifying an anion-spacer-urea molecule in a stepwise fashion. This class of compounds is known as supramolecular self-associating amphiphiles (SSAs) (Tyuleva et al. 2019). SSAs display potential antibiotic action by selectively binding their anionic component to phospholipids (PE and PG) derived from bacterial membranes (Allen et al. 2020; Medina-Carmona et al. 2020; Boles et al. 2022). This class of compounds has shown promising inhibitory efficacy against planktonic clinically relevant MRSA USA300 and *E. coli* DH10β (Tyuleva et al. 2019; Ng et al. 2020; White et al. 2021). Furthermore, it has shown the ability to act as antimicrobial drug delivery systems and enhance antimicrobial activity (White et al. 2020a; White et al. 2020b; Boles et al. 2021; Dora et al. 2021; Ellaby et al. 2022).

Conclusions

Antimicrobial substances have revolutionised the healthcare industry and have significantly contributed to the global population's health by facilitating the management of disease and infection. Antimicrobial substances are classified based on their mechanism of action and generally target the microbial membrane. Notably, classical antimicrobial substances are identified and developed based on their ability to inhibit planktonic and monomicrobial cultures. Since the application (and abuse) of antimicrobials, drug-resistant pathogens have utilised innate, acquired and induced microbial resistance mechanisms to inhibit antimicrobial efficacy. Some of the formidable resistance mechanisms utilised by pathogenic microorganisms include various genotypic and phenotypic aspects; such as being resistant to more than one mechanism of action utilised and the ability to form a complex structured community known as a biofilm. Biofilms are cells encapsulated in an extracellular polymeric substance known as an EXM. In addition to the EXM, continuous intra – and intercellular communication systems provide additional resistance mechanisms to the biofilm community. Additionally, the co-inhabitation of pathogenic organisms (known as a polymicrobial biofilm) presents further obstacles to addressing opportunistic pathogens and their related recalcitrant infections.

A potential novel antimicrobial class referred to as SSAs, has been developed by Professor Jennifer Hiscock and her team from the University of Kent. The step-wise modification in the synthesis of these SSAs has produced a library of over ninety compounds. Notably, these compounds have displayed antibacterial activity by inhibiting planktonic clinically-relevant MRSA and *E. coli*. This warrants further investigation to further elucidate the scope of SSA application.

Aims of the project

This project aims to determine the efficacy of novel SSA compounds against *P. aeruginosa* and *C. albicans* mono- and polymicrobial biofilms *in vitro*, to evaluate the efficacy of these compounds as therapeutic enhancers when in combination with other antimicrobials of interest. Furthermore, this study aims to investigate the efficacy of SSAs against *C. albicans in vivo* by using *Caenorhabditis elegans* as the model organism. Finally, this study aims to investigate the potential membrane interaction between SSAs and fungal membranes using a novel lipid assay.

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Chapter 2: Efficacy of supramolecular self-associating amphiphiles against mono– and polymicrobial biofilms

Abstract

In 2019, 4.95 million deaths were directly associated with bacterial antimicrobial resistance (AMR). The repurposing and development of antimicrobial compounds are of immediate interest in the combat of AMR. A novel library of over ninety supramolecular self-associating amphiphilic (SSA) compounds, synthesised by the research group of Professor Jennifer Hiscock, has displayed antimicrobial efficacy against planktonic cells of clinically relevant bacteria, such as methicillin-resistant *Staphylococcus aureus* and *Escherichia coli,* providing an alternative source to be explored for antimicrobial drugs. However, the efficacy of this library was yet to be determined against biofilms of pathogens, such as *Pseudomonas aeruginosa* and *Candida albicans*. Therefore, this study evaluated the efficiency of SSA compounds against the biofilm formation by these pathogens in a mono– and polymicrobial environment. The compounds were screened for biofilm formation inhibition, through optical density measurements. Thereafter, metabolic activity assays were used to determine the biofilm inhibition and eradication potential of the best performing compounds. The morphological effects of the compounds were evaluated through scanning electron microscopy and confocal laser scanning microscopy. Compounds of interest were then evaluated as adjuvants to improve the antibacterial and antifungal activity of colistin and fluconazole, respectively. Moreover, the scope of application was studied by employing SSAs in *Caenorhabditis elegans* infection models. This study identified novel supramolecular amphiphilic compounds that have potential anti-biofilm activity. Furthermore, this study established potential adjuvant activity and, although the SSAs were ineffective in treating infection in *C. elegans*, this work does lay the foundation for developing novel antimicrobial compounds based on structural combinations and structure-activity relationships.

Keywords

Antibiofilm, biofilms, *Caenorhabditis elegans*, *Candida albicans*, colistin, fluconazole, polymicrobial, *Pseudomonas aeruginosa*, structure-activity relationship

Introduction

Candida albicans is a polymorphic commensal yeast, native to the human microbiota (McCullough et al. 1996). In immunocompromised individuals, *C. albicans* is an opportunistic pathogen capable of causing cutaneous to deadly invasive systemic infections (Bhattacharya et al. 2020). *C. albicans*-related candidemia has been identified as the leading cause of hospital-acquired infections (HAI) and bloodstream infections (BSI) (Pfaller and Diekema 2007; Magill et al. 2014; Sharma and Chakrabarti 2023). In 2022, the World Health Organisation (WHO) declared *C. albicans* as one of the top four fungal pathogens in the critical priority group. The ability of *C. albicans* to form biofilms contributes to the success and recalcitrant nature of this pathogen (Hawser and Douglas 1995). *C. albicans* biofilms are known to facilitate antimicrobial resistance in related infections (Ramage et al. 2005; Ruiz-Baca et al. 2021). An important feature of a *C. albicans* biofilm is the presence of an extracellular polymeric substance (EPS) or extracellular biofilm matrix (EBM). The EBM consists of a complex structured network of glycoproteins, carbohydrates, lipids and nucleic acids and directly contributes to pathogenic potential and fitness by providing antimicrobial protection (Martins et al. 2010; Gulati and Nobile 2016).

Candida albicans is rarely identified and isolated alone as *C. albicans* and *Pseudomonas aeruginosa* are the two most commonly co-occurring opportunistic pathogens that colonize the human microbiome. *P. aeruginosa* is an opportunistic pathogen often isolated from the lungs of patients diagnosed with cystic fibrosis (CF). Once chronically infected, *P. aeruginosa* infections are extremely difficult to eradicate and treat, resulting in extreme rates of morbidity and mortality (Hancock and Speert 2000; Rocha et al. 2019; Jurado-Martín et al. 2021). In 2017, *P. aeruginosa* was declared as one of the top bacterial pathogens in the critical priority group (Tacconelli et al. 2018; Asokan et al. 2019). Moreover, *P. aeruginosa* has been grouped with pathogens displaying multi-drug and extensively drug-resistant properties (Rice 2008; Boucher et al. 2009; Pendleton et al. 2013). Similar to *C. albicans* and other ESKAPE pathogens, *P. aeruginosa* utilizes various virulence factors to facilitate pathogenic potential and fitness (Kunz Coyne et al. 2022).

A virulence factor of interest is the ability to form biofilms. Notably, the sophisticated process of biofilm formation relies upon various quorum-sensing molecules to establish a mature and formidable biofilm. Furthermore, the co-inhabitation of these pathogens produces interkingdom biofilms (Phuengmaung et al. 2022). The interaction between *C. albicans* and *P. aeruginosa* has been described in great depth (Ovchinnikova et al. 2012; Bandara et al. 2013; Fourie 2016; Fourie and Pohl 2019; Fourie et al. 2021; van Dyck et al. 2021). In a polymicrobial biofilm, the related pathogenic constituents display complex interactions that facilitate enhanced antimicrobial resistance properties and promote chronic infections (Timpel et al. 2000; Rodrigues et al. 2019; Khan et al. 2021).

The ability of microbes to circumvent antimicrobial substances has been observed since their discovery. After almost nine decades of widespread antimicrobial use, pathogens from all kingdoms have become increasingly resistant to antimicrobial application (Landecker 2016; Browne et al. 2020). In 2019, predictive statistical models estimated that approximately 4.95 million recorded deaths were directly related to AMR. Importantly, the ESKAPE pathogens alone contributed approximately 100 000 deaths to these statistics (Vos et al. 2020; Antimicrobial Resistance Collaborators 2022; Murray et al. 2022).

Antimicrobial resistance develops through various mechanisms such as membrane permeability alterations, promoted efflux pumps, drug target-site modifications, the establishment of alternative metabolic pathways to circumvent antimicrobial action and the establishment of biofilm formation (Mcdermott et al. 2003; Liwa and Jaka 2015; Aminov 2017; Wicaksono et al. 2021). Thus, alternative strategies are required to address the global threat of polymicrobial biofilms (Gow et al. 2022).

Alternative antimicrobial strategies include the use of naturally derived antimicrobial peptides (AMPs). These molecules display amphiphilic (both hydrophobic and hydrophilic components) characteristics (Lombardo et al. 2015). Based on this principle, Faustino et al. (2009) designed novel anion-spacer-urea-based molecules with amphiphilic and potential surfactant properties. From their work, Hiscock et al. (2016) developed a novel class of compounds with the ability to self-assemble into higher order structures known as supramolecular self-associating amphiphiles (SSAs). The basic structure of these compounds (Figure 1) is a lipophilic phenyl ring attached to a central ureido or thioureido group, which is attached to a carboxylate or sulfonate (White et al. 2020b). These salts are synthesized in a stepwise-modification fashion to form aggregates that can permeate the cell membrane through lipid interactions (Medina-Carmona et al. 2020; Boles et al. 2021) and have proven antibiotic action against planktonic cells of clinically relevant bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* USA300 and *Escherichia coli* DH10β (Allen et al. 2020; Ng et al. 2020; White et al. 2021).

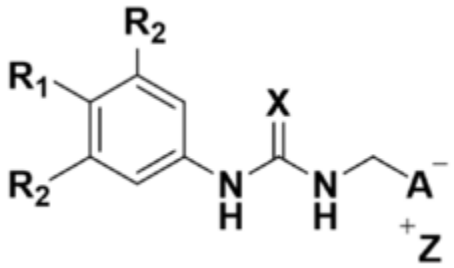


Figure 19. Supramolecular self-associating amphiphile (SSA) backbone structure. Where R = any group, X = oxygen or sulphur, A^- = carboxylate or sulfonate, and Z^+ = counter cation (Blackholly et al. 2016; Hiscock et al. 2016a)

This novel class of compounds requires further study to better understand their scope of application and efficacy against biofilm-forming organisms, as well as to establish a structure-activity relationship and improve SSA design. Therefore, this study aims to evaluate the efficacy of SSAs against *P. aeruginosa* and *C. albicans* mono- and polymicrobial biofilms *in vitro*. Furthermore, this study aims to determine the application of SSAs as therapeutic enhancers in combination with currently available and marketed antimicrobials. Lastly, this study aims to investigate the application of SSAs against *C. albicans* infections *in vivo* by using *Caenorhabditis elegans*.

Materials and methods

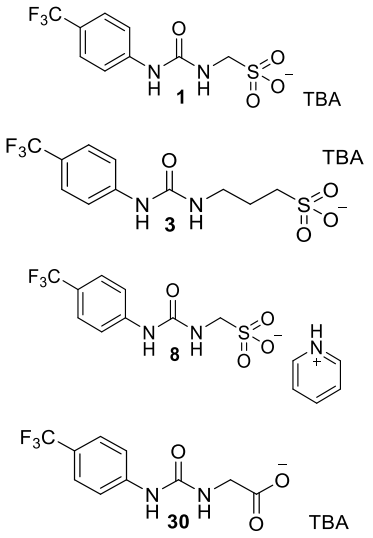
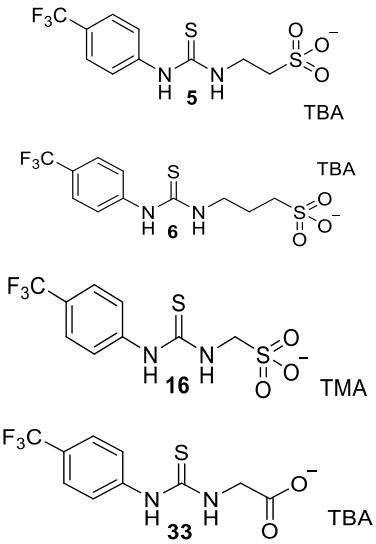
Supramolecular self-associating amphiphile stock solution

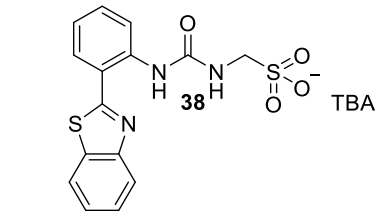
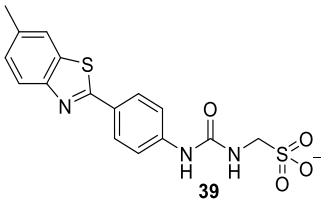
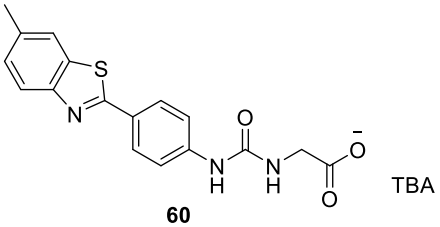
The self-associating amphiphiles (SSAs) used in this study are grouped according to their structural similarities (Table 1). All SSA stock solutions were prepared in 25% ethanol in milli-Q water to obtain a

final concentration of 25.6 mM (Tyuleva et al. 2019). The compounds were solubilized with sonication in three cycles (005-power, high frequency) (Scientech Ultrasonic Cleaner) at 50 °C for 50 minutes. The solutions were stored at room temperature until required for use.

Compounds in group 1 (**SSA 1**, **3**, **8** and **30)** contain trifluoro phenyl rings, attached to a central ureido group and a sulfonate or carbamoyl glycinate moiety, while compounds in group 2 (**SSA 5**, **6**, **16** and **33)** contain a central thiourea group instead of the ureido moiety. Compounds in group 3 (**SSA 38**, **39** and **60)** differ from the other SSAs in that they have a benzothiazole group attached to the phenyl ring. The counter cation of these SSAs is generally tetrabutylammonium (TBA), however, **SSA 8** utilizes a pyridine molecule and **SSA 16** a tetramethylammonium (TMA) molecule as their respective counter cations.

Table 1. Supramolecular self-associating amphiphile compounds, grouped according to their chemical similarities, used in this study

Group 1 Urea SSAs	
Group 2 Thiourea SSAs	

Group 3 Benzothiazole-appended SSAs	 38 TBA  39 TBA  60 TBA

Strain maintenance and culture conditions

The reference strains *Pseudomonas aeruginosa* PAO1 and *Candida albicans* SC5314 were used in this study. The strains were stored at -80 °C in nutrient broth (NB) (1 g.L^{-1} meat extract, 2 g.L^{-1} yeast extract, 5 g.L^{-1} peptone, 8 g.L^{-1} sodium chloride) supplemented with 25% or 15% (v/v) glycerol. Before each assay, the *P. aeruginosa* and *C. albicans* strains were cultivated from the frozen stocks onto nutrient agar (NA) (1 g.L^{-1} malt extract, 2 g.L^{-1} yeast extract, 5 g.L^{-1} peptone, 8 g.L^{-1} sodium chloride, 20 g.L^{-1} agar) and yeast malt extract (YM) agar (3 g.L^{-1} malt extract, 3 g.L^{-1} yeast extract, 5 g.L^{-1} peptone, 10 g.L^{-1} glucose). For every experiment, a fresh (pre-inoculum) culture of *P. aeruginosa* PAO1 was prepared by transferring a single colony from the plates into 10 mL NB and incubating at 37 °C with shaking at 150 rpm for 24 hours. Similarly, a fresh (pre-inoculum) culture of *C. albicans* SC5314 was prepared in 5 mL yeast nitrogen base (YNB) broth media (6.7 g.L^{-1} yeast nitrogen base, 10 g.L^{-1} glucose) and incubated at 30 °C for 24 hours. For all assays, filter-sterilized (0.22 µm nitrocellulose filter, ABLUO®, GVS, United States of America) RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich®, United Kingdom) at pH 7.0, was used.

***In vitro* mono– and polymicrobial biofilm formation**

Biofilms were developed as described previously (Stepanovic et al. 2000; O'Toole 2011; Fourie et al. 2017). A schematic diagram explaining the process of biofilm formation is depicted in Figure 2. Briefly, after incubation, the prepared pre-inoculum was centrifuged (5000 rpm, 4°C, 5 minutes) (F-35-6-30 rotor, Centrifuge 5430R, Eppendorf®, United States of America) for three cycles. After each cycle, the supernatant was discarded and the pellet washed with 10 mL phosphate-buffered saline (PBS) (0.2 g.L^{-1} potassium chloride, 0.2 g.L^{-1} potassium dihydrogen phosphate, 1.15 g.L^{-1} di-sodium hydrogen phosphate, 8 g.L^{-1} sodium chloride) (Oxoid, United Kingdom) at pH 7.3. After the final cycle, the pellet was resuspended in 10 mL PBS and standardised to an OD_{595} of 0.5 for *P. aeruginosa* and 1 x 10^6

cells.mL^{-1} (after counting with a hemacytometer) for *C. albicans*. The standardised cell suspensions were dispensed into 96-well flat-bottom culture plates (Greiner Bio-One, Germany) (250 µL total volume per well) together with a two-fold dilution series of each SSA to achieve a final concentration range of 0.08 – 2.56 mM. Cell-free solvent controls and negative controls were included with the final and maintained ethanol concentration as 2.5 %. The microtiter plates were incubated for 48 hours at 37 °C. To cultivate polymicrobial biofilms of *P. aeruginosa* and *C. albicans*, the methods described for the monomicrobial models were used. However, when standardising the cell solutions for the inoculation of a 96-well culture plate, *P. aeruginosa* and *C. albicans* were adjusted to OD_{595} of 0.1 and 2.0 x 10^6 cells.mL^{-1}, respectively, and equal volumes of each culture was added. Unless otherwise stated, all experiments were performed in technical triplicates and biological duplicates.

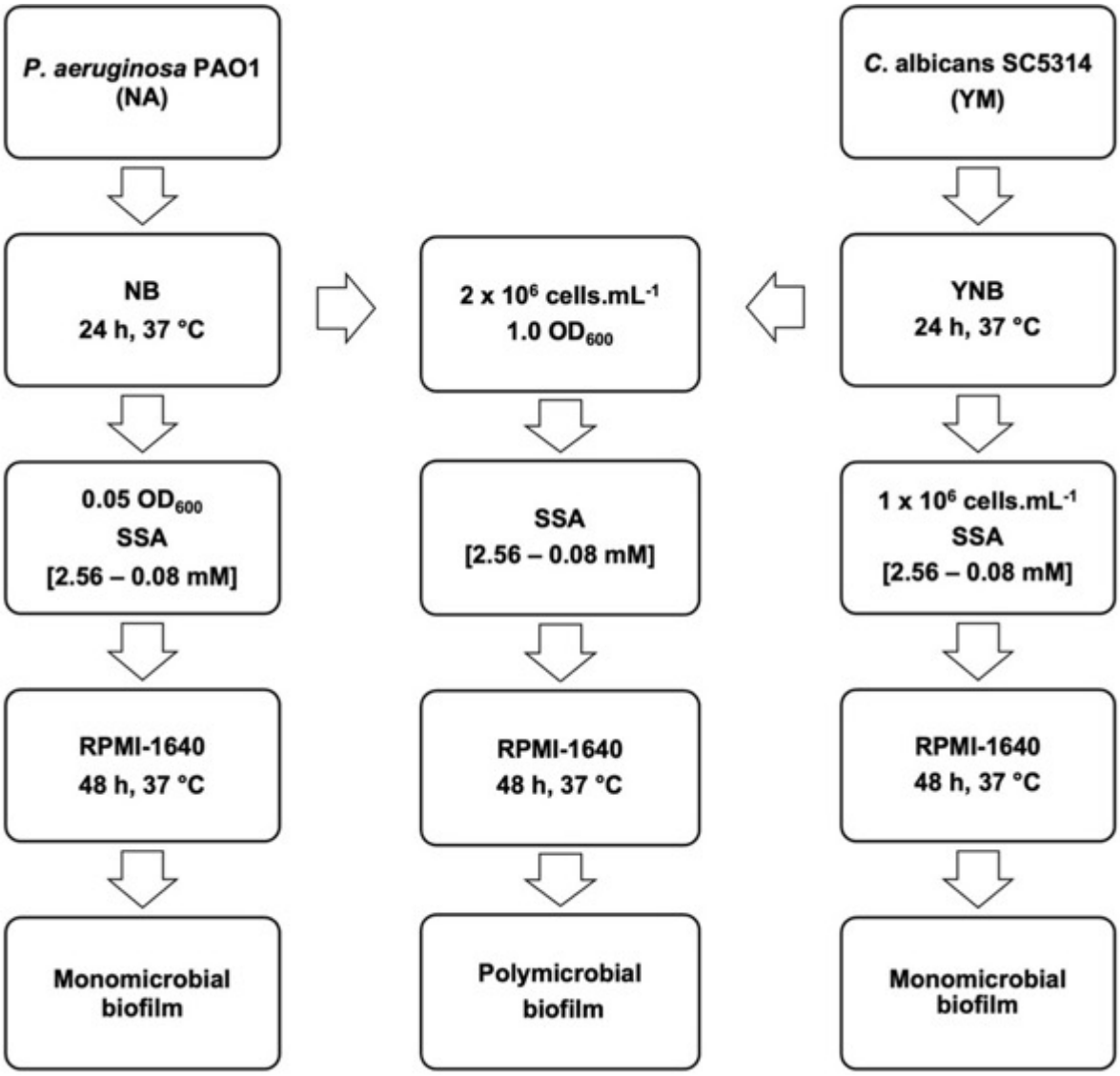


Figure 20. Schematic diagram outlining the process of mono- and polymicrobial biofilm formation for *Pseudomonas aeruginosa* PAO1 and *Candida albicans* SC5314

Preliminary SSA antibiofilm screening

In order to evaluate potential antibiofilm activity, *P. aeruginosa* PAO1 and *C. albicans* SC5314 mono– and polymicrobial biofilms were cultivated as described. Before incubation, an initial OD (at a wavelength of 595 nm) (EZ Read 800 Research, Biochrom, England) was measured to establish a baseline. After 48-hour incubation at 37 °C, the OD of each well was again measured and compared to the initial reading to determine biofilm formation and the percentage inhibition relative to the negative control was calculated. Furthermore, when considering the antimicrobial potential of quaternary ammonium compounds, the effect of TBA chloride (0.08 – 2.56 mM) was evaluated in mono- and polymicrobial biofilms.

Metabolic activity assay of biofilms

Pseudomonas aeruginosa PAO1 and *C. albicans* SC5314 mono– and polymicrobial biofilms were cultivated as described above in the presence of selected SSAs. After incubation, an indirect and semi-quantitative measure of biofilm formation was calculated using a 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl-)-2H-tetrazolium-5-carboxanilide (XTT) colourimetric reduction assay (Mosmann 1983; Braga et al. 2008; Wilson et al. 2017). Briefly, 1 $g.L^{-1}$ of XTT salt (Sigma-Aldrich®, United Kingdom) was dissolved in PBS, filter-sterilised through a 0.22 µm nitrocellulose filter (ABLUO®, GVS, United States of America), aliquoted and stored at -20 °C. Before each XTT assay, an aliquot of XTT stock was thawed and menadione (Sigma-Aldrich®, United Kingdom) (from 10 mM in acetone) was added to a final concentration of 1 mM. The supernatant from the 96-well plates was discarded, the wells washed twice with 200 µL PBS and 50 µL of XTT-menadione solution was introduced to each well. The plates were incubated for 3 hours at 37 °C in the dark and the absorbance was measured at 492 nm, using a microtitre plate reader (EZ Read 800 Research, Biochrom, England).

Scanning electron microscopy (SEM)

Mono– and polymicrobial biofilm models were prepared in a flat bottom 6-well plate (Greiner Bio-One, Germany) in 2 mL of RPMI-1640 medium containing selected SSAs and a sterile polymer disc (Isopore, Merck, Germany). After incubation, the polymer disc was aseptically removed and placed in primary fixative, 3 % (v/v) glutardialdehyde (Merck, Germany) in phosphate buffer (pH 7.0), overnight. The biofilms were washed twice with PBS and fixed with a secondary fixative, 1 % (v/v) osmium tetroxide (Merck, Germany), for 2 hours at room temperature, followed by a second wash step. The biofilms were dehydrated in an ethanol series (50 % for 20 min, 70 % for 20 min, 95 % for 20 min, 100 % for 1 hour) and air-dried in a desiccator. The biofilms were then subjected to critical point drying (Samdri®-795 Critical Point Dryer, Tousimis, United States of America) and coated with gold, using a scanning electron microscopy coating system (EM ACE600, Leica, Austria) for 30 minutes. The biofilms were examined using a JSM-7800F Extreme-resolution Analytical Field Emission Scanning Electron Microscope (ZEISS, Germany).

Preformed biofilm inhibition

The efficacy of the SSAs against mature biofilms was also evaluated using a modified microtitre biofilm eradication assay. *C. albicans* SC5314 biofilms were cultivated as previously described for 48 hours at

37 °C in the wells of a 96-well plate (Greiner Bio-One, Germany). After mature biofilm formation, selected SSAs (final concentration of 2.56 mM) were added to the biofilms, incubated for 10, 20, 60 and 120 minutes, and OD_{595} measured (Haney et al. 2018).

Antimicrobial adjuvant activity of SSAs

In order to determine the adjuvant activity of the SSAs, it was first necessary to determine the BMIC of the antimicrobials, colistin (Sigma-Aldrich®, Merck, Germany) and fluconazole (Sigma-Aldrich®, Merck, Germany) (Matuschek et al. 2018; Bibi et al. 2021). For this, biofilms were prepared as previously described, and exposed to a concentration range of each antimicrobial. The range for colistin and fluconazole was 512 – 0.25 $\mu g.mL^{-1}$. The MBICs of each of the antimicrobials were determined by measuring the optical density at 595 nm and determining the relevant percentage inhibition.

This MBIC was used in combination with selected SSAs in a modified double-dose response (checkerboard) experiment (Figure 3) to determine if combination therapy is more effective against biofilm formation (Hsieh et al. 1993; Bellio et al. 2021). Biofilms were prepared as described with a serial 2-fold dilution of each selected SSA and, colistin or fluconazole. The final concentrations were 0.64 – 5.12 mM for the SSA candidates, 1 – 8 $\mu g.mL^{-1}$ for colistin and 0.0625 – 0.5 $\mu g.mL^{-1}$ for fluconazole. Cell free and negative controls were included. The test plates were incubated at 37 °C for 48 hours and evaluated using optical density (OD_{595}) and XTT assays.

***Caenorhabditis elegans* propagation**

Caenorhabditis elegans glp-4; sek-1 hermaphrodites, obtained from the *Caenorhabditis elegans* Genetics Centre at the University of Minnesota, were maintained on Nematode Growth Medium (NGM) (2.5 $g.L^{-1}$ peptone, 3 $g.L^{-1}$ sodium chloride and 17 $g.L^{-1}$ agar) supplemented with 1 mL 5 $mg.mL^{-1}$ cholesterol, 1 mL 246.5 $mg.mL^{-1}$ magnesium sulfate, 1 mL 147 $mg.mL^{-1}$ calcium chloride and 298.43 $mg.mL^{-1}$ potassium phosphate buffer and spotted with *Escherichia coli* OP50, at 15 °C. After incubation, *C. elegans glp-4; sek-1* nematodes were transferred from stock plates to freshly prepared NGM plates seeded with *E. coli* OP50. These plates were stored at 15 °C and monitored daily for 6 days (Breger et al. 2007; Peleg et al. 2008; Pukkila-Worley et al. 2009; Porta-de-la-Riva et al. 2012).

E. coli OP50 was stored as aliquots at -80 °C and thawed on ice to inoculate onto Luria-Bertani (LB) (5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ sodium chloride, 10 $g.L^{-1}$ tryptone, 15 $g.L^{-1}$ agar) for 24 hours at 37 °C. After 24-hour incubation, a single colony of *E. coli* OP50 was inoculated in LB broth and incubated for 24 hours at 37 °C. A lawn of *E. coli* OP50 was then inoculated on fresh NGM agar plates and incubated for 24 hours at 37 °C before they were used for *C. elegans* propagation.

***Caenorhabditis elegans* synchronization**

Gravid nematode adults were washed off NGM plates using M9 buffer (0.25 $g.L^{-1}$ magnesium sulfate, 3 $g.L^{-1}$ monopotassium phosphate, 5 $g.L^{-1}$ sodium chloride, 6 $g.L^{-1}$ disodium phosphate) and centrifuged (F-35-6-30 rotor, Centrifuge 5430R, Eppendorf®, United States of America) for 2 minutes at 1500 rpm at room temperature. The supernatant was removed, and the washing step was repeated four times. After washing, 5 mL bleaching solution (250 µL 10 M sodium hydroxide, 1 mL bleach, 3.75 mL dH_2O) was added to the pellet and manually slowly shaken for four minutes. The solution was then centrifuged

for 30 seconds at 1100 rpm at room temperature and the pellet (containing embryos) was washed three times with 10 mL M9 buffer. Finally, the embryos were resuspended in 2 mL M9 buffer and incubated for 24 hours at 25 °C. After incubation, the embryos were plated onto fresh NGM agar plates (seeded with an *E. coli* OP50 lawn) and incubated at 25 °C until L4 nematodes were observed (Breger et al. 2007; Peleg et al. 2008; Pukkila-Worley et al. 2009; Porta-de-la-Riva et al. 2012).

Toxicity assay

Sixty synchronized L4 nematodes per replicate were inoculated in a 6-well plate, containing M9 buffer and the selected SSA compound (2.56 and 1.28 mM) and incubated at 25 °C. The nematodes were examined daily to measure mortality until all nematodes died. If nematodes were unresponsive to manual disturbance or were immobile, they were scored dead and removed from the liquid media. A negative control without SSA was included. This experiment was performed in triplicate (Breger et al. 2007; Peleg et al. 2008; Pukkila-Worley et al. 2009; Porta-de-la-Riva et al. 2012).

Liquid medium pathogenesis assay

Candida albicans SC5314 was inoculated onto Yeast Extract Peptone Dextrose (YPD) (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 24 $g.L^{-1}$ agar) agar plates and incubated for 24 hours at 30 °C. After incubation, a single colony of *C. albicans* SC5314 was inoculated into YPD broth and incubated for 24 hours at 30 °C and standardized to an OD_{595} of 0.8. The standardised cell solution (100 µL) was inoculated onto a Brain Heart Infusion (BHI) (2 $g.L^{-1}$ dextrose, 2.5 $g.L^{-1}$ disodium phosphate, 7.8 $g.L^{-1}$ brain extract, 9.7 $g.L^{-1}$ heart extract, 15 $g.L^{-1}$ agar), to cultivate a *C. albicans* SC5314 lawn, which was incubated for 24 hours at 37 °C.

Synchronised washed L4 nematodes were placed in the centre of the BHI-*C. albicans* plates and incubated for 4 hours at 25 °C. The infected nematodes were washed with 5 mL M9 buffer and transferred to a sterile 15 mL tube and washed four times by centrifugation (6 000 rpm for 5 minutes at 4 °C) (F-35-6-30 rotor, Centrifuge 5430R, Eppendorf®, United States of America) with 5 mL M9 buffer. The resultant nematode pellet was transferred to a sterile Petri dish containing M9 buffer. From the Petri dish, 60 L4 nematodes were isolated and transferred to 2 mL liquid media (90 µL kanamycin 50 $mg.mL^{-1}$, 382 µL BHI, 1528 µL M9 buffer) supplemented with the respective SSA at a concentration of 1.28 or 2.56 mM in a 6-well plate. A negative control without SSA was included. The plate was incubated at 25 °C and monitored daily to determine mortality rates as described above. This was done in triplicate (Breger et al. 2007; Peleg et al. 2008; Pukkila-Worley et al. 2009; Porta-de-la-Riva et al. 2012).

Statistical analyses

For all obtained values regarding biofilm formation an average and standard deviation were calculated. Moreover, data were analysed using a student *t*-test to establish statistically significant differences between data sets. A *p*-value of ≤ 0.05 was considered significant. The antimicrobial adjuvant activity of SSAs could only be performed once. *Caenorhabditis elegans* survival was assessed using the Kaplan-Meier method. Differences in the data were determined using the log-rank test using OASIS 2 and statistical analysis was performed using two-way ANOVA with Bonferroni corrections (Han et al. 2016).

Results and Discussion

SSA antibiofilm screening

The use of amphiphilic supramolecular materials presents an opportunity to design novel antimicrobial compounds (Brahmachari et al. 2010; Lombardo et al. 2015). Hiscock et al. (2016) established a novel library of over 90 anion-spacer-urea molecules with potential antimicrobial activity against planktonic bacteria. To evaluate the efficacy of this novel class of compounds against biofilms, it is first necessary to determine which SSAs display the most promising antibiofilm activity. This was achieved by measuring optical density (Stepanović et al. 2000; Hou et al. 2019). Figure 3 depicts the OD_{595} values of the different biofilms. A horizontal line value (HLV) was calculated using average values of the negative control from all experiments. Measurements below the baseline are indicative of potential antibiofilm activity.

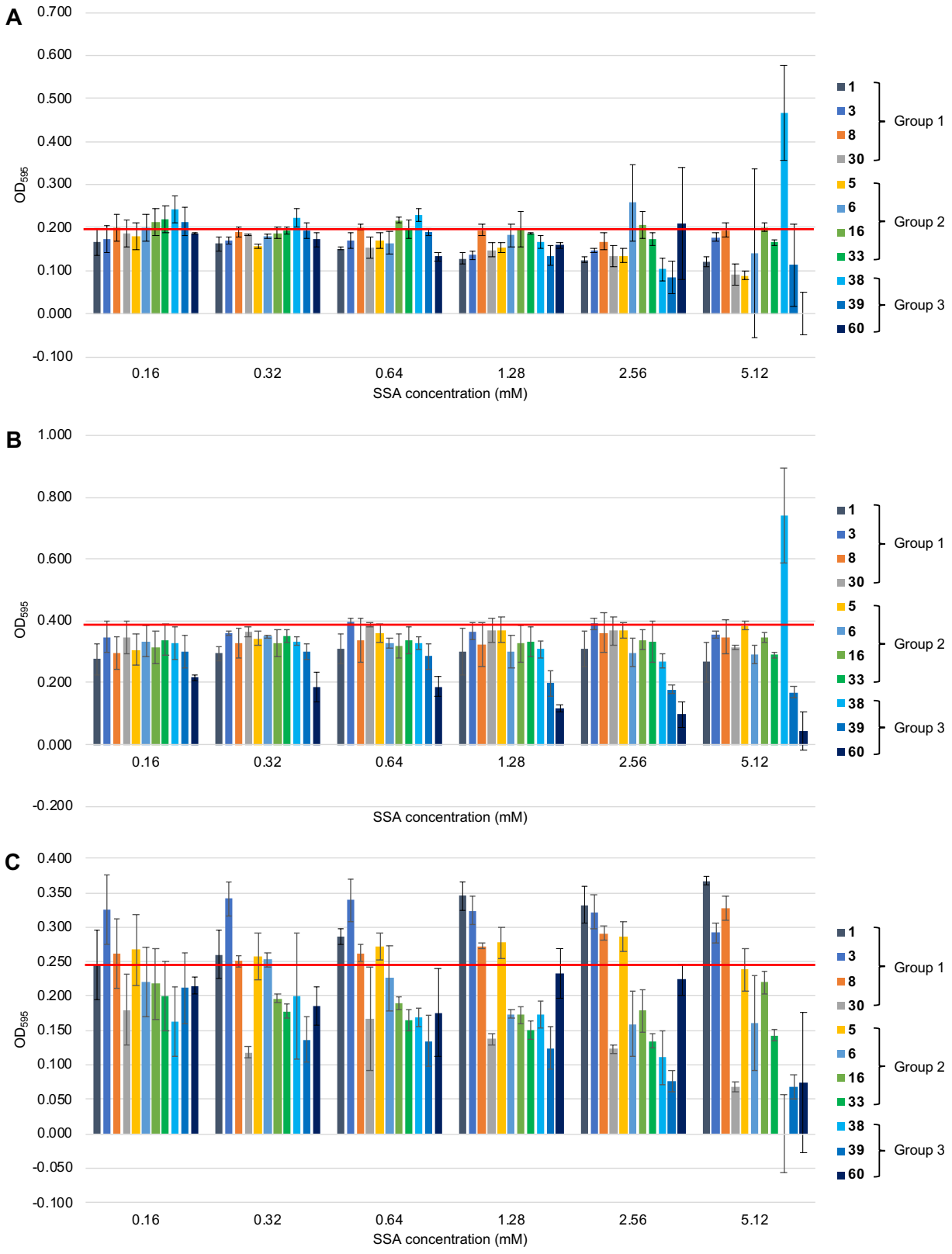


Figure 21. Optical density measurements of biofilms after 48-hour incubation at 37 °C. A horizontal line displays the average negative control for the biofilm models. A) *Pseudomonas aeruginosa* with a horizontal line value of 0.189. B) *Candida albicans* with a horizontal line value of 0.391. C) Polymicrobial biofilm with a horizontal line value of 0.243. Values are the mean of nine repetitions and the standard deviations are indicated by the error bars

Most SSAs displayed some level of inhibition against *P. aeruginosa* (Fig. 3.A) and *C. albicans* (Fig 3.B) mono-microbial biofilm formation. Notably, **SSA 38** presented challenges with solubility and displayed poor inhibitory action. Interestingly, the majority of the SSAs from group 1, except for **SSA 30**, were not effective at inhibiting polymicrobial biofilm formation. However, the other two groups were able to inhibit formation of polymicrobial biofilms (Fig 3.C). By evaluating the performance of all these SSAs across the dilution range, a generalised minimum biofilm inhibitory concentration (MBIC) for the SSAs was selected as 2.56 mM. This is in the range of published MIC_{50s} for a variety of SSAs (White et al. 2021; Rutkauskaite et al. 2023). It should be noted that this generalised MBIC is reflective of the performance of the tested SSAs as a whole and does not necessarily represent the MIC for each individual compound. Table 2 presents the SSAs with the highest percentage inhibition values at MBIC selected for further investigation.

Table 2. Percentage biofilm-formation inhibition of the selected compounds, at the generalised minimum biofilm inhibition concentration of 2.56 mM. Percentage inhibition values were calculated by comparing optical density measurements to a standardised negative control. Standard deviation is indicated in parenthesis

	% Inhibition of biofilm formation		
SSA	***P. aeruginosa***	***C. albicans***	**Polymicrobial biofilm**
30	26 (+/- 4.36)	69 (+/- 8.10)	18 (+/- 4.32)
5	24 (+/- 3.68)	46 (+/- 5.89)	46 (+/- 4.53)
6	18 (+/- 17.10)	84 (+/- 3.14)	25 (+/- 6.60)
33	35 (+/- 4.69)	64 (+/- 3.23)	28 (+/- 2.14)
39	21 (+/- 12.10)	82 (+/- 5.73)	42 (+/- 11.09)

From Table 2, **SSA 30** (group 1) was more inhibitory against biofilm formation of *C. albicans* (69 %) than against *P. aeruginosa* (26 %). However, when employed against a polymicrobial biofilm, the compound displayed only ~18 % inhibition. The decrease in inhibitory potential is unsurprising as polymicrobial biofilms display formidable antimicrobial resistance mechanisms (Orazi and O'Toole 2020) and can show altered responses to antimicrobial substances (O'Brien et al., 2022). SSAs from group 2 also displayed higher activity against *C. albicans* than *P. aeruginosa*. This selectivity is especially evident for **SSA 6**, which displayed the highest inhibition of ~84 % against *C. albicans* biofilm formation, but was less effective than **SSA 5** in inhibiting polymicrobial biofilm formation at only ~25 %. In group 3, **SSA 39** not only displayed a very high percentage inhibition of *C. albicans* biofilm formation (82 %), but also significantly inhibited polymicrobial biofilm formation (42 %) with activity against *P. aeruginosa* biofilms comparable to the other SSAs.

In line with these observations, the modification of the hydrophilic group to a carbamoyl glycinate in group 1 (as seen in **SSA 30**) improved action against both *P. aeruginosa* and *C. albicans*. From group 2, for the SSAs with a sulphonate hydrophilic group the length of the alkane chain corresponds to increased activity and selectivity towards *C. albicans* biofilms. Notably, the carbamothioyl glycinate moiety of **SSA 33** also displays improved action against the bacteria. These observations complement Allen et al. (2020), who observed the incorporation of a carboxylate rather than a sulfonate group promoted inhibitory action against Gram-negative bacteria. From group 3, the addition of a

benzothiazole moiety to the general structure cause solubility problems (**SSA 38**). However, the strategic addition of this moiety (such as in **SSA 39**) promotes antimicrobial action when combined with a sulphonate group, preferentially against *C. albicans*. When considering the structural modifications of **SSA 60** and observations made by Allen and co-workers, the addition of a carboxylate did not improve inhibitory function in the biofilm models.

Metabolic activity antibiofilm assay

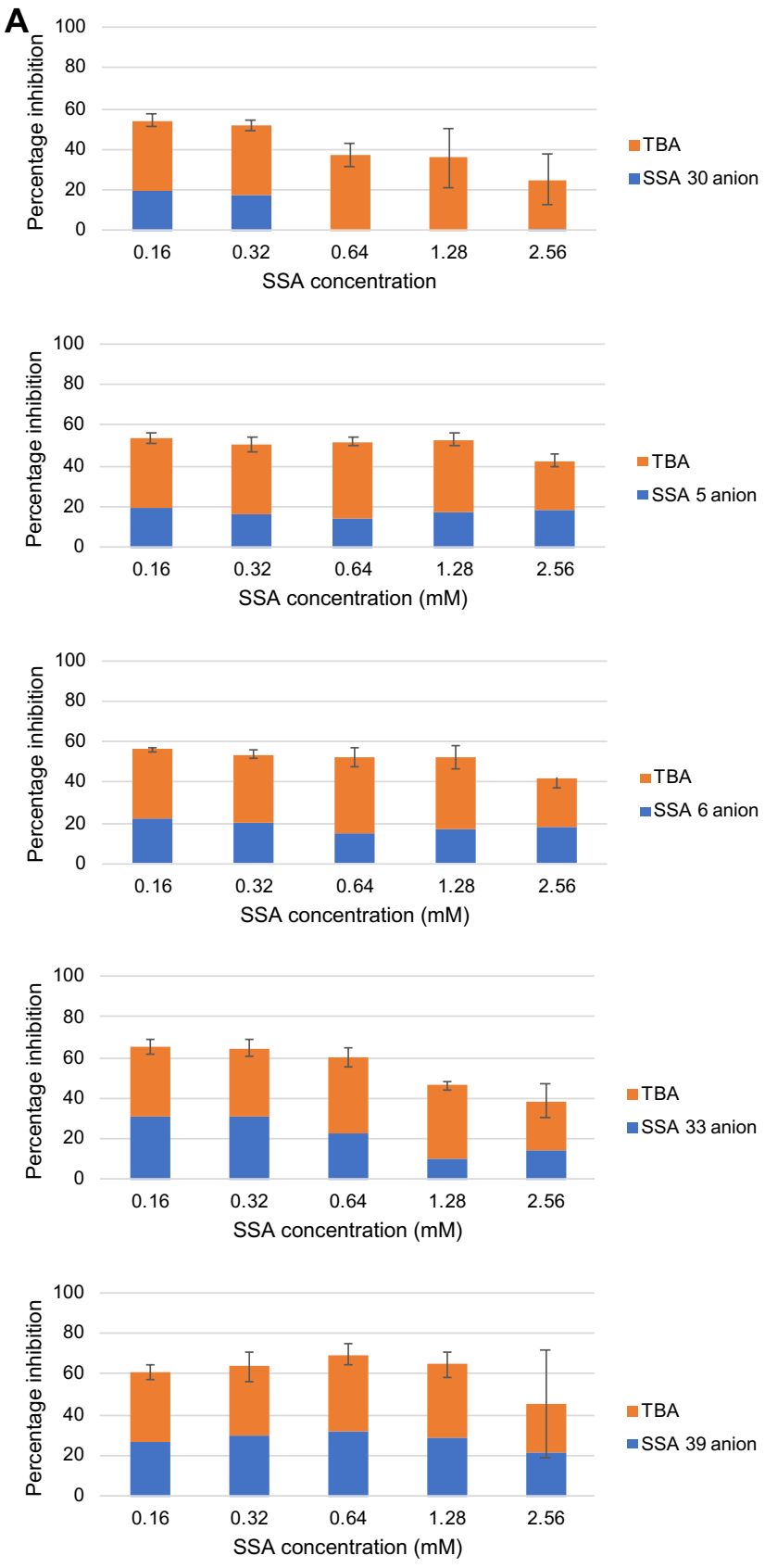
Since optical density measurements, as applied to the screening of SSAs, measured both planktonic and biofilm cells in the wells, the specific activity against only biofilms cells was assayed using the XTT assay, which employs washing steps to remove non-adherent cells. Moreover, the metabolic activity assay is of interest as it determines the influence of the SSA candidates on the viability of the biofilm cells. The XTT assay utilizes the conversion of XTT salt into formazan in the presence of metabolically active cells. The primary mechanism of conversion relies on mitochondrial succinoxidase – and cytochrome P450 systems. The ability of cells to convert XTT to the formazan product indicates a direct relationship between colourimetric signal strength and cell viability (Hawser 1996; da Silva et al. 2008; Dhale et al. 2014).

Figure 4 display the metabolic inhibitory action of the SSA candidates, against the biofilm models at a concentration range of 0.16 – 2.56 mM. From group 1, **SSA 30** shows a dose-dependent response against *P. aeruginosa* and polymicrobial biofilms, with decreasing action as the compound concentration increases (Figures 4.A and 4.C). In contrast, the opposite is observed for the *C. albicans* biofilm (Figure 4.B). The decrease in inhibitory action, with increasing concentration, alludes to a selective interaction between the SSA candidate and the respective biofilm membranes.

From group 2, **SSA 33** displays a similar response profile to **SSA 30**, which may be attributed to the similarity in structural composition (i.e. carbon skeleton and carboxylate). The only difference between **SSA 30** and **33** is a sulphur substitution of the carbonyl carbon from the central urea moiety, which did not impact the antimicrobial activity. Notably, both these substances indicate poor inhibitory action in a polymicrobial biofilm at 2.56 mM (Figure 4.C). Furthermore, **SSA 5** and **6** share similar response profiles in most biofilm models, with the exception of **SSA 6** employed against *C. albicans* biofilms at 2.56 mM.

From group 3, **SSA 39** maintains promising metabolic inhibitory action against the biofilms at most of the concentrations. It is also noteworthy to consider the poor inhibitory action of **SSA 39** against polymicrobial biofilms at lower concentrations, in comparison to the high performing inhibition at 2.56 mM (Figure 4.C). It is interesting to note that **SSA 39** forms hydrogel structures, which may warrant further investigation into the inhibition observed (White et al. 2020a).

The contribution of both the anion and counter cation to the observed inhibitory action is significant as the counter cation used in the synthesis of the SSA candidates is the quaternary ammonium compound, TBA, which has inherent antimicrobial characteristics (Kull et al. 1961; Caratzoulas et al. 2006; Obłąk et al. 2013; Jennings et al. 2016; Marinescu and Popa 2022).


A
Percentage inhibition
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0.16
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SSA concentration
TBA
SSA 30 anion
Percentage inhibition
SSA concentration (mM)
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SSA 5 anion
Percentage inhibition
SSA concentration (mM)
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SSA 6 anion
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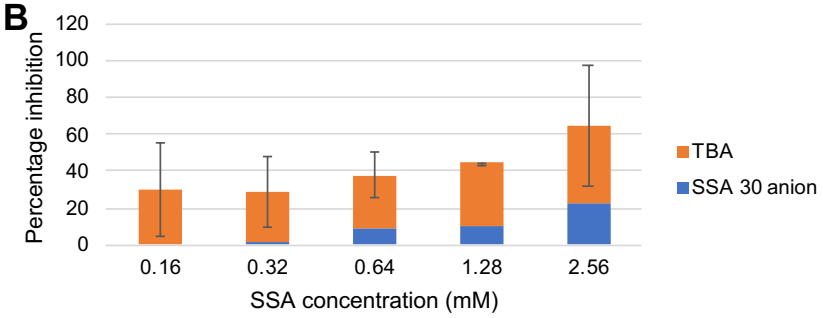
Percentage inhibition
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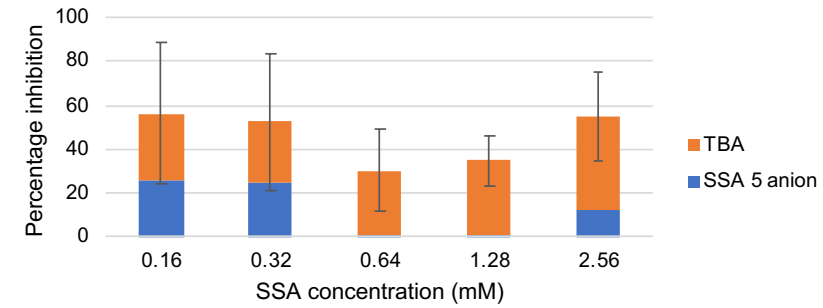
SSA concentration (mM)
TBA
SSA 30 anion


Percentage inhibition
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SSA concentration (mM)
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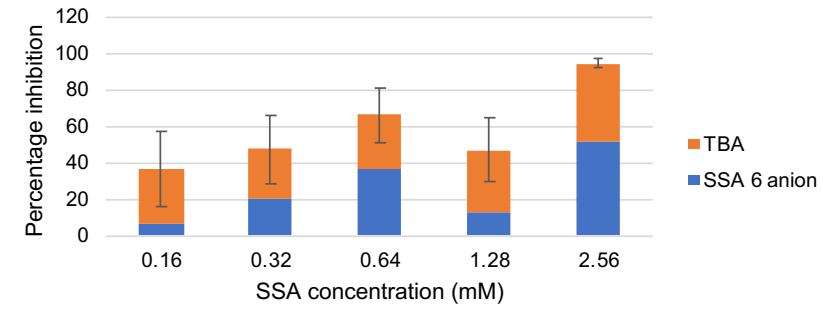
Percentage inhibition
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SSA concentration (mM)
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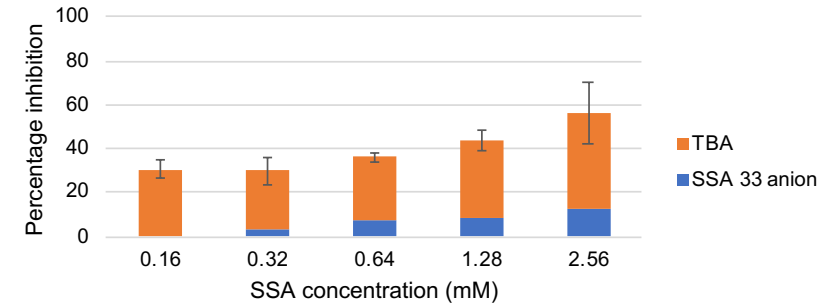
SSA 6 anion


Percentage inhibition
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SSA concentration (mM)
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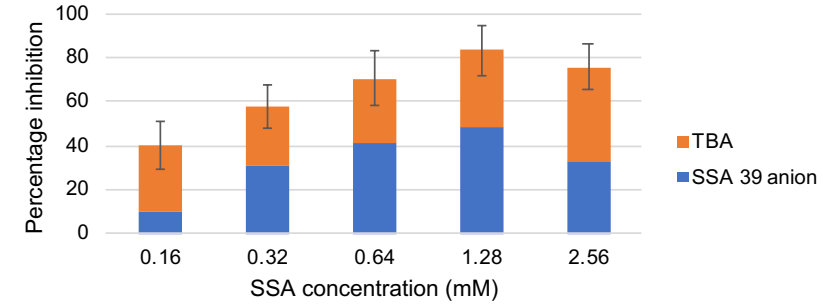
SSA 33 anion


Percentage inhibition
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SSA concentration (mM)
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SSA 39 anion

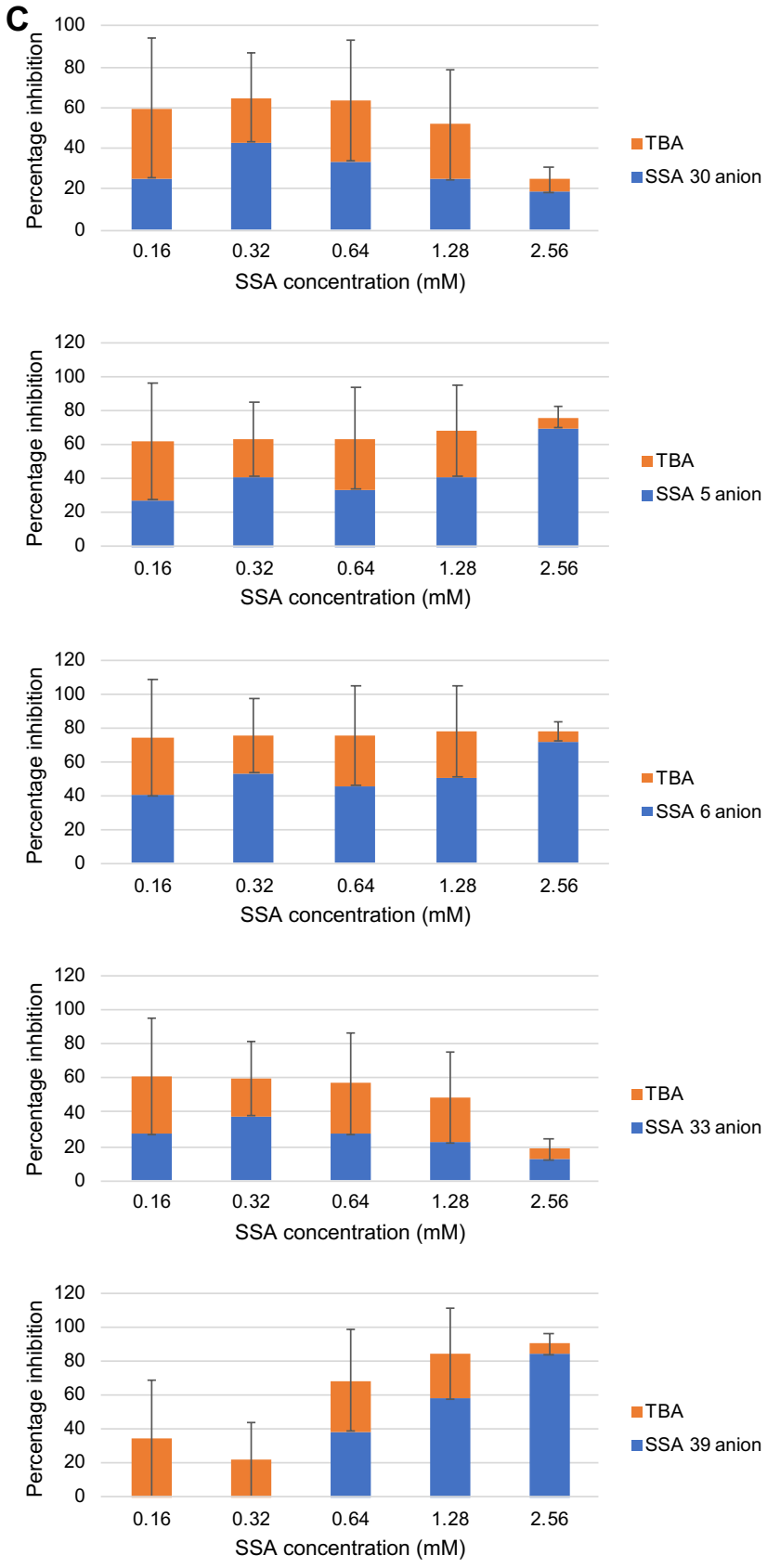

Figure 22. Percentage metabolic inhibitory action of SSA candidates against the respective biofilm models at a concentration range of 2.56 - 0.16 mM where A) *P. aeruginosa*, B) *C. albicans* and C) polymicrobial biofilms. Values are the mean of five repetitions. The standard deviations are indicated by error bars and are representative of the total inhibition observed

For *P. aeruginosa*, the contribution of the anion to the inhibition is generally more evident at lower concentrations (Figure 4.A), with the anions of **SSA 6**, **33** and **39** contributing mostly to the overall inhibition. The inhibitory contribution of the anion of **SSA 39** remains significant in all concentrations employed. Against *C. albicans* biofilms, most SSA anions (with the exception of the **SSA 5** anion) have an increased contribution as the concentration increases (Figure 4.B), indicating their relatively important role in the inhibition observed. For the polymicrobial biofilm (Figure 4.C) a mixed response between those of *P. aeruginosa* and *C. albicans* is seen. Generally, the anionic component of the SSAs contribute significantly to the observed inhibitory action. **SSA 39**, however, shows a dependency to the TBA cation at lower concentrations. Importantly, the complex interaction between the species may also contribute to the overall inhibition seen for the polymicrobial biofilms. For instance, the production of *P. aeruginosa* metabolites such as quorum-sensing molecules alters the fungal cell wall, inhibits fungal hyphal cells and ultimately compromises the pathogen's fitness (Fourie et al. 2016; Phuengmaung et al. 2022). Similarly, the production of farnesol quorum-sensing molecules by *C. albicans* inhibits *P. aeruginosa* virulence factor production and inhibits its pathogenic potential (Cugini et al. 2007). In line with the antagonistic relationship between the pathogens, the compromised fitness of biofilm cells may facilitate the improved inhibitory action observed, especially for **SSA 39**.

Influence of SSAs on the morphology of biofilms cells

Figure 5 depicts scanning electron micrographs of *P. aeruginosa* and *C. albicans* mono- and polymicrobial control biofilms incubated for 48 hours. Importantly, a *C. albicans* monomicrobial biofilm, with smooth yeast, pseudohyphae and hyphae morphologies, is visible. However, only *C. albicans* yeast cells with very short hyphae are visible, confirming that *P. aeruginosa* inhibits hyphal formation *in vitro* (Sudbery et al. 2004; McAlester et al. 2008; Lindsay et al. 2012; Fourie and Pohl 2019; Lewis et al. 2019).

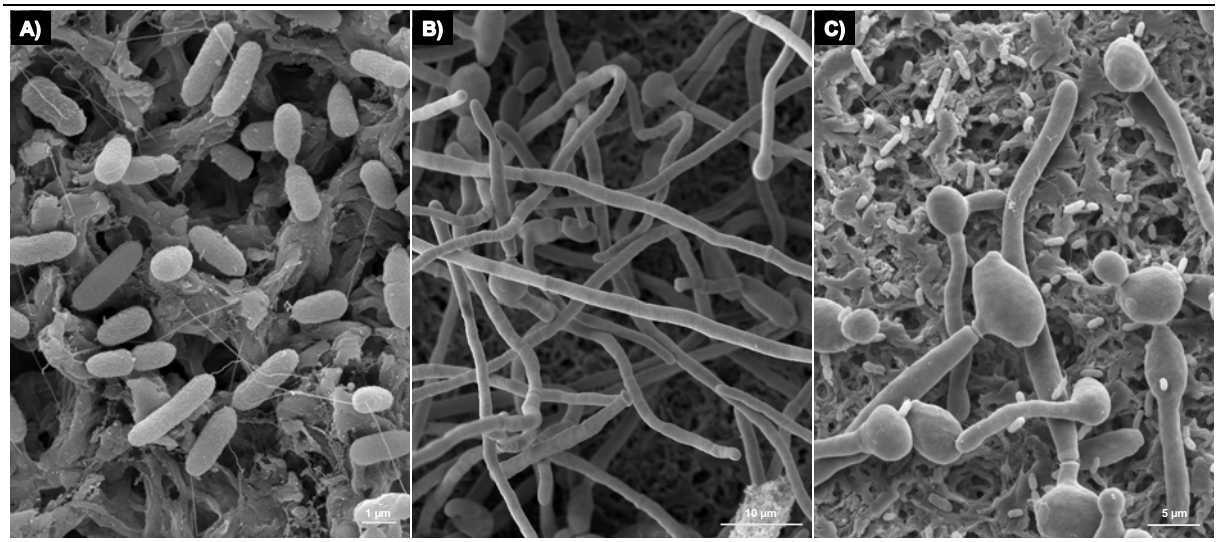

Figure 5. Scanning electron microscopy micrograph of biofilm controls cultivated for 48 hours at 37 °C. A) *P. aeruginosa* monomicrobial biofilm; B) *C. albicans* monomicrobial biofilm; C) polymicrobial biofilm

Figure 6 shows the effect of the selected SSA from group 1 (**SSA 30**) on the biofilm morphology. SEM analysis shows no observable difference in the monomicrobial biofilms (Figures 6.A and 6.B), a few fungal vesicle-like structures are observed in the polymicrobial biofilm (Figure 6.C).

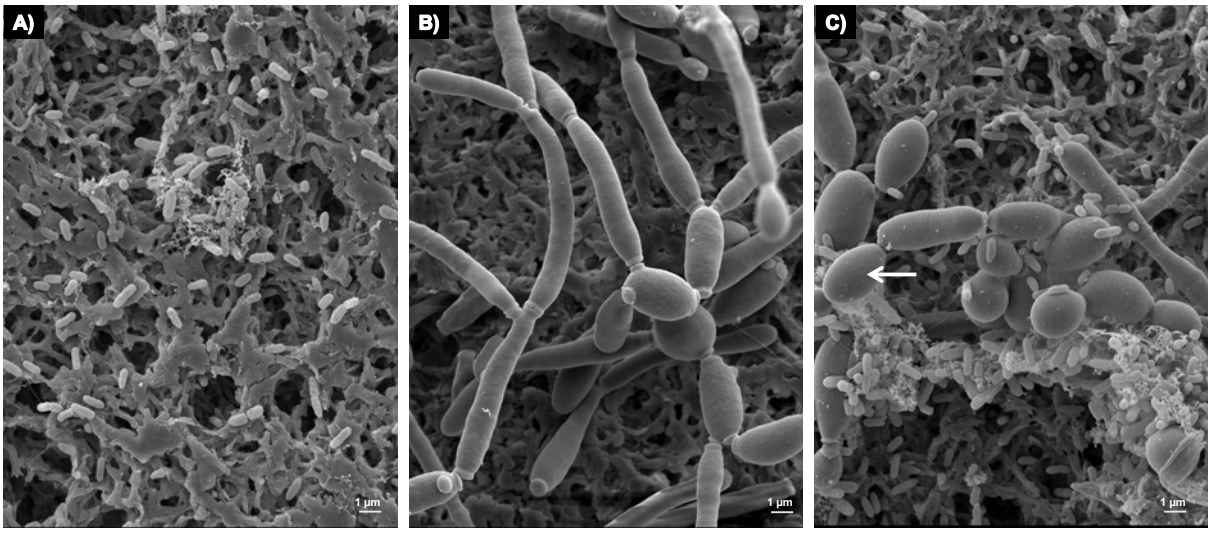

Figure 6. Scanning electron micrograph of biofilms cultivated in the presence of 2.56 mM supramolecular self-associating amphiphile **30** at 37 °C for 48 hours. A) *P. aeruginosa* biofilm; B) *C. albicans* biofilm C) polymicrobial biofilm and arrow indicates the presence of vesicle-like surface structures

It was found that the compounds in group 2 display promising antibiofilm capabilities (Table 2). Morphological differences may be expected in the relevant biofilms. In the *P. aeruginosa* biofilm exposed to **SSA 5**, (Figure 7.A) an excess of extracellular material, possibly EXM, can be observed. Furthermore, Figure 7.B displays abnormalities on the fungal cell surfaces, which are reminiscent of vesicles (Rizzo et al. 2020a; Liebana-Jordan et al. 2021; Zarnowski et al. 2021). These two micrographs may therefore indicate that **SSA 5** could cause increased secretion of substances by both *P. aeruginosa* and *C. albicans*. As expected, **SSA 6** produced very similar results to **SSA 5**. Some extracellular material is visible in the *P. aeruginosa* biofilm (Figure 7.D) and the vesicle-like structures are visible on the hyphae of the *C. albicans* biofilm (Figure 7.E).

The extracellular matrix of a biofilm can be described as a complex organ continuously interacting with and responding to its immediate environment (Chaudhuri et al. 2020). The production of extracellular material is directly correlated to the success of biofilm cells. The EXM of a biofilm imparts spatial context for intercellular signalling events and thereby determines cellular behaviour, proliferation, migration and, ultimately, its survival (Kim et al. 2011). To facilitate the multifaceted interactions between the EXM and biofilm cells, the EXM is constantly undergoing modifications whereby components are overproduced, degraded or modified in response to external stimuli (Yue 2014). In line with this, Yu et al. (2021) observed that self-induced cell lysis promotes biofilm formation in response to external pressures. Their work showed that the presence of additional extracellular DNA contributed to biofilm formation and provided additional protection to antibiofilm substances. When referring to the presence of excess cellular material, as observed in Figures 7.A and 7.D, it may be argued that the *P. aeruginosa* biofilm cells overproduced EXM-materials as a response to cellular stress induced by **SSA 5** and **6**, respectively (Yu et al. 2018; Martins et al. 2021).

The presence of extracellular vesicles (EVs) in fungi was identified and described as ‘protoplasts’ by Gibson and Peberdy in 1972. From this initial finding, various publications have explored the structure and composition of EVs (Gibson and Peberdy 1972; Rizzo et al. 2021). EVs have gained a significant

interest in recent years, due to their contribution to virulence-associated mechanisms. It is important to note that studies on fungal EVs are hindered by the limited understanding of their related biogenesis, composition and cargo specificity profiles (Rizzo et al. 2021). However, literature does indicate a complex interaction between EVs and related pathogens (Albuquerque et al. 2008; Gehrmann et al. 2011; Vallejo et al. 2012; Peres da Silva et al. 2015; Leone et al. 2018; Ikeda et al. 2018; Zhao et al. 2019; Rizzo et al. 2020b). *C. albicans* EVs play roles in cellular organisation, carbohydrate and lipid metabolisms, response to environmental stress and related pathogenesis. Furthermore, Bitencourt et al. (2022) explored the involvement of fungal vesicles in intracellular communication, which resulted in a yeast-to-hypha transition response, prompted after EV production (Bitencourt et al. 2022). Our findings presented in Figures 7.B, 7.E and 7.H support these results with the possible production of fungal EVs in response to cellular stress induced by the SSAs in group 2 (Vargas et al. 2015; Zamith-Miranda et al. 2018; Bielska and May 2019).

Figure 7.C displays a polymicrobial biofilm exposed to **SSA 5**, with completely inhibited hyphal growth. This may be due to the combined effects of the SSA and *P. aeruginosa* as discussed above. Notably, yeast cells here also display possible cell surface modifications with rough surfaces, in comparison to the smooth surface in the control. Figure 7.F shows the morphological effect of **SSA 6** on polymicrobial biofilms, where it is evident that filamentation of *C. albicans* is also considerably inhibited. Similar results are also seen for **SSA 33** (Figure 7.G-I). These findings may suggest that group 2 SSAs display selective morphological inhibitory action in a polymicrobial biofilm.

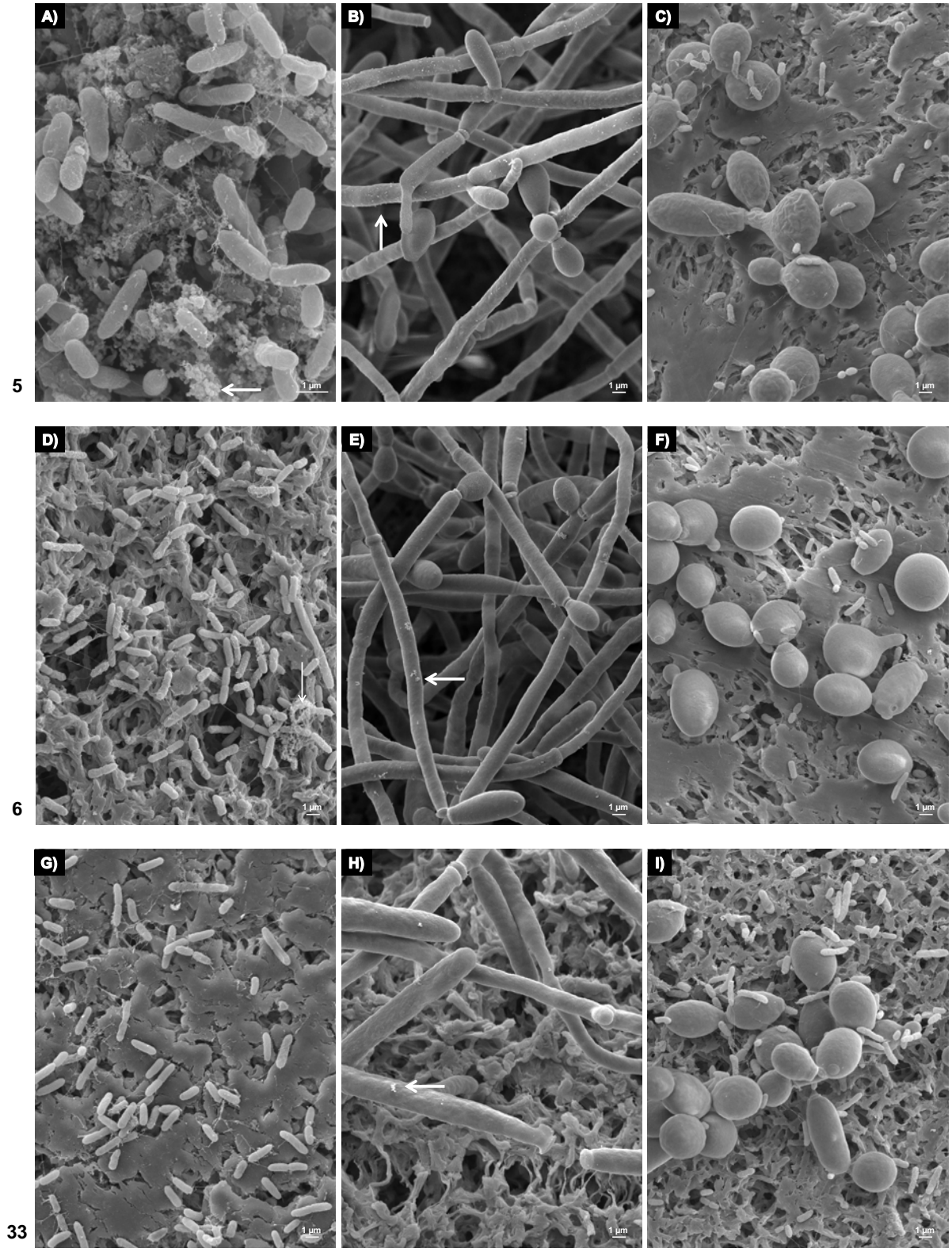

Figure 7. Scanning electron micrographs of mono- and polymicrobial biofilms cultivated at 37 °C for 48 hours in the presence of supramolecular self-associating amphiphile candidates **5** (A-C), **6** (D-F) and **33** (G-I) at 2.56 mM. Arrows indicate the presence of vesicle-like surface structures

Although the group 3 SSA, **SSA 39**, did not cause any significant morphological changes in the *P. aeruginosa* (Figure 8.A) or polymicrobial biofilms (Figure 8.C), an interesting effect was observed on the morphology of *C. albicans* biofilm cells (Figure 8.B). From this micrograph, it is evident that

pseudohyphae and hyphae are significantly inhibited, compared to the control and other SSA experimental groups. This unique morphological inhibitory action complements the observed metabolic inhibition and warrants further investigation.

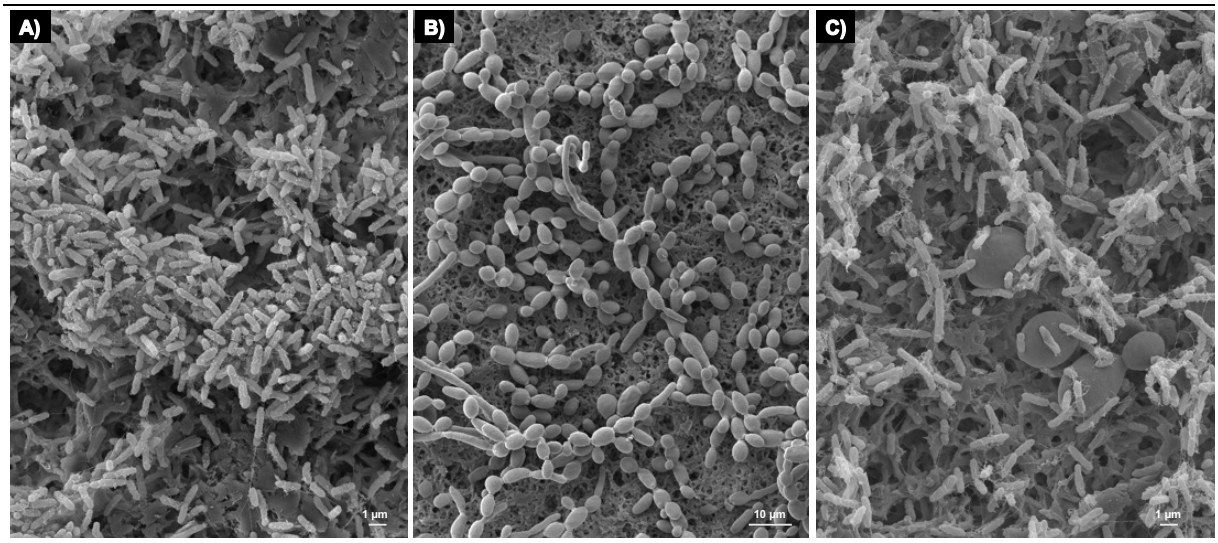

Figure 8. Scanning electron micrographs of mono- and polymicrobial biofilms under different magnifications cultivated for 48 hours at 37 °C. A) *P. aeruginosa* biofilm; B) *C. albicans* biofilm and C) polymicrobial biofilm incubated with 2.56 mM of compound 39

To evaluate the ability of **SSA 39** to inhibit *C. albicans* filamentation during fungal biofilm formation, a sub-MBIC concentration of 1.28 mM was employed in the same manner as described above. Figure 9 indicates the dose dependent effect of **SSA 39** on filamentation in *C. albicans*, with the sub-MBIC still allowing for hyphal formation.

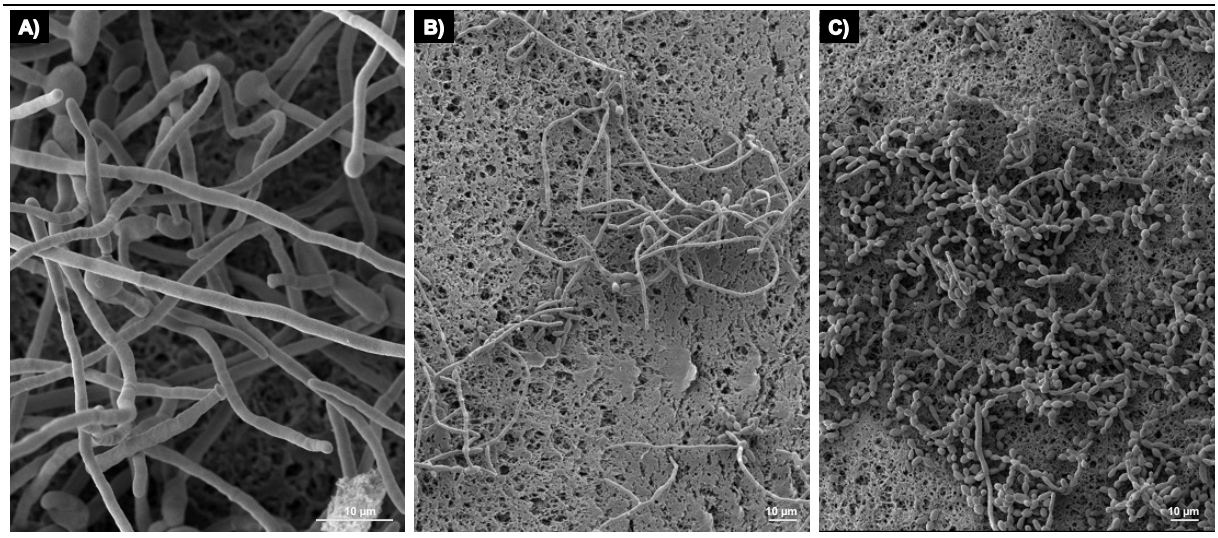

Figure 9. Scanning electron micrographs of *C. albicans* biofilms under different magnifications cultivated at 37 °C for 48 hours. A) Control (untreated) biofilm; B) biofilms cultivated in 1.28 mM of compound 39 and C) biofilms cultivated in 2.56 mM of compound 39

Eradication of preformed biofilms

The complex tertiary composition of a biofilm EXM avails various antimicrobial defence mechanisms through physical, electrostatic and microenvironment considerations (Bridier et al. 2010; Martin et al.

2021; Bridier and Briandet 2022). These formidable resistance and defence mechanisms are employed at the attachment phase of biofilm formation and increase as the biofilm matures (Patel 2005). In line with this, a decrease in antibiofilm activity is expected. Figure 10 shows the optical density measurements of *P. aeruginosa* and *C. albicans* mono- and polymicrobial biofilms exposed to the SSAs for different incubation times. Similar to the screening procedure, negative control optical density measurements were used to calculate a HLV for each respective biofilm model. Optical density measurements below the HLV indicate potential inhibitory action. From this, the selected SSAs were not able to eradicate preformed biofilms of *P. aeruginosa* (Figure 10.A) or polymicrobial biofilms (Figure 10.C), with very large standard deviations observed. However, some eradication of *C. albicans* mature biofilms was seen for all the SSAs, except **SSA 39** (Figure 10.B)**.** Interestingly, this activity was not dependent on contact time.

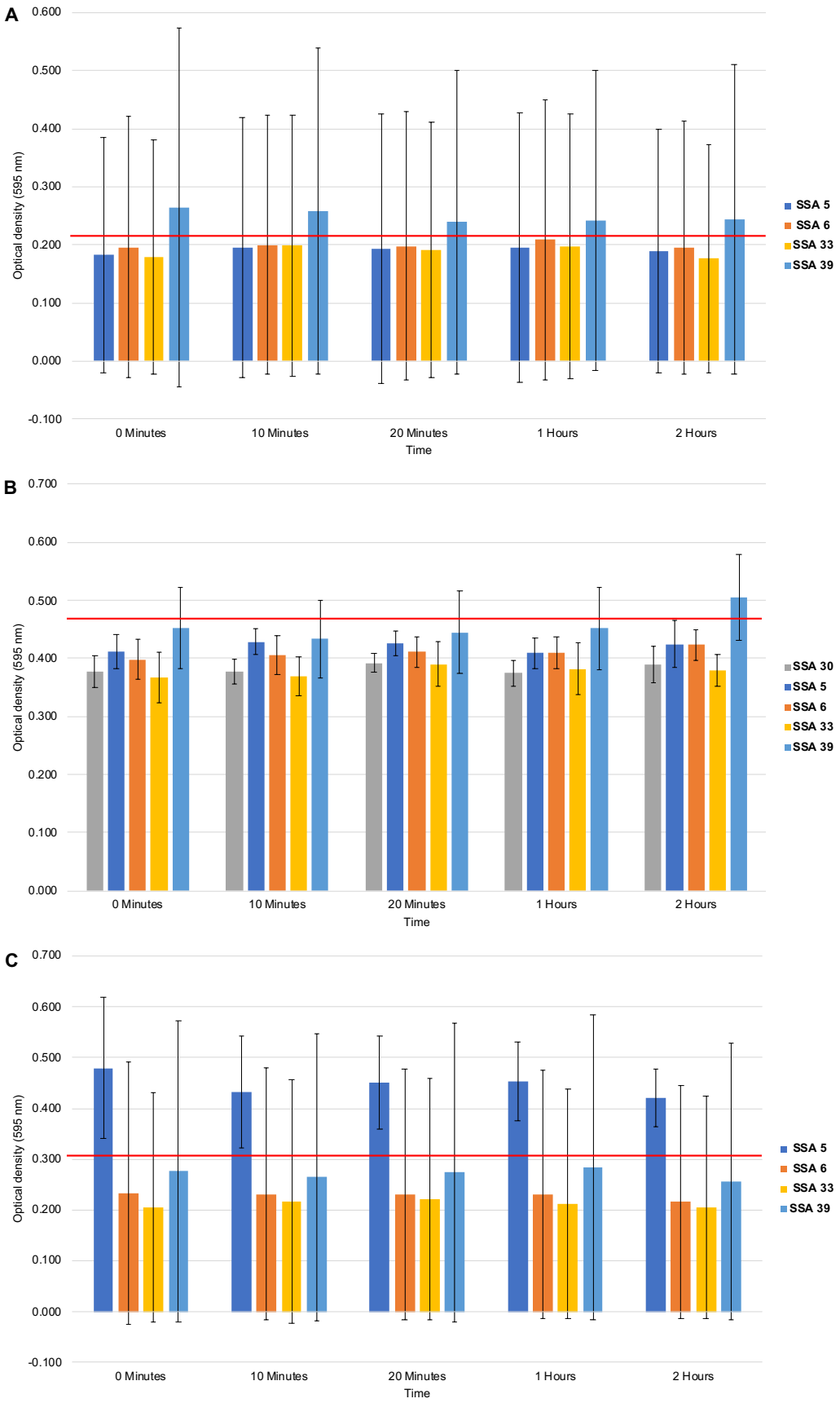

Figure 10. Preformed mono- and polymicrobial biofilms cultivated at 37 °C for 48 hours challenged with supramolecular self-associating amphiphiles for different incubation times. A) *P. aeruginosa*, B) *C. albicans* and C) polymicrobial biofilm. Values are the mean of six repetitions and the standard deviations are indicated by the error bars

XTT assay was performed on *C. albicans* preformed biofilms to determine the metabolic inhibitory action of the SSAs (Figure 11). The most significant and reproducible results were seen after 2 hours contact time, with **SSA 5** and **6** from group 2. This confirms the ability of these SSAs to penetrate the EXM and affect the *C. albicans* cells within a mature biofilm. Although **SSA 39** could inhibit *C. albicans* biofilm formation, it was less effective at eradicating mature biofilms, possibly indicating that this SSA cannot penetrate mature *C. albicans* EXM effectively.

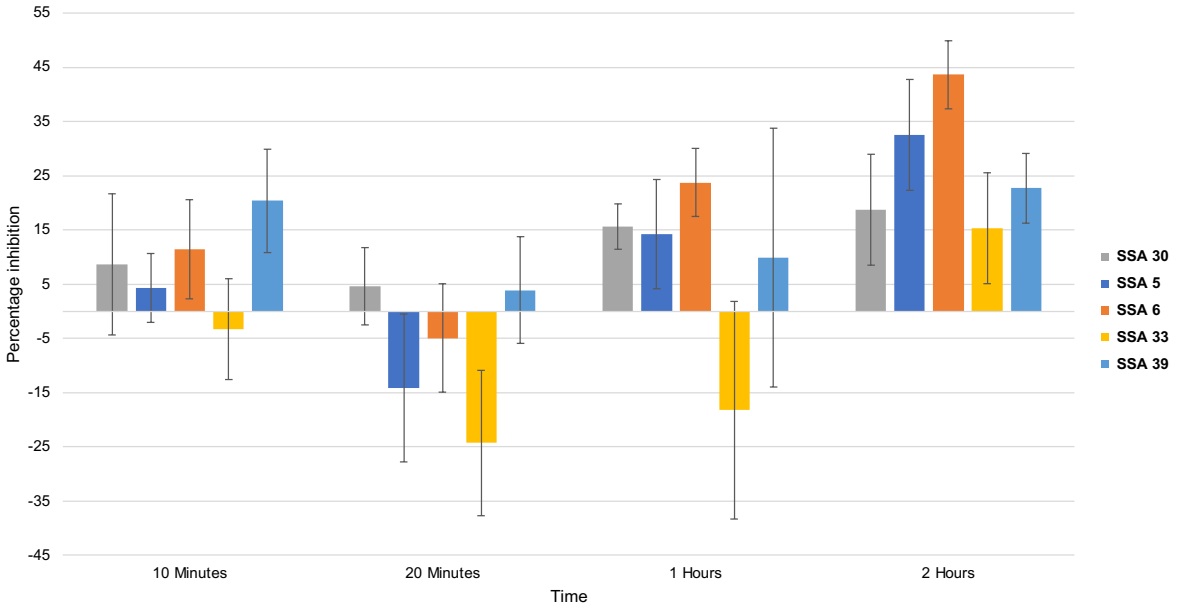

Figure 11. Metabolic activity assay percentage inhibition employed at a concentration of 2.56 mM against preformed *C. albicans* biofilm cultivated for 48 hours at 37 °C. Values are the mean of six repetitions and the standard deviations are indicated by the error bars

SSAs as antimicrobial potentiators

Determination of the minimum biofilm inhibitory concentrations of colistin and fluconazole

The combined use of antimicrobial compounds presents various benefits, broadening the antimicrobial spectrum, providing synergistic interactions may result in improved inhibitory activity (Grassi et al. 2017; Hawas et al. 2022). Also, in the case of polymicrobial infections, the combined use of antibacterials and antifungals can compromise the complex interkingdom tertiary structure (Baronia and Ahmed 2014; Pletz et al. 2017). The use of drug delivery systems such as amphiphilic compounds displayed improved antimicrobial action against biofilms (Smith 2005; Samimi et al. 2018; Vinothini and Rajan 2018; Albayaty et al. 2021; Mota Fernandes et al. 2021). The amphiphilic self-aggregating nature of SSAs have been applied as next-generation drug delivery vehicles (Lu et al. 2018; White et al. 2020b). To determine if the addition of SSAs to standard antimicrobials, colistin and fluconazole, can potentiate the action of these drugs against biofilms, it was needed to first determine the MICs of the two drugs against the various biofilms.

Figure 12 displays the percentage inhibition measurements of biofilms cultivated in the presence of colistin. A distinct and obvious minimum biofilm inhibitory concentration where approximately 50 % of the sample is inhibited ($MBIC_{50}$) is observed and identified as 2 $\mu g.mL^{-1}$ for *P. aeruginosa* (Figure 12.A). These data support current knowledge of colistin reference minimum inhibitory concentrations (MICs)

for Gram-negative bacteria and correlate to EUCAST breakpoints in colistin MICs, where a colistin MIC of ≤ 2 μg.mL^{-1} represents susceptible isolates and a MIC of > 2 μg.mL^{-1} represents resistant isolates (Hengzhuang et al. 2011; Matuschek et al. 2018). These results are supported by the metabolic activity assay (Figure 12.A) with 84 % inhibition at 2 μg.mL^{-1}. To be able to determine the ability of SSAs to potentiate the activity of colistin against polymicrobial biofilms, the inhibition of *C. albicans* biofilms also needed to be evaluated. Figure 12.B shows an apparent 53% inhibition in biomass. However, the inhibition data of metabolic activity is less reproducible (Figure 12.B), with consistent inhibition only observed at >256 μg.ml^{-1}. This could be due to the selective antibacterial nature of colistin. Although the exact mechanism of action for colistin is unknown, it is speculated that the substance shares a mechanism of action with polymyxin B due to their structural similarity. Notably, polymyxin B has been shown to have antifungal activity when used in combination with fluconazole (Zhai et al. 2010). Colistin binds to the anionic phosphate groups of lipid A moieties of lipopolysaccharides (LPS) of Gram-negative bacteria. Thereafter, colistin competitively displaces Mg^{2+} and Ca^{2+} cations from the membrane lipids, resulting in membrane destabilisation (Andrade et al. 2020; El-Sayed Ahmed et al. 2020). The hypothesised mechanism of action for colistin may explain the large standard errors observed.

When looking at the effect of colistin against polymicrobial biofilms, an inhibitory profile similar to that of *P. aeruginosa* is produced with an $MBIC_{50}$ of 2 μg.mL^{-1} (Figure 12.C). At this concentration, colistin displays 62% inhibitory action against the polymicrobial biofilm, which may suggest that colistin targets the bacterial constituent in these biofilms. The efficacy of colistin against the metabolic activity in the polymicrobial biofilm is corroborated by the XTT assay (Figure 12.C), with 2 μg.mL^{-1} colistin causing 78% biofilm metabolic inhibitory action. However, the complex interaction between *P. aeruginosa* and *C. albicans* also needs to be remembered as this may have unknown consequences for colistin action. Furthermore, a study by O'Brien et al. (2022) proved that modulations in a *P. aeruginosa*-*C. albicans* polymicrobial environment caused heritable resistance against colistin and that polymicrobial cultivation increased the frequency of colistin-resistant isolates.

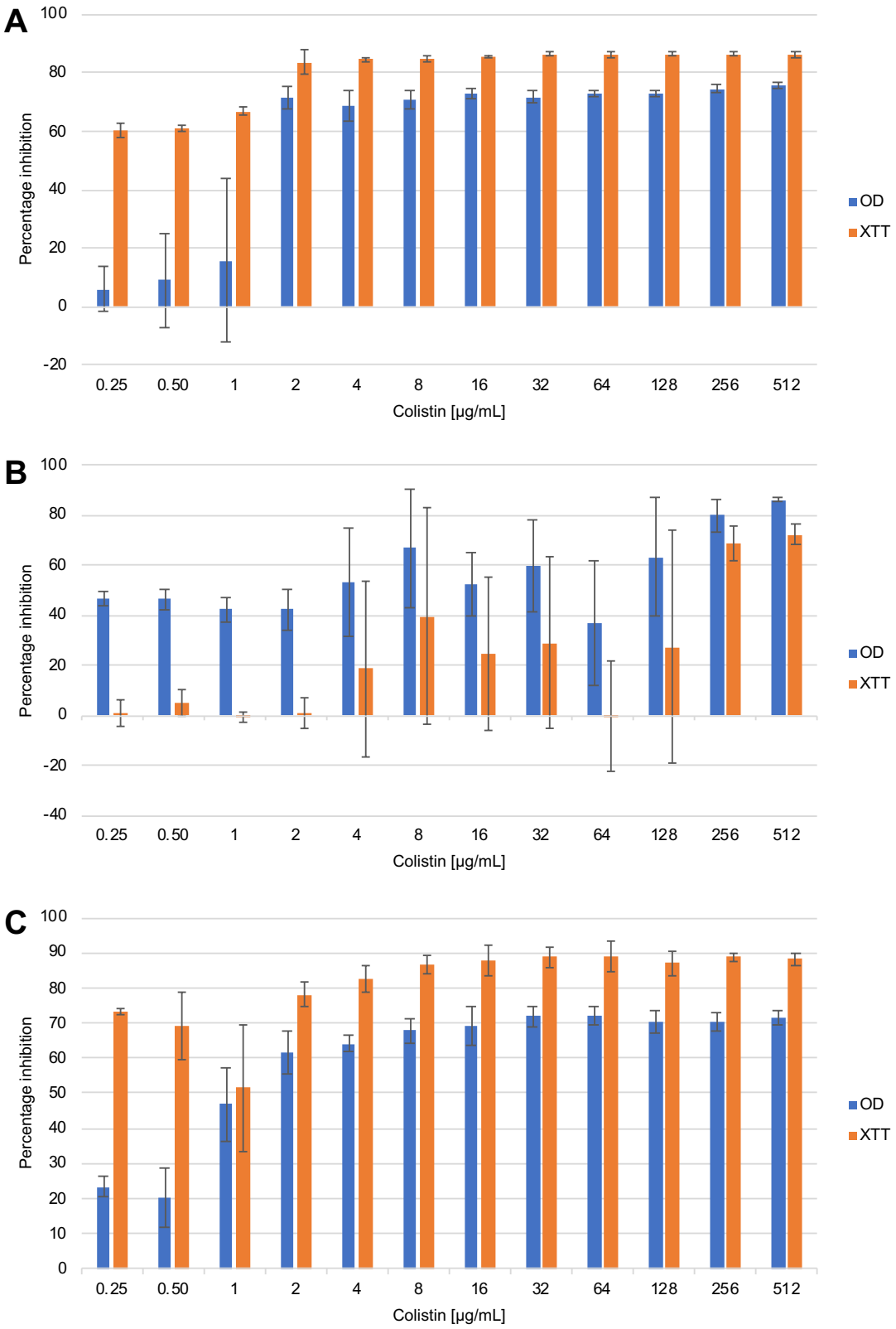

Figure 12. Graphs indicating percentage inhibition values of biomass, and metabolic activity of biofilms cultivated for 48 hours at 37 °C. A) *P. aeruginosa*, B) *C. albicans* and C) polymicrobial biofilms. Values are the mean of six repetitions and the standard deviations are indicated by the error bars

Fluconazole is known to display inhibitory action in a concentration range of 0.25 – 256 $\mu g.mL^{-1}$ (Nagy et al. 2018); thus, the same concentration range was used to determine the MIC of fluconazole in this study (Figure 13). As expected, fluconazole was unable to successfully inhibit bacterial biofilm formation as measured by biomass and metabolic activity and against *C. albicans*, fluconazole had a MIC_{50} of 0.25 $\mu g.mL^{-1}$ (with 65 % inhibition of biomass and 67 % inhibition of metabolic activity). This concentration correlates with known MIC breakpoints (European Committee of Antimicrobial Susceptibility Testing 2022).

Figure 13.C shows the percentage inhibition for fluconazole when employed against a polymicrobial biofilm model. Interestingly, the data does not display the successful inhibition of biomass, but does show a reduction in metabolic activity of the biofilm. The observed lack of biomass inhibition may be attributed to the presence of *P. aeruginosa* and the complex interaction it shares with *C. albicans*. Furthermore, research has suggested that the presence of a *P. aeruginosa* quorum-sensing molecule, N-(3-Oxododecanoyl)-L-homoserine lactone, contributes to *C. albicans* fluconazole resistance by circumventing changes in sterol biosynthesis, promoting drug efflux action and ultimately maintaining the cell membrane's integrity (Bandara et al. 2020).

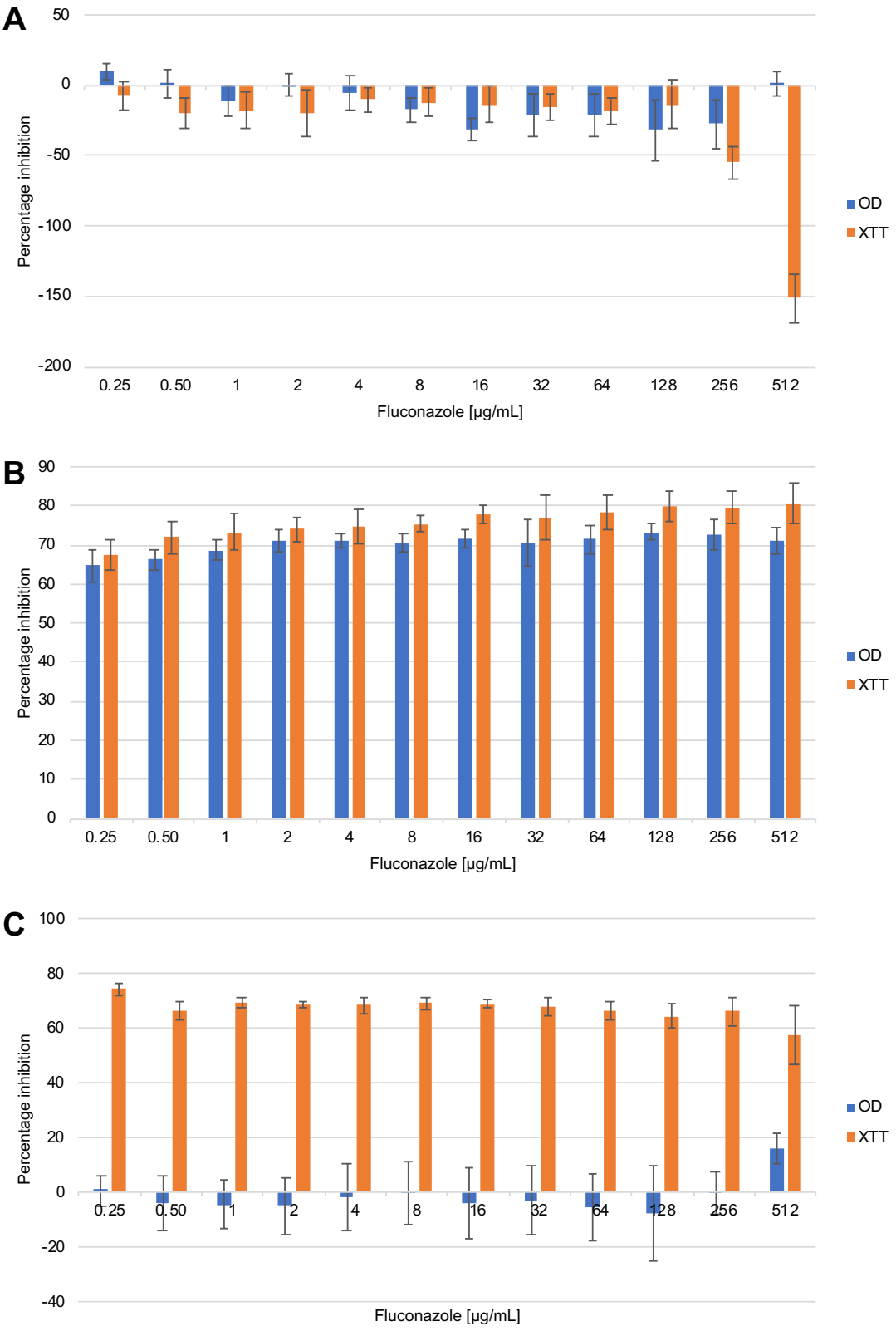

Figure 13. Graphs indicating percentage inhibition values of biomass and metabolic activity of biofilms cultivated for 48 hours at 37 °C. A) *P. aeruginosa*, B) *C. albicans* and B) polymicrobial biofilms. Values are the mean of six repetitions and the standard deviations are indicated by the error bars

Biofilm inhibition by a combination of SSAs and antimicrobial drugs

To determine the efficacy of SSAs as potentiating agents, an adapted checkerboard assay was employed against *P. aeruginosa* and *C. albicans* mono- and polymicrobial biofilms, using the XTT assay for metabolic activity. Table 3 shows the percentage inhibition of metabolic activity for the selected compounds against *P. aeruginosa* biofilm formation. **SSA 30** and **5** improve the antimicrobial action of colistin when used in combination, as seen by the decrease in MBIC. **SSA 30** displays the most promising combinational activity at ~91%. The other SSAs show a decrease in maximum inhibitory action, compared to colistin alone. However, it is important to remember the inhibition observed is at a lower concentration for both colistin and the SSAs.

Table 3. Percentage inhibition of metabolic activity of SSAs, colistin and a combination against *P. aeruginosa* biofilms. Standard deviations are indicated in parenthesis

	SSA		Colistin		SSA + Colistin	
	MBIC (mM)	Inhibition (%)	MBIC (μg.mL^{-1})	Inhibition (%)	MBIC (mM/μg.mL^{-1})	Inhibition (%)
SSA 30	2.56	25 (+/- 12.14)	2	84 (+/- 4.24)	0.64/1	91
SSA 5	2.56	43 (+/- 3.47)	2	84 (+/- 4.24)	1.28/1	86
SSA 6	2.56	42 (+/- 5.31)	2	84 (+/- 4.24)	0.64/1	78
SSA 33	2.56	39 (+/- 8.24)	2	84 (+/- 4.24)	0.64/1	66
SSA 39	2.56	46 (+/- 26.62)	2	84 (+/- 4.24)	0.64/1	60

The combination of colistin and SSAs increases the antibiofilm activity against *C. albicans* (Table 4). **SSA 6** shows a ~100% metabolic inhibitory action at sub-MBIC concentrations and **SSA 39** displays ~94% inhibitory action at a sub-MBIC of 0.64 mM. These data suggest a complementary action between the SSA and colistin against *C. albicans* biofilms.

Table 4. Percentage inhibition of metabolic activity of SSAs, colistin and a combination against *C. albicans* biofilms. Standard deviations are indicated in parenthesis

	SSA		Colistin		SSA + Colistin	
	MBIC (mM)	**Inhibition (%)**	**MBIC (μg.mL^{-1})**	**Inhibition (%)**	**MBIC (mM/μg.mL^{-1})**	**Inhibition (%)**
SSA 30	2.56	22 (+/- 49.29)	4	19 (+/- 35.16)	2.56/1	94
SSA 5	2.56	55 (+/- 20.29)	4	19 (+/- 35.16)	1.28/1	47
SSA 6	2.56	95 (+/- 2.07)	4	19 (+/- 35.16)	1.28/1	100
SSA 33	2.56	56 (+/- 13.86)	4	19 (+/- 35.16)	2.56/1	67
SSA 39	2.56	76 (+/- 10.40)	4	19 (+/- 35.16)	0.64/1	94

In polymicrobial biofilms (Table 5), SSAs from groups 1 and 2 display a decrease in inhibitory action, relative to colistin alone, respectively. However, **SSA 39** showed increased inhibitory activity (~83%) at a sub-MBIC concentration for both the SSA and colistin.

Table 5. Percentage inhibition of metabolic activity of SSAs, colistin and a combination against polymicrobial biofilms. Standard deviations are indicated in parenthesis

	SSA		Colistin		SSA + Colistin	
	MBIC (mM)	**Inhibition (%)**	**MBIC (μg.mL^{-1})**	**Inhibition (%)**	**MBIC (mM/μg.mL^{-1})**	**Inhibition (%)**
SSA 30	2.56	25 (+/- 8.35)	2	78 (+/- 3.62)	2.56/1	58
SSA 5	2.56	76 (+/- 2.50)	2	78 (+/- 3.62)	2.56/2	54
SSA 6	2.56	78 (+/- 4.88)	2	78 (+/- 3.62)	2.56/2	68
SSA 33	2.56	18 (+/- 6.46)	2	78 (+/- 3.62)	2.56/1	32
SSA 39	2.56	90 (+/- 1.12)	2	78 (+/- 3.62)	1.28/1	83

Fluconazole is known as the first line of defence against fungal infections and disease (Charlier et al. 2006) and is not inhibitory against *P. aeruginosa*. Furthermore, the combination of fluconazole and SSAs generally did not increase the inhibition beyond that seen for the SSAs alone (Table 6). For **SSA 30**, the **SSA 30**-fluconazole combination (at 2.56 mM/ 0.25 μg.mL^{-1}) increased inhibitory action, compared to the SSA alone. In contrast, even at twice the MBIC concentration combination, **SSA 33** display no inhibition against *P. aeruginosa*, possibly due to an unfavourable interaction between the two compounds.

Table 6. Percentage inhibition of metabolic activity of SSAs, fluconazole and a combination against *P. aeruginosa* biofilms. Standard deviations are indicated in parenthesis

	SSA		**Fluconazole**		**SSA + Fluconazole**	
	MBIC (mM)	**Inhibition (%)**	**MBIC (μg.mL^{-1})**	**Inhibition (%)**	**MBIC (mM/μg.mL^{-1})**	**Inhibition (%)**
SSA 30	2.56	25 (+/- 12.14)	0.25	0 (+/- 9.50)	2.56/0.25	63
SSA 5	2.56	43 (+/- 3.47)	0.25	0 (+/- 9.50)	2.56/0.125	45
SSA 6	2.56	42 (+/- 5.31)	0.25	0 (+/- 9.50)	2.56/0.125	42
SSA 33	2.56	39 (+/- 8.24)	0.25	0 (+/- 9.50)	5.12/0.50	0
SSA 39	2.56	46 (+/- 26.62)	0.25	0 (+/- 9.50)	1.28/0.125	43

Table 7 displays an increase in inhibitory concentration for the combination of most SSAs with fluconazole against *C. albicans* biofilms. **SSA 30** is able to lower the BMIC of fluconazole to 0.0625 μg.mL^{-1}, while increasing the percentage inhibition to ~75 %. Although **SSA 6**, **33** and **39** can reduce the BMIC of fluconazole to the same level with an increase in percentage inhibition, this was only seen at high concentration of the SSAs (5.12 mM).

Table 7. Percentage inhibition of metabolic activity of SSAs, fluconazole and a combination against *C. albicans* biofilms. Standard deviations are indicated in parenthesis

	SSA		Fluconazole		SSA + Fluconazole	
	MBIC (mM)	Inhibition (%)	MBIC ($\mu g.mL^{-1}$)	Inhibition (%)	MBIC ($mM/\mu g.mL^{-1}$)	Inhibition (%)
SSA 30	2.56	22 (+/- 49.29)	0.25	68 (+/- 3.98)	2.56/0.0625	75
SSA 5	2.56	55 (+/- 20.29)	0.25	68 (+/- 3.98)	0.64/0.0625	57
SSA 6	2.56	95 (+/- 2.07)	0.25	68 (+/- 3.98)	2.56/0.0625	87
SSA 33	2.56	56 (+/- 13.86)	0.25	68 (+/- 3.98)	5.12/0.0625	96
SSA 39	2.56	76 (+/- 10.40)	0.25	68 (+/- 3.98)	5.12/0.0625	84

For polymicrobial biofilms, most of the SSAs could also reduce the BMIC of fluconazole and significantly increase the percentage inhibition (Table 8). However, the concentration of SSA needed generally is 2.56 or 5.12 mM.

Table 8. Percentage inhibition of metabolic activity of SSAs, fluconazole and a combination against polymicrobial biofilms. Standard deviations are indicated in parenthesis

	SSA		Fluconazole		SSA + Fluconazole	
	MBIC (mM)	Inhibition (%)	MBIC ($\mu g.mL^{-1}$)	Inhibition (%)	MBIC ($mM/\mu g.mL^{-1}$)	Inhibition (%)
SSA 30	2.56	25 (+/- 8.35)	0.25	74 (+/- 2.32)	2.56/0.125	69
SSA 5	2.56	76 (+/- 2.50)	0.25	74 (+/- 2.32)	5.12/0.125	92
SSA 6	2.56	78 (+/- 4.88)	0.25	74 (+/- 2.32)	5.12/0.0625	79
SSA 33	2.56	18 (+/- 6.46)	0.25	74 (+/- 2.32)	0.64/0.125	58
SSA 39	2.56	90 (+/- 1.12)	0.25	74 (+/- 2.32)	5.12/0.250	95

The application of SSAs to improve antimicrobial inhibitory action presents the opportunity to investigate alternative treatment options and strategies against opportunistic pathogens, such as *P. aeruginosa* and *C. albicans*, and their related biofilms (White et al. 2020b; Boles et al. 2021; Dora et al. 2021). In combination with the last resort antibacterial agent, colistin, SSAs may be potentiating agents. From these data, **SSA 30** shows an inhibitory action against *P. aeruginosa* biofilms. Whereas **SSA 39** suggests the most potential against *C. albicans* and polymicrobial biofilms, with the lowest MBIC concentrations required for the most inhibitory action. In contrast, when used in combination with fluconazole, the SSAs generally did not improve the antimicrobial inhibitory action against *P. aeruginosa* biofilms. **SSA 30**, however, showed potential combined action at a MBIC fluconazole concentration of 0.25 $\mu g.mL^{-1}$.

For both the *C. albicans* and polymicrobial biofilms, the SSAs required a double MBIC concentration to improve fluconazole inhibitory action with two exceptions being observed. **SSA 5** displayed potential inhibitory action at sub-MBIC concentrations of both the SSA and fluconazole. **SSA 33** displayed similar results against the polymicrobial biofilm but only inhibited biofilm formation at 58%. From these results, it may be speculated that the SSAs display possible potentiating abilities. However, further investigation is required to establish the reproducibility of the data and to determine the scope of combinational-application.

***Caenorhabditis elegans* toxicity and infection assays**

The use of the model organism *Caenorhabditis elegans* as an experimental system provides various advantages to evaluating the efficacy of antimicrobials. The small hermaphroditic nematode utilizes simple cultivation and maintenance conditions and rapid reproduction times produce invariant lineages. The organism displays a sophisticated innate immune system in its epithelial cells, which act as the primary line of defence against pathogens and infection. In addition, through the response systems, *C. elegans* facilitates the understanding of vertebrate innate immune response systems in a host-pathogen interaction (Kurz and Ewbank 2000; Markaki and Tavernarakis 2010).

Based on the promising effects of **SSA 39** in the various *in vitro* experiments, this compound was selected for use in *in vivo* studies to determine the toxicity of the compound in an animal model (Porta-de-la-Riva et al. 2012). Moreover, **SSA 39** displayed selective *C. albicans* inhibitory action and was employed in a *C. elegans* infection assay.

Figure 14 and Table 9 display the toxicity screening, (assessed using the Kaplan-Meier method) and log-rank test results (using OASIS 2 with appropriate statistical analyses). From these results, it is evident that control nematodes maintain a high survival percentage. Fluconazole (at a final concentration of 0.25 $\mu g.mL^{-1}$) killed ~30% of the nematodes over the incubation period. However, at final concentrations of 1.28 mM and 2.56 mM, *C. elegans* responded negatively in a dose-dependent manner to **SSA 39.** From these data, it is evident that at concentrations with antimicrobial potential, **SSA 39** is toxic to *C. elegans*.

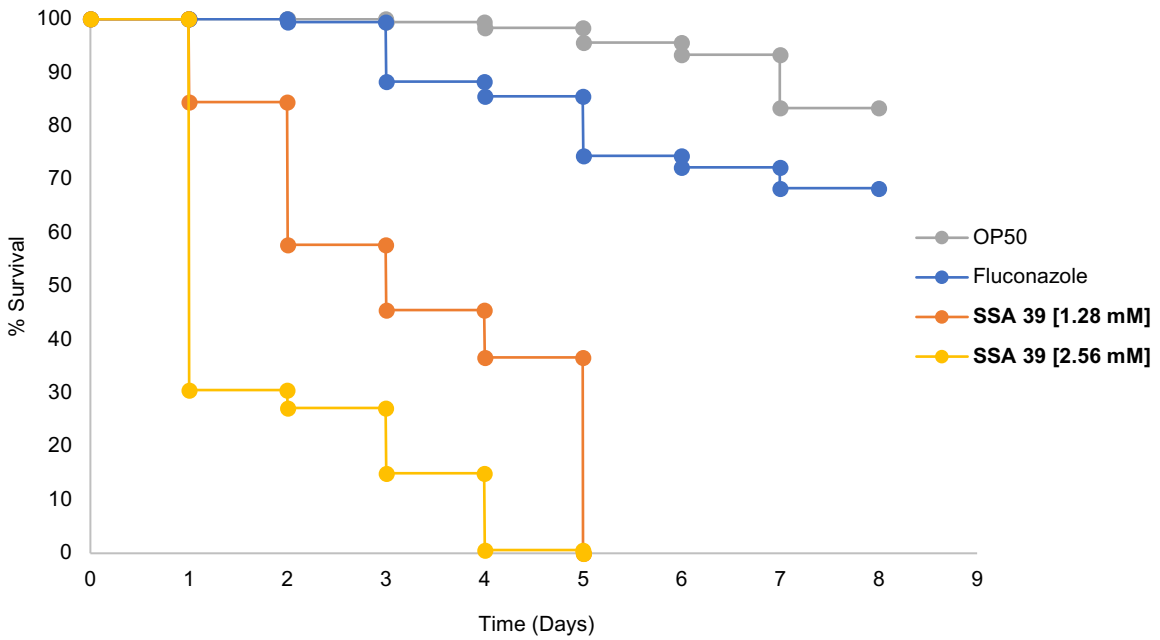

Figure 14. *Caenorhabditis elegans* Kaplan-Meier graph is indicative of nematode percentage survival. A control group (nematodes incubated after being fed *E. coli* OP50) was used for substance toxicity comparison

Table 9. *Caenorhabditis elegans* toxicity assay data. The median lifespan of each test group (with standard error) is indicated with days to reach 50 % mortality. Bonferroni values are included for the log-rank test of comparative differences in survival

Conditions	Median lifespan (Days)	Standard error (S.E)	Days to reach 50 % survival	Log-rank test *p*-value (Bonferroni *p*-value)
***E. coli* OP50**	6.20	0.34	7	0
Fluconazole	4.42	0.30	5	0.0267 *
SSA 39 [1.28 mM]	3.77	0.24	4	< 0.0007 *
SSA 39 [2.56 mM]	1.72	0.15	1	< 0.05 *

Asterisk (*) indicate a significant difference from the experimental models compared to the *E. coli* OP50 control group.

From personal communication, the Hiscock group has shown that an *E. coli* OP50 seeded agar plate (0.10 mM **SSA 39**) reports limited toxicity against L4 nematodes (unpublished data, 2023). Furthermore, current literature shows that **SSA 39** at a final concentration of 1.25 μM, is not toxic to normal human dermal fibroblasts when used in combination with the anticancer substance, cisplatin (Dora et al. 2021). In addition, preliminary toxicity studies investigating adamantane-appended SSAs (related to group 1 and 3 compounds) showed that the substances were not toxic against human erythrocytes, and a concentration of > 5 mM was required to induce 10 % haemolysis. Also, preferential action against MRSA bacterial cells over human erythrocytes was displayed at the final concentration. Furthermore, Rutkauskaite et al. investigated the toxicity of these adamantane-appended compounds against the *Galleria mellonella* moth larvae model at a concentration of 5 mM. In this study, a group 3-related

adamantane-appended substance was acutely toxic, displaying a 60 % death rate within 24 hours, with the remainder of larvae surviving the five-day experiment (Rutkauskaite et al. 2023).

Although the SSA was toxic to the nematodes, the ability of **SSA 39** to inhibit infection by *C. albicans* was determined. From Figure 15, it is evident that *C. elegans'* survival was significantly reduced when infected with *C. albicans* SC5314. It is known that *C. albicans* accumulates in the nematode intestinal lumen (Pukkila-Worley et al. 2009). Fungal cells are ingested and survive the mechanical pharynx crushing, resulting in intestinal and anal swelling and finally, death (Elkabti et al. 2018). When considering the effect of fluconazole on *C. albicans*, it is expected that an increase in nematode survival would be observed. Fluconazole increase the days to reach 50% mortality by one day. This contrasts with the case where *C. elegans* is exposed to *C. albicans* infection and **SSA 39**. At the highest SSA concentration, the combination decreased the median lifespan, and the time to reach 50% mortality. This shows that the toxicity of the SSA enhances the mortality due to infection. At a concentration of 1.28 mM, a slight decrease in lifespan is seen, without a decrease in time to reach 50% mortality (Table 10).

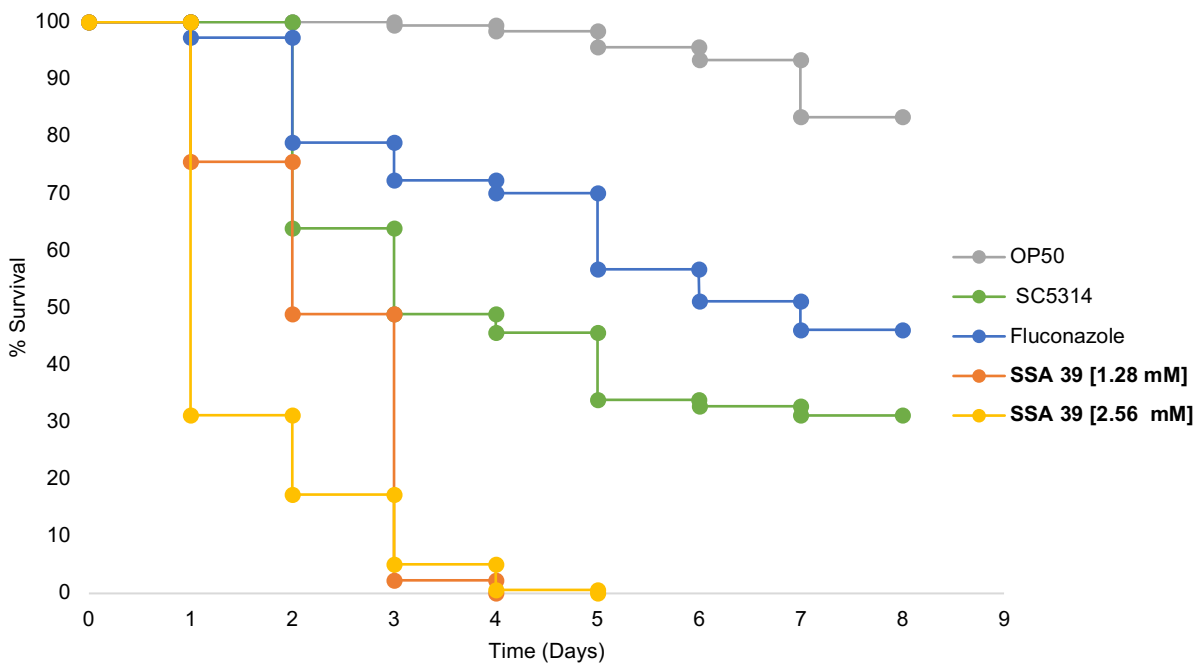

Figure 15. *Caenorhabditis elegans* Kaplan-Meier graph of nematode infection assay percentage survival. Nematodes infected with *C. albicans* indicated an expected decrease in the percentage of survival

Table 10. *Caenorhabditis elegans* infection assay statistical analyses. The median lifespan of each test group (with standard error) is indicated with days to reach 50 % mortality. Bonferroni P-values are included for the log-rank test of comparative differences in survival

Conditions	Median lifespan (Days)	Standard error (S.E)	Days to reach 50 % survival	Log-rank test *p*-value (Bonferroni *p*-value)
***E. coli* OP50**	6.20	0.34	7	0
***C. albicans* SC5314**	3.02	0.21	2	< 0.05 *
Fluconazole	3.72	0.33	3	< 0.05 *
SSA 39 [1.28 mM]	2.25	0.11	2	0.0324 *
SSA 39 [2.56 mM]	1.53	0.12	1	< 0.05 *

Asterisk (*) indicate a significant difference from the experimental models compared to the *E. coli* OP50 control group.

Conclusions

This study evaluated the potential action of novel SSA compounds against mono- and polymicrobial biofilms formed by the opportunistic pathogens *P. aeruginosa* and *C. albicans*. From this study it can be concluded that SSAs indeed have antibiofilm activity and that the specificity towards either *P. aeruginosa* or *C. albicans* may be influenced by the specific structures of the different SSAs. Structure-activity relationship (SAR) is an important tool in the design of novel antimicrobial substances. By analysing the SAR of antimicrobial substances, critical structural features may be identified to facilitate novel antimicrobial compound design with improved activity (King et al. 1996; Woo et al. 2002). Furthermore, structure-activity relationship (SAR) modulations may be extrapolated in terms of the anionic and cationic inhibitory effects. The data presented alludes to preferential interactions between the SSAs and different microbial membranes. A degree of specificity is observed between bacterial and fungal membranes based on structural composition of the SSAs employed. In addition, the ability of the different SSAs to inhibit specific monomicrobial biofilms, does not necessarily predict their ability to inhibit polymicrobial biofilms of the two species. This is expected from the complex nature of the polymicrobial interactions. It can also be concluded that certain groups of SSAs may be more successful in penetrating the EXM and inhibiting mature *C. albicans* biofilms. Interestingly, **SSA 39**, which was able to inhibit biofilm formation in *C. albicans*, was less effective against pre-formed biofilms. This phenomenon needs further study.

The tested SSAs also displayed varying abilities to act as antimicrobial potentiating agents for colistin and fluconazole. This feature should also be investigated further, especially regarding the mechanism of interaction between the different compounds and how these combinations interact with the different cells. Unfortunately, the selected SSA (**SSA 39**) was found to be toxic to *C. elegans* at the concentrations tested and did not protect the nematodes from infection by *C. albicans*. It is proposed that lower concentrations should be evaluated in future, as well as the effects of combination treatment. This data provides additional information regarding structural characteristics influencing antimicrobial activity in order to supplement the design and evolution of the novel library of compounds.

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Chapter 3: Elucidating membrane interactions of supramolecular self-associating amphiphiles

Abstract

Multidrug-resistant pathogens have established effective mechanisms to combat currently available and marketed drugs. The need for novel antimicrobial therapeutic strategies is of immediate interest to global public health. To this end, a novel library of supramolecular self-associating amphiphiles (SSAs) has displayed potential antimicrobial activity against clinically relevant pathogens. This library of compounds has a hypothesized antimicrobial mechanism of action through interaction with bacterially derived phospholipids. In addition, some have been shown to be inhibitory to fungal biofilm formation. However, further elucidation is required to determine if this is also relevant against fungal pathogens. The fluorescent property of SSA 39 allowed the microscopic investigation of the interaction between this SSA and *Candida albicans* SC5314 biofilms, using confocal scanning laser microscopy and indicated a potential membrane interaction. This study also used a novel phospholipid nanodisc coordination assay to study the interaction between SSA 39 and phospholipids isolated from *Candida albicans* NCPF3645 and *Candida auris* WT1912. To do this, membrane phospholipids were isolated to synthesize a mimetic membrane system (nanodiscs) belted with styrene-maleic acid (SMA). After synthesis and purification, phospholipid nanodiscs were characterised and their size was determined by dynamic light scattering (DLS) techniques through measuring the Brownian motion of molecules in solution. The interaction between the phospholipid nanodiscs and SSA 39 was evaluated, using a solution-state ^{1}H nuclear magnetic resonance (NMR) spectroscopy titration technique. The titration results validated the observed SSA-membrane interaction and contribute to the identification of a hypothesized mechanism of action employed by this library of compounds.

Keywords

Candida albicans, *Candida auris*, antibiofilm, phospholipids, nanodisc, membrane mimetic systems

Introduction

The establishment of antimicrobial resistance (AMR) and the growing threat of multidrug-resistant pathogens is of immediate interest to the health of the global public (Domalaon et al. 2019). Antimicrobial resistance has developed through various mechanisms, which include changes in the permeability of cell membranes, upregulation of efflux pumps, acquisition of alternative metabolic pathways to circumvent drug inhibition and mutations to target sites (Mcdermott et al. 2003). Some pathogens have developed resistance mechanisms to more than three antimicrobial categories, known as multidrug resistance (MDR) (Basak et al. 2016). Approaches to addressing AMR and MDR include revisiting infection-prevention and control strategies, revising drug policies, repurposing currently available antimicrobials and the development of novel drugs (Tanwar et al. 2014).

A current antimicrobial mechanism of action is to compromise the integrity of the cell membrane. Antibacterial classes such as β-lactams (penicillin) and polymyxin (colistin) interact with the bacterial cell wall resulting in the disruption of the tertiary structure. β-lactam antibacterials, bind to penicillin-binding proteins thereby preventing the transpeptidation of peptidoglycan strands, which in turn prevents cell wall synthesis (Yotsuji et al. 1988; Lam et al. 2020; Moges et al. 2021). Polymyxin antibacterials interact with the bacterial cell membrane by binding to the anionic phosphate groups of lipid A moieties (of lipopolysaccharides in the bacterial cell wall) which results in cation displacement and membrane disruption (Petrosillo et al. 2008; Aghazadeh et al. 2016; Yousfi et al. 2019; Andrade et al. 2020). Similarly, the antifungal class azole (fluconazole) and polyenes (amphotericin B) interact with the fungal cell membrane, resulting in fungal inhibitory action. Azoles inhibit the fungal cytochrome P450 14α-demethylase enzyme, which prevents ergosterol biosynthesis, and in turn, disrupts the fungal cell membrane (Hargrove et al. 2017; Nishimoto et al. 2020; Ahmadi et al. 2022). Polyenes selectively bind to ergosterol in the fungal cell membrane and induce pore formation, compromising the fungal membrane (Dutcher 1968; Carolus et al. 2020; Schwarz et al. 2022).

Novel antimicrobial drug development strategies utilise targets such as quorum-sensing molecules, cell division machinery and virulence factors (Belete 2019). When considering the mechanisms of action employed by currently available antimicrobial substances, it is evident that microbial phospholipid membranes are promising targets for fighting antimicrobial resistance (Mingeot-Leclercq and Décout 2016). Microbial membranes are formed from amphiphilic lipids, which generally consist of glycerophospholipids (GPLs). GPLs are composed of a glycerol backbone, two fatty acids, a phosphate moiety and a variable headgroup (Figure 1) (Sohlenkamp and Geiger 2016).

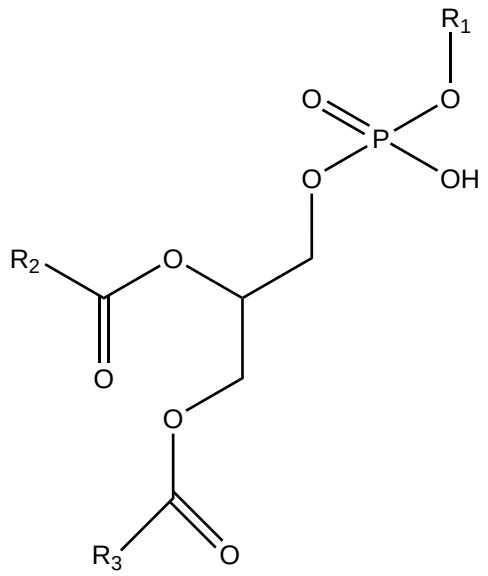

Figure 23. Basic structural backbone of a glycerophospholipid where; R_1 = hydrophilic residue substitution and R_2 and R_3 = hydrophobic fatty acid residue substitutions

It is established that there is no standard blueprint for microbial membrane compositions. The composition of a microbial membrane is directly related to the culture conditions, life cycle phase and environmental factors, however, some general features are found in specific groups of microbes. Bacteria, such as *Pseudomonas aeruginosa*, contain three main GPLs namely phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC) (Figure 2) (Comerci et al. 2006; Tashiro et al. 2011; Sohlenkamp and Geiger 2016).

In comparison, fungal membranes differ significantly from bacterial membranes not only in GPL composition but also in the presence of membrane sterols (de Kroon et al. 2013; Sant et al. 2016). The main phospholipids of *C. albicans* are PC, PE, phosphatidylinositol (PI) and phosphatidylserine (PS), PG (Figure 2), phosphatidic acid (PA), lysoPC, lysoPE and lysoPG (Khandelwal et al. 2018). It is noteworthy that the ratio of these GLPs also differs between organisms (Perczyk et al. 2020; Shahi et al. 2020). After PC, PE has generally been identified as the second most abundant GPL in the *C. albicans* phospholipid membrane (Mahto et al. 2014; Khandelwal et al. 2016). PE provides a structural advantage to the fungal membrane by forming membrane curvatures which are important for functions such as facilitating opposing membrane-fusion events (Siegel and Epand 1997; Siegel 1999).

GLPs may provide specific targets for the development of novel antimicrobial compounds, which are aimed at disrupting the phospholipid membrane (Hitchcock et al. 1987). To do this, stable phospholipid bilayer models are needed to determine the biologically relevant interactions between GLPs and the potential drugs *in situ*. This bilayer facilitates the formation of distinct hydrophilic and lipophilic sections, which results in amphiphilic behaviour (Faustino et al. 2009; Rai et al. 2016; Pinheiro and Faustino 2017).

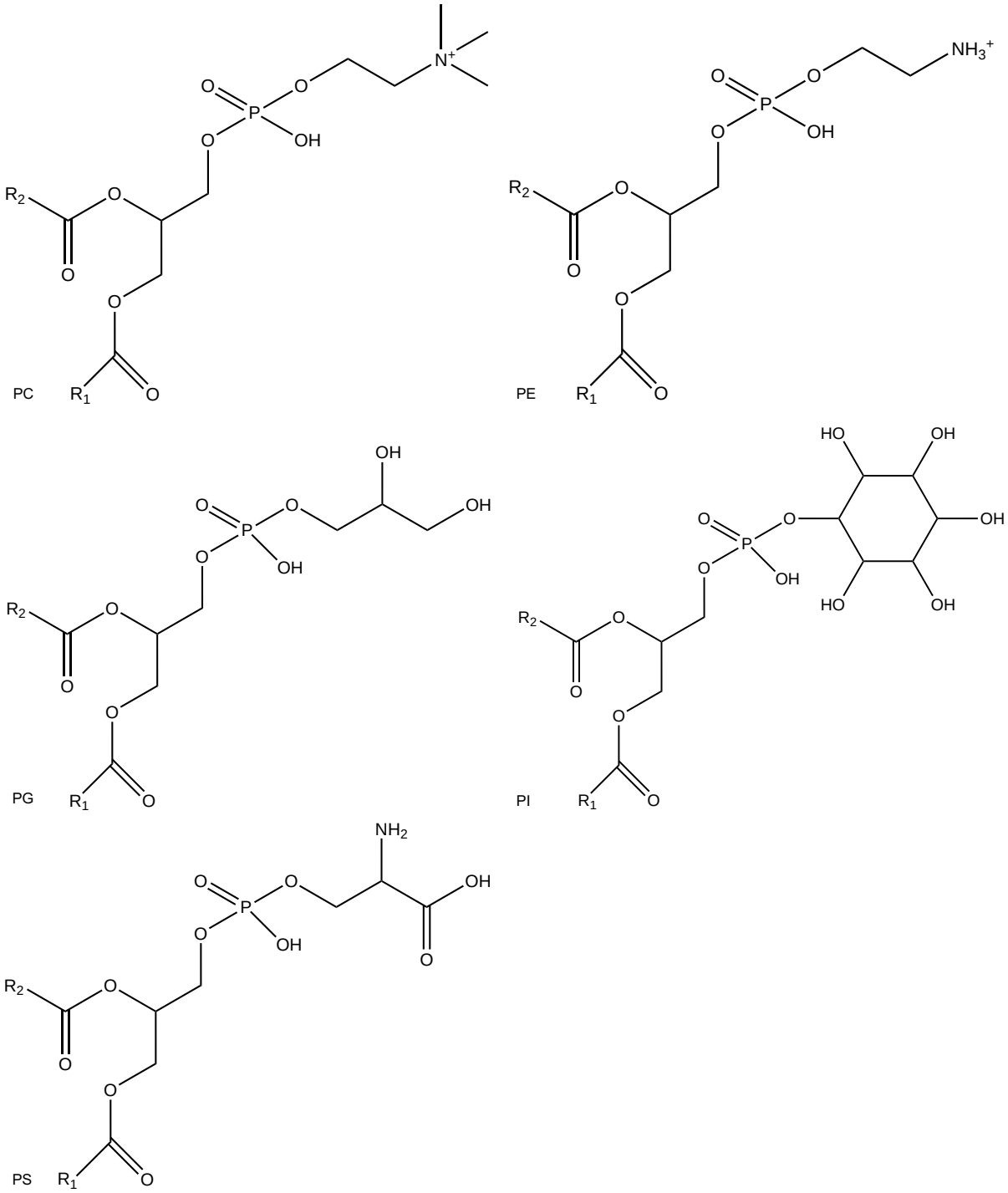

Figure 2. Structure of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS)

Thus, the ability of amphiphiles to self-assemble into superstructures presents the opportunity to design novel compounds with the inherent ability to interact with microbial phospholipid membranes, and potentially employ antimicrobial action (Debnath et al. 2010; Hill et al. 2014; Blackholly et al. 2016). Faustino et al. (2009) developed hydrogen bond donor (HBD)-anion compounds with amphiphilic characteristics to function as surfactants. From their findings, these original constructs were modified to produce anion-spacer-urea-based molecules (Pittelkow et al. 2009). Hiscock et al. (2016) refined

these molecules and established a novel class of sulfonate-urea compounds (Figure 4) with the ability to self-assemble into hydrogen-bonded nanostructures. This class of molecules deemed supramolecular self-associating amphiphiles (SSAs), are synthesized by modifying the basic anion-spacer-urea molecule and counter cation in a stepwise fashion.

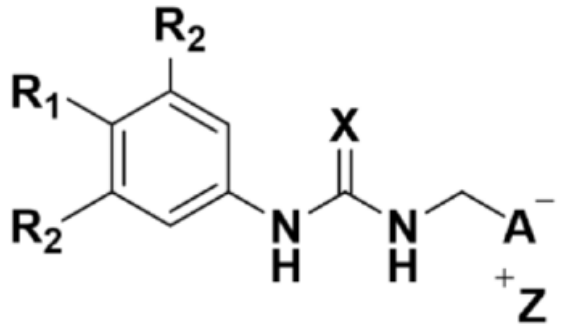

Figure 4. General supramolecular self-associating amphiphile structure where, $R_{1/2}$ = possible moiety modification sites, X = oxygen or sulphur and A = possible moiety modification site. The structure of the relevant sodium, potassium, pyridinium or tetraalkylammonium counter cation ($^{+}$Z) has been omitted for clarity (Blackholly et al. 2016; Hiscock et al. 2016a; Hiscock et al. 2016b)

Furthermore, these compounds were shown to have an antibacterial response against clinical *Staphylococcus aureus* and *Escherichia coli* strains (Tyuleva et al. 2019; Ng et al. 2020). Furthermore, from Chapter 2, SSAs indicated potential application as antifungal substances. Townshend et al. (2020) hypothesized that the potential mechanism of action is based on a selective interaction with phospholipids commonly found in the bacterial membrane, which is facilitated through a complimentary SSA-head group interaction. They hypothesized that the interaction was based on the amphiphilic nature of the SSAs combined with the hydrogen bond donating and accepting groups within the SSA-phospholipid nanodisc molecular scaffold (Figure 5) (Boles et al. 2022).

Bayburt et al. (2002) devised nanoparticulate phospholipid bilayer discs (nanodiscs) that were assembled from phospholipids and stabilised by an amphipathic protein (membrane scaffold protein) or polymer belt, such as styrene-maleic acid (SMA) (Hall et al. 2018). These bilayer discs can be used to study membranes and membrane proteins by mimicking biological systems in a controlled environment (Bayburt et al. 2002; Nath et al. 2007; Ravula et al. 2018). Thus, nanodiscs present the opportunity to elucidate the interaction between potential antimicrobial SSAs and membrane systems (Denisov and Sligar 2016; Townshend et al. 2020). This property was used by Boles et al. (2022) to investigate the membrane coordination, permeation, and lysis properties of selected SSAs**.**

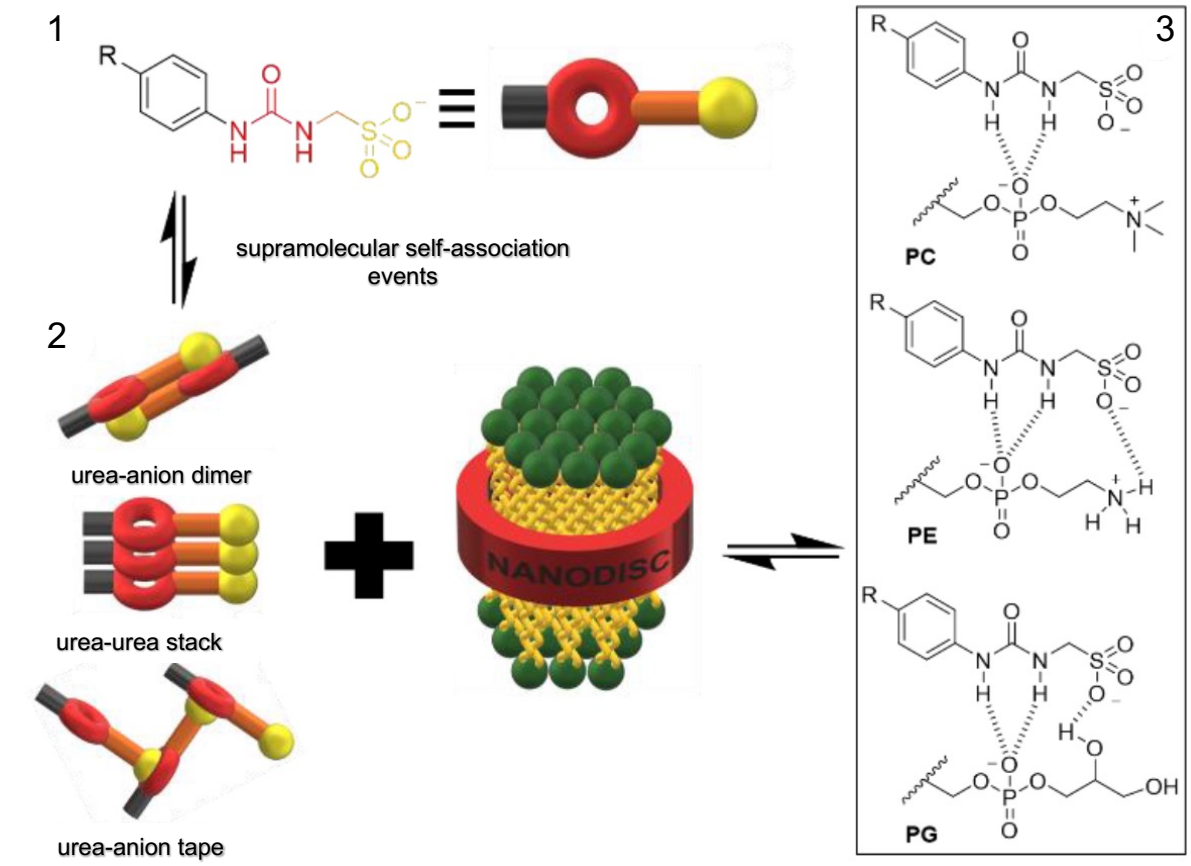

Figure 5. Illustration of 1) a representative structure of the anionic SSA component. 2) the hydrogen-bonded self-associative events observed for the anionic component in a solid state. 3) hypothesized hydrogen bonding interactions of the anionic component with different phospholipid headgroups (Townshend et al. 2020)

In Chapter 2, SSA 39 displayed the potential to inhibit biofilm formation, with a degree of specificity toward *Candida albicans* SC5314, by inhibiting pseudohyphae and hyphae formation. Thus, it was of interest to evaluate the application of the nanodiscs, composed of fungal-membrane phospholipids, to investigate the potential interaction between SSA 39 and fungal membranes. This interaction is significant as it presents a novel opportunity to understand the scope of nanodisc technology as well as SSA-membrane interactions. Therefore, this study aims to investigate the application of fungal phospholipid nanodiscs, and to understand membrane lipid coordination events with the novel class of antifungal compounds.

Materials and methods

Supramolecular self-associating amphiphile stock solution

The SSA used in this study was SSA 39, which has a tetrabutylammonium (TBA) counter cation (Figure 6). The SSA stock solution was prepared in an EtOH:H_2O (1:19) solution as per Boles et al. (2022) to obtain a concentration of 5 mM and was stored at 4 °C. SSA 39 contains a hydrogen bond donating/accepting urea functionality, linked with a methyl spacer to a sulfonate functionality this hydrophilic functionality. It also acts as the hydrogen bond donating group which is known to coordinate with the urea group creating a hydrogen-bonded complex. On the opposite side of the urea functionality,

there is a hydrophobic methyl-substituted benzothiazole group, which is also planar in nature and capable of undergoing pi-pi stacking interactions.

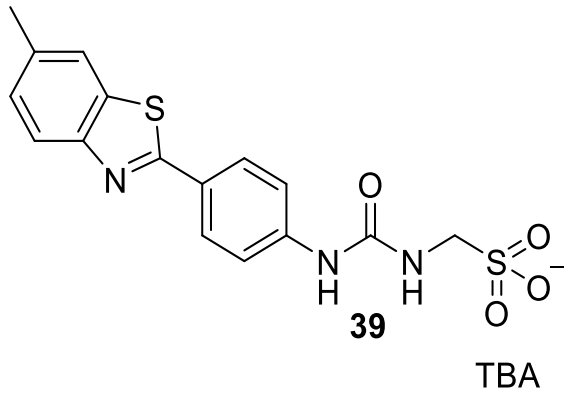

Figure 6. SSA 39, TBA = tetrabutylammonium

Evaluating SSA-membrane interaction using confocal laser scanning microscopy (CLSM)

Strain maintenance and culture conditions

Candida albicans SC5314 was stored at -80 °C in a nutrient broth (NB) (1 g.L^{-1} meat extract, 2 g.L^{-1} yeast extract, 5 g.L^{-1} peptone, 8 g.L^{-1} sodium chloride) supplemented with 15 % (v/v) glycerol. Before use, *C. albicans* SC5314 was cultivated from the frozen stocks onto yeast malt extract (YM) agar (3 g.L^{-1} malt extract, 3 g.L^{-1} yeast extract, 5 g.L^{-1} peptone, 10 g.L^{-1} glucose, 16 g.L^{-1} agar) and then maintained on YM agar plates and stored at 4 °C. For the relevant experiment, a fresh (pre-inoculum) culture of *C. albicans* SC5314 was prepared by transferring a single colony from the plates into 5 mL yeast nitrogen base (YNB) broth media (10 g.L^{-1} glucose, 16 g.L^{-1} yeast nitrogen base) and incubating aerobically at 30 °C for 24 hours. For all experimental procedures, filter-sterilized (0.22 µm nitrocellulose filter, ABLUO®, GVS, United States of America) RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich®, United Kingdom) at pH 7.0 was used. Unless otherwise stated, all washing steps were performed either with sterile milli-Q water or phosphate-buffered saline (PBS) (0.2 g.L^{-1} potassium chloride, 0.2 g.L^{-1} potassium dihydrogen phosphate, 1.15 g.L^{-1} di-sodium hydrogen phosphate, 8 g.L^{-1} sodium chloride) (Oxoid, United Kingdom) at pH 7.3 (± 0.2 at 25 °C).

Biofilm formation and microscopy

Biofilms were developed as described previously, with minor modifications (Stepanovic et al. 2000; O'Toole 2011; Fourie et al. 2017). *Candida albicans* SC5314 was cultivated on YM agar for 24 hours at 30 °C. A single colony of *C. albicans* SC5314 was inoculated into a test tube containing 10 mL of YNB broth (3 g.L^{-1} malt extract, 3 g.L^{-1} yeast extract, 5 g.L^{-1} peptone, 10 g.L^{-1} glucose) and incubated for 24 hours at 30 °C. After incubation, the cells were harvested by centrifugation at 5000 rpm (F-35-6-30 rotor, Centrifuge 5430R, Eppendorf®, United States of America) for 5 minutes at 4 °C. The supernatant was discarded, and the pellet washed three times with 10 mL of phosphate-buffered saline (PBS) (0.2 g.L^{-1} potassium chloride, 0.2 g.L^{-1} potassium dihydrogen phosphate, 1.15 g.L^{-1} di-sodium

hydrogen phosphate, 8 g.L^{-1} sodium chloride) (Oxoid, United Kingdom) solution. The cells were enumerated with a haemocytometer and standardised to 1 x 10^{6} cells.mL^{-1}.

The standardised cell solution was dispensed onto a sterile glass coverslip in a 6-well plate (Greiner Bio-One, Germany) containing filter-sterilized (0.22 µm nitrocellulose filter, ABLUO®, GVS, United States of America) RPMI-1640 medium (with L-glutamine and sodium bicarbonate (pH 7.0)) (Sigma-Aldrich®, United Kingdom) and SSA 39 at a final concentration of 2.56 mM. The plate was incubated for 48 hours at 37 °C to promote biofilm formation. After incubation, the glass coverslip disc was fixed to a microscope slide and the biofilms were examined using a ZEISS LSM 900 with AiryScan 2 laser scanning microscope (ZEISS, Germany) at an excitation/emission wavelength of 365/461 nm (using the DAPI filter setting). This was possible due to the inherent fluorescent properties of SSA 39.

Evaluating phospholipid membrane interactions

(performed in collaboration with Ms Kira Hilton - University of Kent, Dr Charlie Hind - UK Health and Security Agency and Ms Mahnoor Hassan - King's College London)

Strains used and culture conditions

Candida albicans NCPF3645 and *Candida auris* WT1912 were used in this study due to the availability at the University of Kent in the following phospholipid nanodisc studies. *C. albicans* NCPF3645 and *C. auris* WT1912 are from the same genus as *C. albicans* SC5314 and considering the potential similarity in membrane composition, was of interest to examine in this study. Pre-inoculums were cultivated on yeast peptone dextrose (YPD) (10 g.L^{-1} yeast extract, 15 g.L^{-1} agar, 20 g.L^{-1} peptone) agar plates at 37 °C for 24 hours. After incubation, a single colony was suspended in 3 mL YPD broth and incubated at 37 °C with shaking at 180 rpm for 24 hours. These cultures acted as inoculums for 500 mL YPD broth in a 2 L Erlen-Meyer flask, which was incubated at 30 °C with shaking at 180 rpm for 24 hours. Cells were harvested by centrifugation (7 500 rpm for 10 minutes at 4 °C) and most of the supernatant was decanted. A small volume of supernatant (~ 2 mL) was retained and the pellet was resuspended by vortexing. The cell solution was aliquoted into 15 mL conical tubes, boiled at 98 °C for 25 minutes and sonicated in a water bath at 60 °C for 30 minutes to facilitate cell lysis. Finally, the samples were 'snap-frozen' using liquid nitrogen and placed at -80 °C for lyophilisation of the biomass. Samples were stored at -80 °C until use. To determine whether cell lysis was successful, the lysate was plated onto a YM agar plate and incubated at 37 °C for 24 hours. If no growth was observed, cell lysis was considered successful.

Isolation of membrane fraction

Lyophilised *C. albicans* NCPF3645 and *C. auris* WT1912 samples were thawed over ice and transferred to a glass beaker and resuspended in 20 mL lysis buffer (50 mM sodium phosphate monobasic (Fluorochem, United Kingdom), 300 mM sodium chloride (Thermo Fischer Scientific, United Kingdom)) at pH 8. While maintaining the beaker on ice, the samples were sonicated (Soniprep 150, MSE, United Kingdom) at 85 % power for 10 minutes. The samples were transferred to sterile centrifuge tubes and centrifuged (Ja-25.50 rotor, Avanti™ J-30 I, Beckman Coulter, United States of America) at 10 000 rpm at 4 °C for 15 minutes. The pellet was discarded, and the supernatant was transferred to sterile ultracentrifuge tubes. The supernatant was subjected to ultracentrifugation (70-Ti rotor, Optima™ LE-

80K Ultracentrifuge, Beckman Coulter, United States of America) at 40 000 rpm at 4 °C for 60 minutes. The supernatant produced was discarded and the pellet, containing the membrane fraction, was resuspended in 1 mL phosphate buffer [50 mM sodium phosphate monobasic (Fluorochem, United Kingdom), 300 mM sodium chloride (Thermo Fischer Scientific, United Kingdom)] (Bligh and Dyer 1959; Hara and Radin 1978; Chen et al. 1981; Araujo et al. 2013).

Phospholipid Folch extraction

To extract the phospholipids from the membrane fraction pellets, the Folch extraction method was utilised with a 2:1 chloroform:methanol ($CHCl_3$:MeOH) solution (Folch et al. 1957). The extraction mixture (membrane pellet and solvents) was incubated at room temperature for 60 minutes on a magnetic stirrer until dissolved. This was followed by a wash step with H_2O and the phases were allowed to separate at room temperature. The lower organic phase, containing the phospholipids, was collected and dried using a rotary evaporator at 40 °C (Rotavapor R-114, Buchi, Switzerland). The phospholipids were stored at -20 °C in the dark to prevent light-sensitive oxidation (Yavlovich et al. 2010).

Phospholipid nanodisc synthesis

The isolated phospholipids were resuspended in a 3:1 $CHCl_3$:MeOH solution and transferred to a 50 mL round bottom flask. The isolated phospholipids solution was dried using a rotary evaporator (Rotavapor R-114, Buchi, Switzerland) at 40 °C to remove excess MeOH. The solution was then dried under a nitrogen stream overnight. After overnight drying, 1 mL of phosphate at pH 7.4 was added. The solution was sonicated (40 kHz, Bransonic 1210R-MTH Ultrasonic Cleaner, Emerson Electric, United States of America) at room temperature for 60 minutes. After sonication, styrene-maleic anhydride (SMA) (provided by Dr Jose Luis Ortega-Roldan from the University of Kent) was added in a 5:1 SMA to lipid ratio and incubated at 37 °C for 60 minutes. The solution was dialysed overnight, using a 10 K Da membrane (SnakeSkin™ Dialysis Tubing 10 K MWCO 16 mm, Thermo Fischer Scientific, United States of America) in 5 L phosphate buffer at pH 7.4, to remove excess SMA. The phospholipid-SMA solution was then concentrated to 0.5 mL using a 10 K Da concentrator through centrifugation at 4 000 rpm (A-4-81 rotor, Centrifuge 5810R, Eppendorf®, United States of America) at 4 °C for 5 minutes. Thereafter, gel filtration chromatography and a size exclusion column (SEC) (Superdex™ 200 Increase 10/300 GL, ÄKTA purifier, Amersham Pharmacia Biotech, United Kingdom) with the same phosphate buffer (pH 7.4) were used to verify and monitor the presence of SMA at an absorbance of 260 nm. Furthermore, the SEC was used to remove any remaining free SMA from the solution.

Phospholipid nanodisc quantification

NanoDrop Spectrophotometer

To quantify the phospholipid nanodisc concentration after gel filtration, ultraviolet-visible (UV-Vis) spectroscopy was carried out using a NanoDrop spectrophotometer (NanoDrop™ One C, Thermo Fischer Scientific, United States of America) at a wavelength of 260 nm. Calibration was performed with phosphate buffer at pH 7.4. Each measurement was repeated three times to establish an average value. After quantification, the solution was aliquoted into 0.5 mL portions and stored at -20 °C until needed. To determine the nanodisc concentration the following formula was used:

[SMA] = (A_{260} – 0.0279)/0.0083

DLS analysis

Photo correlation spectroscopy (PCS) was performed to quantify the size and size distribution of the nanodiscs in the sample (Stetefeld et al. 2016). The average number weighted particle size distribution of phospholipid nanodiscs was measured using a particle size analyser (Litesizer® 500 BM10, Anton Paar, Austria) and data were processed using KalliopeTM Professional software. A series of 10 runs were recorded at 20 °C, allowing 10 minutes for sample equilibration before the first measurement. Notably, transmission electron microscopy (TEM) analysis may be used to confirm the presence of phospholipid nanodiscs in the sample.

Phospholipid nanodisc ^{1}H NMR adhesion assay

To evaluate membrane coordination activity, phospholipid nanodiscs (in a concentration range of 3.13 – 0.21 µM) were titrated against **SSA 39** (0.05 mM) in sodium phosphate buffer at pH 7.4. The phospholipid solution was supplemented with 112 µL deuterium oxide (D_2O) (5 %) (Cambridge Isotope Laboratories Incorporated, United States of America) and 28 µL 4,4-dimethyl 4-silapentane-1-sulfonic acid (DSS) (0.01 mM) (Thermo Fischer Scientific, United Kingdom) as internal standard.

Proton NMR, with a Carr-Purcell-Meiboom-Gill pulse sequence (CPMG) filter, spectra with a Watergate sequence were determined with a Bruker Avance III 600 Hz spectrometer equipped with a TCIP cryoprobe at 298 K. Furthermore, P1 pulses were 9 µs at 7.9 W. Pre-saturation was applied between acquisitions for 100 ms at 280 mW and acquisition was carried out at 0.851 s. A 1D ^{1}H NMR, with a CPMG filter (300 ms with delays between 180 ° pulses for 1 ms) (Carr and Purcell 1954; Meiboom and Gill 1958) modified with a 3-9-19 Watergate sequence (to suppress the water signal), was obtained for each experimental data point. The CPMG filter was used to suppress NMR resonances of molecular species in the sample with long correlation times, which in this experimental procedure correlated to SSAs coordinated to phospholipid nanodiscs. This facilitated a higher quality NMR spectrum for SSAs interacting with phospholipid nanodiscs. All spectra were automatically phased, then baseline corrected using a polynomial function and calibrated to the centre of a sodium trimethylsilyl propanesulphonate (DSS) peak. The water signal was suppressed in MestreNova software using a convolution method in those experiments with poor water suppression.

Results and Discussion

SSA 39 interacts with *C. albicans* cell surfaces

In Chapter 2, it was established that the novel antimicrobial library of SSAs had potential antibiofilm activity in mono- and polymicrobial biofilm models, with some being more active against the fungal constituent in these models. Interestingly, although SSA 39 could inhibit *C. albicans* biofilm formation, it was less effective at eradicating mature biofilms, indicating that this SSA cannot penetrate mature *C. albicans* EXM effectively. From the scanning electron microscopy (SEM), SSA 39 was of specific interest as it could inhibit filamentation at 2.56 mM. Since SSA 39 is inherently fluorescent, due to the presence of the benzothiazole moiety attached to the phenyl ring, it could be used to evaluate the

interaction of this compound with *Candida albicans* SC5314 biofilms (Figure 7). As expected, only yeast cells were present (Figure 7.1), while some degree of filamentation can still be seen (Figure 7.3). The fluorescence micrographs indicate that SSA 39 can interact with the fungal cell surface at both concentrations (indicated by the blue fluorescence in Figures 7.2 and 7.4). It is also observed that the compound has successfully penetrated a yeast cell and pseudohyphae. Interestingly, a potential interaction between SSA 39 and the extracellular matrix of *C. albicans* is observed (Figure 7.4), which may contribute to the resistance of mature biofilms to these compounds as seen in Chapter 2.

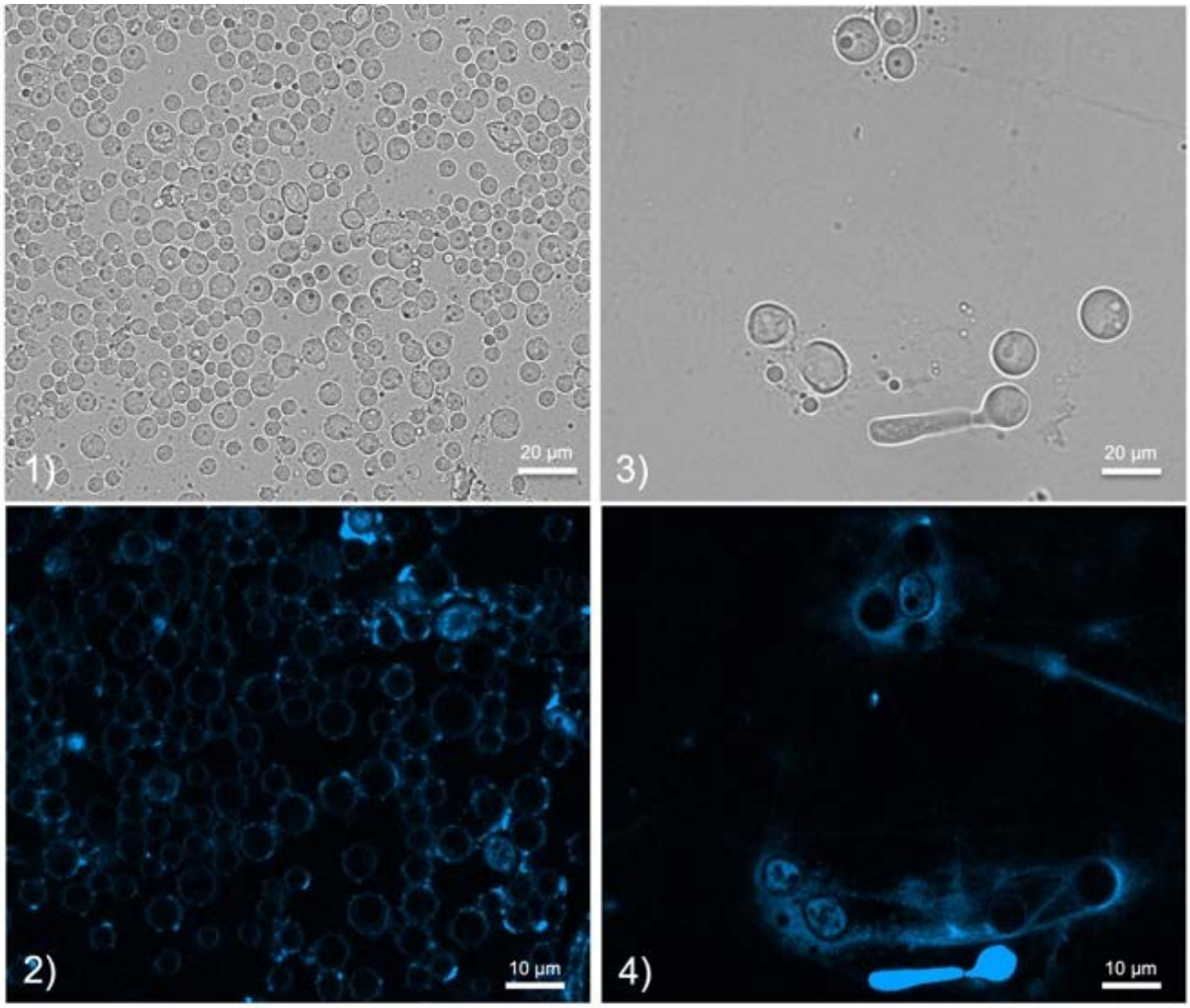

Figure 7. Interaction of SSA 39 with *Candida albicans* SC5314. Light micrograph 1) indicates a biofilm formed in the presence of SSA 39 at 2.56 mM. Micrograph 2) indicates the blue fluorescence of SSA 39 on the yeast cell surfaces. Light micrograph 3) indicates a biofilm cultivated in the presence of 1.28 mM SSA 39. Micrograph 4) indicates blue fluorescence of SSA 39 on the yeast cell surfaces, germ tube and interacting with the extracellular material

Interaction of SSA 39 with fungal phospholipid nanodiscs

Microscopy analysis suggested a possible SSA-fungal membrane interaction. Therefore, it is of interest to determine whether the use of a novel fungal phospholipid nanodisc can be applied to elucidate the potential SSA-fungal membrane interaction observed. To verify the presence and successful synthesis of phospholipid nanodiscs, the use of DLS analysis was employed to measure the size distribution of particles in the solution. It is established that the size of nanodiscs varies depending on the application

and composition of phospholipids used. The most used phospholipid nanodiscs are approximately 10 nm in diameter. However, recent studies have indicated the engineering of nanodiscs up to a size of 90 nm (Hagn et al. 2013; Padmanabha Das et al. 2020).

DLS analysis evaluates the high-frequency fluctuations in scattered light to quantify the Brownian motion of individual particles in solution, which facilitates the measurement of particle size and particle size distribution within a sample (Babick 2019). Figure 8 shows the hydrodynamic diameter distribution obtained from DLS analysis of the respective phospholipid nanodiscs synthesized. Figure 8.1 displays the phospholipid nanodisc produced from *C. albicans* NCPF3645. From the data, it is evident that an approximate phospholipid nanodisc particle size of 9 nm is produced. This is in line with other studies on phospholipid nanodiscs synthesized using the GPL, PC (Boles et al. 2022). Figure 8.2 indicates the phospholipid nanodisc synthesized using *C. auris* WT1912. Interestingly, the average particle size distribution profile shows two distinct peaks at 21 nm and 84 nm. The species corresponding to the peak at 84 nm is likely to be due to a complex aggregation process between the GPLs and nanodiscs in solution. It is noteworthy to mention that phospholipidome analysis has indicated variable and distinctive phospholipid compositions between different *Candida* species (Singh et al. 2010; Zamith-Miranda et al. 2019). In line with these findings, a comparative lipidomic investigation between *Candida albicans* and *Candida auris* extracellular vesicles indicated a differential lipid profile between the organisms. *C. albicans* indicated higher levels of abundance in glycerophospholipids. Whereas *C. auris* indicated higher expression levels of PC and PE (Zamith-Miranda et al. 2021). These data may allude to a different phospholipid nanodisc size of *C. auris* compared to that of *C. albicans*. Furthermore, complex interactions (based on the amphiphilic nature of GPLs) may be present between the phospholipids in the solution, which include the self-association of secondary phospholipid structures (Ritchie et al. 2009; Hagn et al. 2013). This may explain the differences in nanodisc sizes produced.

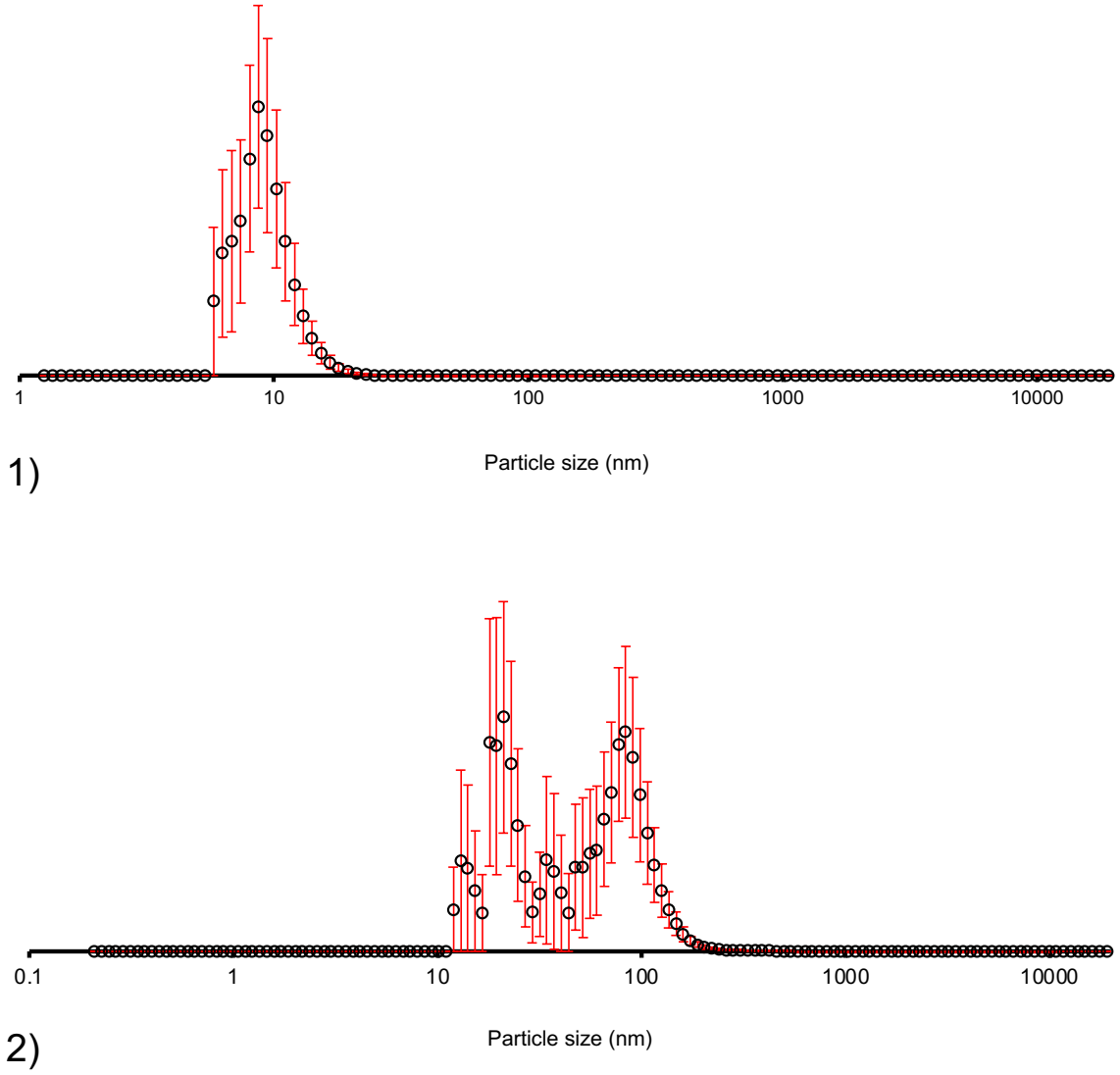

Figure 8. The average number weighted particle size distribution of 1) *Candida albicans* NCPF3645 and 2) *Candida auris* WT1912 nanodiscs (44 µM) in phosphate buffer at pH 7.4, calculated from 10 dynamic light scattering runs at 20 °C

Proton nuclear magnetic resonance (NMR) spectroscopy is an analytical tool used to study the molecular structure and composition of organic and inorganic compounds in a sample. NMR is based on the magnetic properties of atomic nuclei, whereas proton NMR focuses specifically on the atomic protons (Lindon et al. 1999). In NMR spectroscopy, a sample is subjected to a strong magnetic field to which the nuclei protons align. When the magnetic field is altered by a radiofrequency pulse, the protons absorb energy and transition to a higher energy field. This process is referred to as spin-spin coupling or residual coupling. As the protons return to their native state, a unique radiofrequency signal is produced that can be detected and analysed. This unique radiofrequency signal enables the determination of the molecular structure and composition of the sample (Bharti and Roy 2012; Blümich 2016). NMR spectroscopy has a wide range of applications such as structural analysis of organic compounds and identification and quantification of unknown compounds.

When considering the proximity of nuclei protons in a sample, the produced coupling signals can be broadened and obscured by interactions between spins of similar nuclei (Sun et al. 2013; Hatzakis 2019). The Carr-Purcell-Meiboom-Gill pulse sequence nuclear magnetic resonance (CPMG NMR) technique is a powerful tool used to characterise conformational exchange processes in biomolecules under a millisecond timescale. CPMG NMR was developed to overcome inconsistencies in homonuclear decoupling to obtain higher-quality NMR spectra. CPMG NMR is based on different relaxation times of NMR environments that are affected by rates of diffusion which, in turn, is affected by the size of molecules in a sample (Carr and Purcell 1954; Meiboom and Gill 1958; Vallurupalli et al. 2009). From these different relaxation times, the NMR signals of larger molecules in solution are removed. From the coordination of the SSA and phospholipid nanodisc, a large complex formation will be observed. Additionally, D_2O has a distinct magnetic coupling response in NMR analysis. Therefore, D_2O is used as a solvent (instead of H_2O) to produce distinct NMR signals for each hydrogen site in the molecule, known as NMR locking. Also, DSS is used as an internal standard to standardise the NMR spectra obtained. The produced phospholipid nanodiscs were then titrated against the SSA-D_2O-DSS solution while maintaining the SSA concentration (McIntosh 2013; Donaldson et al. 2016).

By integrating the NMR resonance profiles of the SSA anion and counter cation (TBA) to their corresponding SSA concentration (after standardisation to the DSS internal standard), the absolute relative area under the respective spectra can be plotted to evaluate SSA-phospholipid coordination. In line with these techniques, the capability of SSA 39 to coordinate with the appropriate phospholipid membrane was investigated. Also, the percentage coordination between SSA 39 and the respective phospholipid nanodisc can be calculated as a function against phospholipid nanodisc concentration. It is noteworthy to mention that the SSA concentration is kept constant at 5 mM throughout the experimental procedures. The *C. albicans* NCPF3645 ^{1}H NMR CPMG titration (Figure 9) indicates the integrated resonance profile of the anionic and cationic components of SSA 39. From the data, a relatively uniform absolute relative area is observed for the TBA counter cation, whereas a decline is observed for the anionic component of SSA 39. These data show the ability of the anionic component to interact or coordinate with the phospholipid nanodiscs, but not the TBA counter cation.

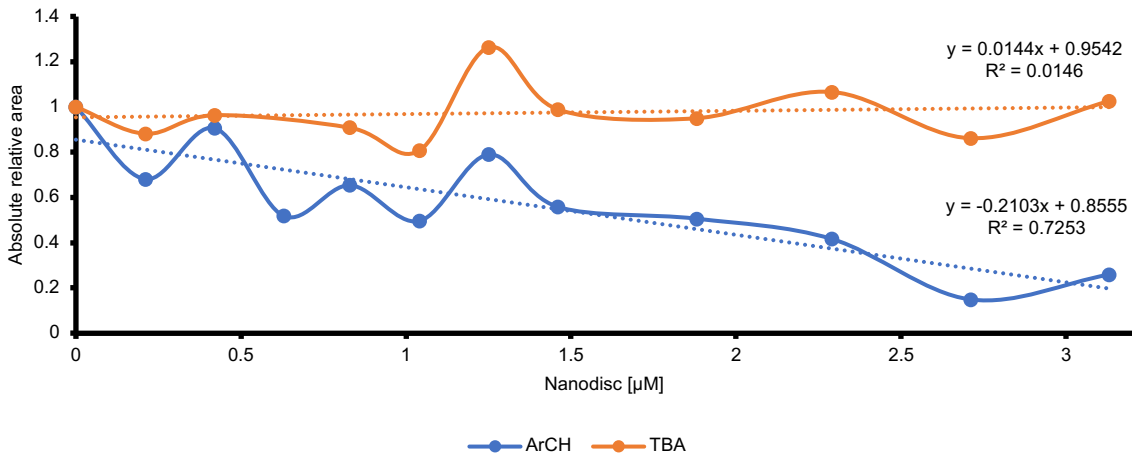

Figure 9. Graph illustrating the integrated ^{1}H NMR CPMG resonance profile of SSA 39 (5 mM) when titrated with *C. albicans* NCPF3645 phospholipid nanodiscs

When considering the absolute relative area data presented in Figure 9, it is expected that the anionic component of SSA 39 will have a high percentage coordination with the *C. albicans* NCPF3645 nanodisc at a high concentration. Figure 10 supports these expectations and indicates a gradual increase in percentage compound coordination at higher concentrations of phospholipid nanodiscs. At a concentration of 3.13 μM, a percentage interaction of 74% is observed. Notably, a high percentage compound coordination of 85% is observed at a concentration of 2.71 μM. These data suggest that a strong interaction and coordination event is observed between SSA 39, and the phospholipids presented in the nanodisc structure.

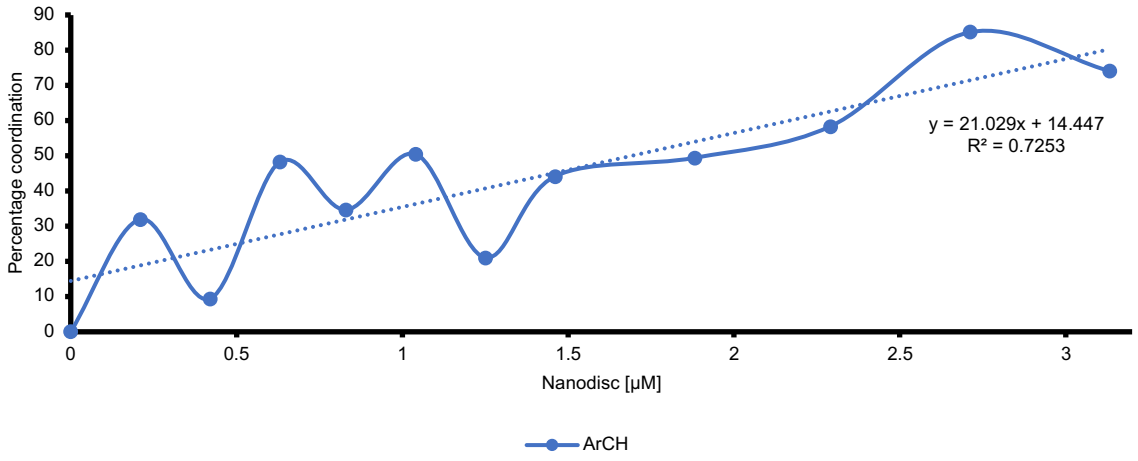

Figure 10. Graph illustrating the percentage interaction of the anionic component of SSA 39 (5 mM) with the *C. albicans* NCPF3645 phospholipid nanodisc structure

As shown above, different phospholipid profiles are observed between organisms and often also between different strains of the same organism (Benamara et al. 2011; de Kroon et al. 2013). Therefore, it is important to evaluate the potential interaction between SSA 39 and the phospholipid nanodiscs synthesised with *C. auris* WT1912.

The *C. auris* WT1912 ^{1}H NMR CPMG titration (Figure 11) indicates the integrated resonance profile of the anionic and cationic components of SSA 39. From the data, it can be seen that a decrease in the absolute relative area is observed for both the TBA counter cation and the anionic component of SSA 39 with increasing concentration of nanodiscs. This does suggest a complex interaction between the TBA counter cation as well as the anion of SSA 39 with the phospholipid nanodisc.

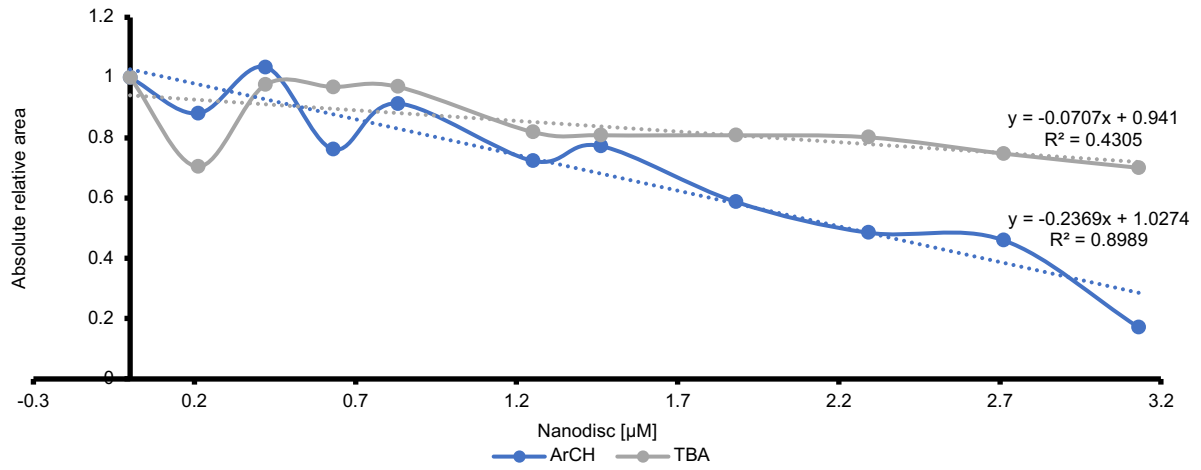

Figure 11. Graph illustrating the integrated ^{1}H NMR CPMG resonance profile of **SSA 39** (5 mM) when titrated with *C. auris* WT1912 phospholipid nanodiscs

Furthermore, Figure 12 indicates the percentage interaction data for the anionic component of SSA 39 and the phospholipid nanodiscs, synthesized with *C. auris* WT1912. From these data, an 83% interaction is between the anionic component of SSA 39 and the nanodiscs, at a concentration of 3.13 μM.

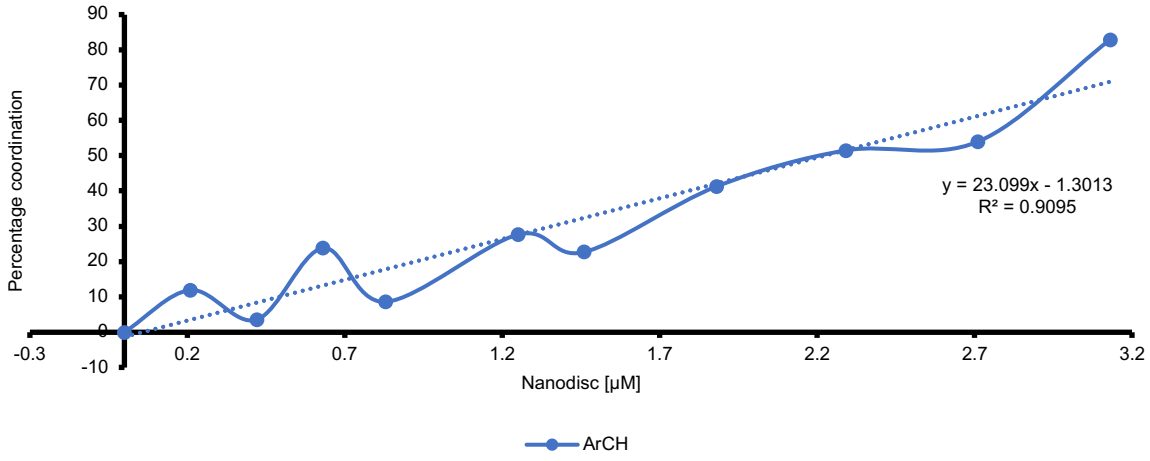

Figure 12. Graph illustrating the percentage interaction of the anionic component of SSA 39 (5 mM) with the *C. auris* WT1912 phospholipid nanodisc structure.

Based on these findings, it may be argued that there is an interaction between SSA 39 and the related phospholipid nanodiscs. Based on previous studies (White et al. 2020; Boles et al. 2022), several SSA-phospholipid headgroup complexation events, facilitated through a combination of multiple hydrogen bond formations and/or electrostatic interactions, may be hypothesized. For all the phospholipids, these

interactions can occur as a result of hydrogen bond formations and favourable electrostatic and covalent events between the central urea group (from the SSA) and the oxygen of the phosphate moiety (of the phospholipid) (Figure 13). In addition, this also occurs between the sulphonate and the tertiary ammonium. Similarly, an additional bond may form between the sulfonate moiety and the glycerol of PG and the inositol of PI.

For PC, a potential interaction may be facilitated between the sulfonate moiety (from the SSA) and the tertiary ammonium group (from PI) (White et al. 2020). This bond formation may result in a stronger interaction between the SSA and PC. However, sterically-hindered formations may be observed between these groups and result in weaker complexation events.

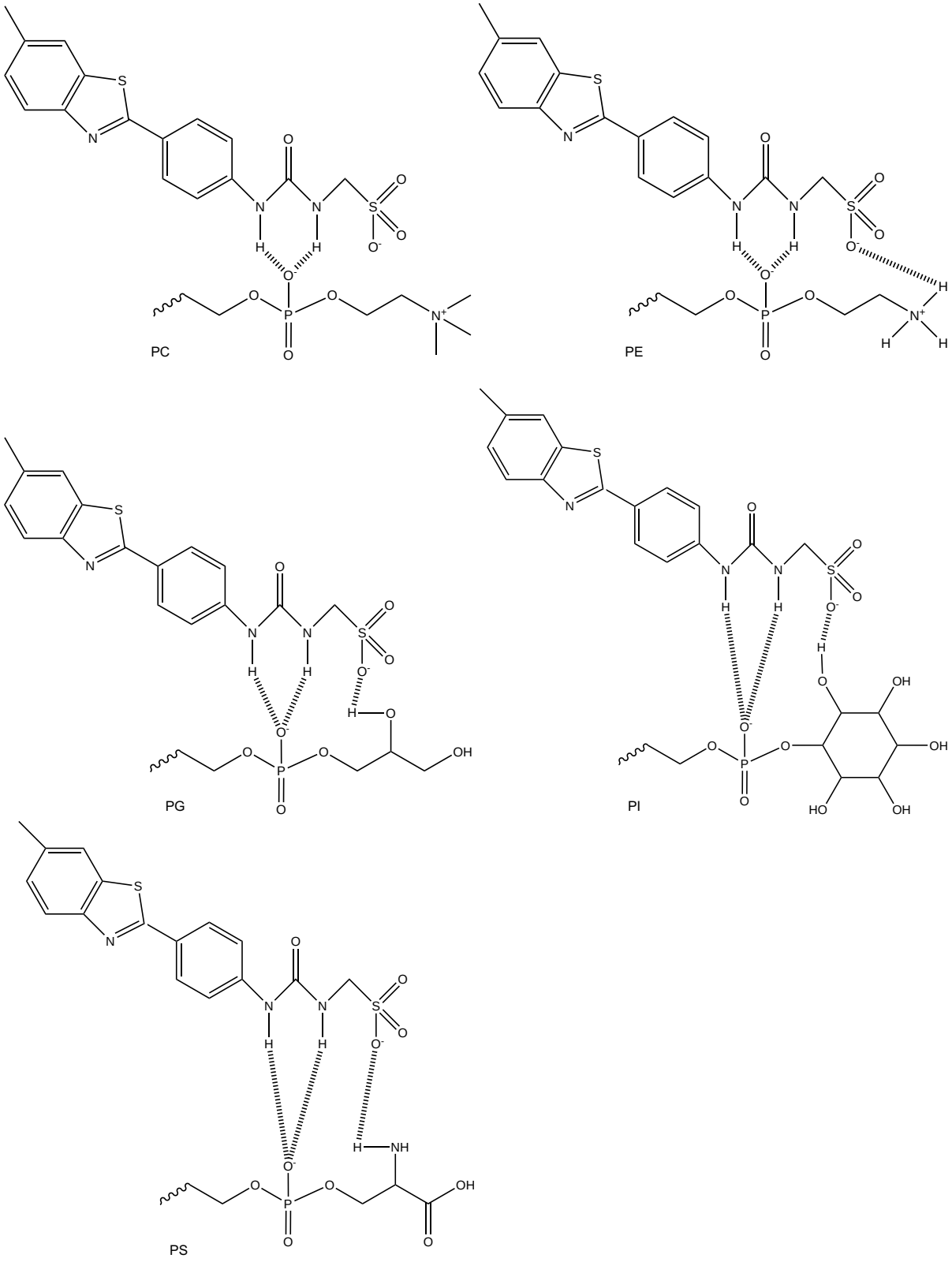

Figure 13. Diagram illustration of the possible complexation events between phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylserine and SSA 39 (White et al. 2020)

Conclusions

In Chapter 2, SSA 39 displayed promising activity against *Candia albicans* SC5314 biofilms. This study evaluated the interaction between SSA 39 and fungal membranes using both CLSM and a synthetic membrane system, nanodiscs. CLSM analysis indicated compound localisation at the surface of yeast cells as well as the ability of the compound to penetrate yeast and pseudohyphae. From these findings, phospholipid nanodiscs were synthesized using *Candida albicans* NCPF3645 and *Candida auris* WT1912. This novel technique indicated the potential to suspend fungal membrane phospholipids in a synthetic system for interaction studies. Moreover, the use of a novel phospholipid nanodisc ^{1}NMR CPMG adhesion assay indicated the potential percentage coordination activity between the anionic component of SSA 39 and the phospholipid nanodiscs. Based on these results, as well as studies by White et al. (2020) and Boles et al. (2022), we hypothesize potential SSA-phospholipid headgroup complexation events for the fungal GLPs, PI and PS. Future research should focus on elucidating the fungal membrane phospholipid composition of clinically relevant opportunistic pathogens and determining the selectivity of the SSA library against relevant phospholipid combinations.

The misuse of currently available antimicrobial compounds has established a niche of opportunistic pathogens with multi-drug resistant mechanisms such as biofilm formation. Alternative measures are of immediate interest to circumvent antimicrobial resistance. To do so, alternative strategies include drug repurposing and designing novel compounds aimed at biofilm formation. The application of synthetic membrane systems facilitate the study of microbial membrane components in a stable and controlled environment. This technology provides the opportunity to optimise antimicrobial drug target sites and design novel antimicrobial compounds.

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Chapter 4: General discussion and conclusions

Introduction

Pseudomonas aeruginosa and *Candida albicans* are two opportunistic pathogens that can cause a variety of infections in humans, particularly in individuals with weakened immune systems or other underlying medical conditions. While both microorganisms are typically harmless in healthy individuals, they can cause significant infections in immunocompromised individuals. One of the most concerning aspects of *P. aeruginosa* and *C. albicans* is their ability to develop antimicrobial resistance. *P. aeruginosa* can develop resistance to a wide range of antibiotics, while *C. albicans* can develop resistance to antifungal drugs (Pendleton et al. 2013; Trejo-Hernández et al. 2014; de Oliveira et al. 2020; Talapko et al. 2021). Both pathogens are also known for their ability to form biofilms, which can contribute to the persistence of infections over time. Furthermore, *P. aeruginosa* and *C. albicans* can form polymicrobial biofilms when they coexist in the same environment, and are commonly found together in biofilms associated with various infections, including ventilator-associated pneumonia, cystic fibrosis, and catheter-associated infections. The interaction between these species is complex and not yet fully understood. It has been suggested that the two microorganisms can form antagonistic relationships, but other studies have suggested that their interaction can also be mutualistic, leading to increased virulence and the production of harmful metabolites (McAlester et al. 2008; Ovchinnikova et al. 2012; Bandara et al. 2013; Fourie et al. 2016; Phuengmaung et al. 2020).

The interaction between *P. aeruginosa* and *C. albicans* has important clinical implications, particularly in the context of biofilm-associated infections. Biofilms are notoriously difficult to treat with conventional antibiotics, and the presence of multiple microorganisms in a biofilm can further complicate treatment. Therefore, understanding the nature of the interaction between these microorganisms and developing novel strategies to disrupt their interactions may have significant therapeutic implications (Koch and Høiby 1993; Aaron et al. 2002; Francolini and Donelli 2010; Sherrard et al. 2014; Floyd et al. 2017; Granchelli et al. 2018; Reece et al. 2021).

The emergence of antimicrobial resistance is a growing concern in public health, and the development of novel antimicrobial therapies and strategies is essential to combat this problem (Coates et al. 2011; Belete 2019). Traditional antibiotics have been the cornerstone of antimicrobial therapy for decades, but the widespread use and misuse of these drugs have contributed to the emergence and spread of antimicrobial resistance. Therefore, there is a need to explore new avenues for antimicrobial therapy, including the development of alternative agents that target microbial pathogens in new ways. One promising area of research for the development of novel antimicrobial therapies is the use of natural or synthetic molecules that disrupt the integrity of the microbial cell membrane (Raffa et al. 2005; Haldar et al. 2005; Smith 2005; Abusrewil et al. 2020). Amphiphilic molecules have attracted attention due to their ability to self-assemble into ordered structures and interaction with biological membranes. These structures have the potential to act as novel antimicrobial agents by disrupting the lipid bilayer of microbial cells, leading to cell death. The use of amphiphilic molecules as antimicrobial agents has several advantages, including their broad-spectrum activity against various microorganisms, their potential for use in combination therapies, and their ability to overcome antimicrobial resistance (Baktir

et al. 2012; Mayer and Kronstad 2017; Tyuleva et al. 2019; White et al. 2020b; Mota Fernandes et al. 2021).

Supramolecular self-associating amphiphiles (SSAs) are a promising novel class of amphiphilic molecules that have gained attention as potential antimicrobial alternatives. These amphiphilic molecules can self-assemble into complex structures, which are hypothesized to disrupt bacterial membranes (Hiscock et al. 2016; Gumbs et al. 2018; Luo et al. 2021; Yang et al. 2022a; Yang et al. 2022b). The advantage of using SSAs as antimicrobial agents lies in their potential to overcome the limitations of conventional antibiotics. Additionally, SSAs can be designed to have low toxicity, making them a promising avenue for the development of new antimicrobial agents. The development of novel antimicrobial therapies and strategies that incorporate SSAs has the potential to contribute significantly to the fight against antimicrobial resistance and the treatment of infectious diseases (Townshend et al. 2020; Ng et al. 2020; Ellaby et al. 2022; Rutkauskaite et al. 2023). In this study, we evaluated the efficacy and application of novel SSAs when employed against the opportunistic pathogens *P. aeruginosa* and *C. albicans*.

Supramolecular self-associating amphiphiles can be effective against mono- and polymicrobial biofilms

In alignment with current knowledge proving the antibacterial action of SSAs against planktonic cells of Gram-positive and Gram-negative bacteria (White et al. 2021), our studies proved that SSAs do display antibiofilm activity against biofilms of Gram-negative bacteria and significantly, *Candida albicans*. Additionally, the investigation of SSAs employed against mature biofilms showed the ability of most compounds to penetrate the protective extracellular matrix (EXM) and affect *C. albicans* cell within the complex structure. When considering the presence of GLPs in the extracellular matrix, it may be argued that the SSAs interact selectively with EXM components resulting in disassembly (Kim et al. 2011; Yue 2014; Mitchell et al. 2016; Boles et al. 2022; Doolan et al. 2022). Our studies further deduced selective inhibitory action based on structural characteristics through structure-activity relationship investigations. One of the structural findings relates to the importance of the counter cation for the overall antimicrobial activity. This coincides with Allen et al. (2020), who established that the presence of the SSA counter anion results in selectively increased antimicrobial activity against planktonic methicillin-resistant *Staphylococcus aureus* and *Escherichia coli*.

Furthermore, the outer membrane glycerophospholipid composition of *P. aeruginosa* PAO1 mostly contains phosphatidylethanolamine and phosphatidylglycerol, which facilitates a negative net surface charge (van Meer and de Kroon 2011). In contrast, the outer membrane GPL composition of *C. albicans* SC5314 mostly contains phosphatidylcholine and phosphatidylethanolamine, which facilitates a neutral net surface charge (Singh et al. 2010; Zamith-Miranda et al. 2019). The distinct difference in GPL composition and surface charge directly relates to the selective inhibitory action from structural differences observed in the SSAs. Moreover, it is established that differential membrane profiles are observed in different microbial lifecycle phases and environments such as mature biofilms encapsulated in an extracellular matrix (Kohlwein et al. 1996; Lattif et al. 2011; de Kroon et al. 2013; Hilton et al.

2021). The lipophilic and hydrophilic components of the SSAs may infer additional influence on the complex interaction observed between the SSA anion, cation and relevant microbial membrane.

In line with the complex interactions previously mentioned, the tested SSAs displayed interesting results as potentiating agents for the drugs colistin and fluconazole. These data suggest an improved and beneficial interaction between select SSAs and the antibacterial substance. However, this feature should be investigated in greater detail to substantiate any findings. It is proposed to investigate the relationship between the SSAs and both substances to determine the nature of the interaction (Kuhn et al. 2003; Petrosillo et al. 2008; Meletiadis et al. 2010; Grassi et al. 2017; Torres et al. 2018; Bellio et al. 2021). Furthermore, it is of interest to investigate the membrane influence inferred by the SSA-drug complex challenged against the respective mono- and polymicrobial biofilm models. With reference to the toxicity and infection studies performed, although lower concentrations of the selected SSA (SSA 39) was found to be non-toxic in cell culture models (Boles et al. 2021; Dora et al. 2021; White et al. 2021), the concentrations used in this study was toxic to *Caenorhabditis elegans* and could not protect *C. elegans* from *C. albicans* infection.

Supramolecular self-associating amphiphiles interact with *Candida* membranes

From the *in vitro* findings discussed in Chapter 2, SSA 39 was selected to elucidate SSA-membrane interactions. The molecular structure of SSA 39 facilitates intrinsic fluorescent properties that may be exploited to observe relevant SSA-membrane interactions (Allen et al. 2020; White et al. 2020a). White et al. (2020) used a combination of fluorescence and transmitted light microscopy imaging techniques to show that SSA 39 is present at a Gram-positive and Gram-negative cell surface as a spherical aggregate. From their investigation, it is speculated that the SSA coats the bacterial surface, and disrupts susceptible cell membranes, which results in internalisation.

The current study investigated membrane interactions of SSA 39 with yeast (*C. albicans*) membranes, and found that this SSA also interacts with the yeast cell surface as well as the extracellular matrix of the biofilm. To investigate the observed SSA localisation and elucidate potential SSA-membrane interactions, a technique known as nanodisc technology was employed. Nanodiscs are synthetic membrane systems composed of relevant components (in this case GPLs) and a polymer belt (in this case styrene maleic anhydride or SMA) to imitate the phospholipid bilayer environment of cells. From its composition, nuclear magnetic resonance (NMR) titration assays may be performed to deduce membrane component interactions with compounds, such as SSAs (Scheidt and Huster 2008; Denisov and Sligar 2016; Ravula et al. 2018; Medina-Carmona et al. 2020; Padmanabha Das et al. 2020).

To our knowledge, our study is the first to synthesize phospholipid nanodiscs from glycerophospholipids extracted from *C. albicans* NCPF3645 and *C. auris* WT1912 biofilm cells. The synthesized phospholipid nanodiscs were utilised in a novel phospholipid nanodisc ^{1}H NMR CPMG adhesion assay to establish SSA-membrane coordination events (Vallurupalli et al. 2009). From these data, we hypothesize novel interactions and bond formations between SSA 39 and fungal GLPs, phosphatidylinositol and phosphatidylserine. The hypothesized interaction between SSA 39 and GPLs (White et al. 2020a), and the presence of GPLs in the extracellular matrix of biofilms (Kim et al. 2011; Yue 2014; Mitchell et al.

2016; Boles et al. 2022; Doolan et al. 2022) may explain this phenomenon. It is interesting to note that although SSA 39 was able to inhibit *C. albicans* biofilm formation, it was less effective against mature biofilms. This, may in part, be explained by the ability of the GLPs in the EXM to sequester the SSA and prevent interaction with the cell membrane.

Future considerations

In line with the combined efforts of an interdisciplinary consortium chaired by Professor Jennifer Hiscock, our study evaluated supramolecular self-associating amphiphiles as novel anti-biofilm compounds. The novelty of this project elucidated crucial structure-activity relationship information to evolve the novel library of molecules synthesized by the Hiscock group. From the study, future considerations are important to evaluate the efficacy and feasibility of these compounds accurately and thoroughly in a clinical setting. Proposed considerations include evaluating the efficacy of other SSAs in combination and investigating the effect of these combinations as potentiating agents for currently marketed antimicrobials of interest. Furthermore, it is proposed that future research should attempt additional toxicity – and co-infection studies at lower concentrations and in combination with other substances (such as colistin or fluconazole) when challenging animal model infections to elaborate the scope of SSA technology application.

Above all, an important aspect of drug development is understanding the mechanism(s) of actions of the novel antimicrobial compounds and their specific targets. Although evidence points to the interaction between SSAs and cell membranes, factors such as the specificity of the interaction between SSAs and GLPs and other membrane constituents (such as sterols) needs to be further elucidated. One avenue that may be pursued is determining the phospholipid membrane composition of clinically relevant pathogens in different biofilm stages and investigating the selectivity of the SSA library against phospholipid combinations. The investigation of protein-protein interactions may supplement current findings and provide insight into the hypothesized SSA-GPLs coordination events. To do so, the use of pull-down assays are of interest to confirm our hypothesised novel SSA interactions (Louche et al. 2017).

Transcriptome profiling is a powerful tool used to elucidate the genetic regulation and expression patterns of cells. This technology offers invaluable insight to the biological processes involved in cellular and metabolic maintenance (Rani and Sharma 2017; Chong et al. 2018). To complement transcriptomic investigation, interactome studies will facilitate the analysis of interactions, and the consequences of those interactions, between and among proteins and other molecules within a cell (Parrish et al. 2006; Luck et al. 2017). Furthermore, novel imaging nanotechnology called Auger-architectomics, which is based in principle on Auger electron optics linked to scanning electron microscopy (SEM), is used to study drug bio-sensors in nano-detail (Swart et al. 2014). Nano-scanning Auger microscopy (NanoSAM) technology will enable the analysis of cellular elemental compositions and profiles to expose the effect and fate of SSAs on cells (Swart et al. 2010; Swart et al. 2012). In line with these techniques, a thorough and detailed cellular architecture may be composed to unambiguously report the SSA mechanism of action.

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Summary

Antimicrobial resistance (AMR) has become a significant problem, leading to millions of deaths related to AMR-associated infections. Hospitals, which are susceptible to opportunistic pathogens such as *Pseudomonas aeruginosa* and *Candida albicans*, are vulnerable to contamination. Biofilms, which are a noteworthy antimicrobial resistance mechanism, contribute to consistent and recurring infections and display enhanced resistance to various host defence mechanisms. The World Health Organization (WHO) has identified a list of critically important bacterial and fungal pathogens (including *P. aeruginosa* and *C. albicans*) that require immediate research and subsequent development of novel antimicrobial substances.

Supramolecular self-associating amphiphiles (SSAs) are a promising class of amphiphilic molecules that have gained attention as potential antimicrobial alternatives. This study evaluated the efficacy of SSAs against mono- and polymicrobial biofilms formed by *P. aeruginosa* and *C. albicans* and proved that SSAs do indeed have antibiofilm capabilities. However, these capabilities indicated a degree of specificity between biofilm models. From the compounds screened, five showed promising inhibitory action. It is interesting to report, SSA 39, which was able to inhibit biofilm formation, was less effective against pre-formed biofilms. SSA 39 was found to interact with *C. albicans* SC5314 biofilms and fungal membranes, alluding to a potential membrane interaction. The tested SSAs also displayed a varying ability to act as antimicrobial potentiating agents for colistin and fluconazole. Unfortunately, the selected SSA (SSA 39) was found to be toxic to *Caenorhabditis elegans* and did not protect the nematodes from infection by *C. albicans*.

The use of synthetic membrane systems (known as nanodiscs) provides a stable and controlled environment to study microbial membrane components, and may facilitate the design of novel antimicrobial compounds with optimized drug target sites. In line with this, a novel phospholipid nanodisc ^{1}H NMR CPMG adhesion assay was used to hypothesize potential interactions between glycerophospholipids isolated from fungal membranes.

From this study, it is evident that alternative strategies such as drug repurposing and novel compound design are necessary to combat antimicrobial resistance and prevent the growth of multidrug-resistant pathogens. Furthermore, this study contributes by highlighting the need to develop novel strategies to treat biofilm-associated infections, given that biofilm environments can enhance antimicrobial resistance mechanisms. Future considerations should focus on investigating the fungal membrane phospholipid composition of clinically relevant opportunistic pathogens, as well as determining the selectivity of the SSA library against relevant phospholipid combinations. Finally, it is of importance to elucidate the fungal mechanism(s) of actions utilised by SSAs and to report the complete fungal cellular response profile. To do so, transcriptomic investigation, Nano-scanning Auger microscopy (NanoSAM) technology and establishing resultant interactome maps will be used.