Developing novel anti-*Candida* therapies through the understanding of drug-resistance mechanisms and by tailoring new chemicals to be used in anti-biofilm surfaces

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Abstract: The fungi belonging to the *Candida* genus are commensals of the human microbiome, however, when the host immune system is compromised these yeasts can overgrow causing infections that can be life-threatening. Besides, being well adapted as human-infecting microbes, *Candida* cells are able to acquire resistance to usual antifungals. Therefore, it is urgent the discovery of new molecules with antifungal activity. In this work, several camphorimine-based complexes having silver, copper and zinc as metallic centers, were synthesized, characterized and their activity against *Candida* strains, including those resistant to azoles, tested. Two promising Ag(I)-camphorimine complexes (P and Q) were identified. The MIC value calculated for compound P was 15.63 µg/mL either for *C. albicans* and *C. glabrata* while for compound Q was 31.25 µg/mL. Exploring functionalized polycaprolactone coatings with both compounds, it was possible to reduce colonization of stainless steel plates by 62% and 25%, comparing with colonization obtained in non-functionalized plates. These observations strongly suggest these compounds have a strong potential to be used as anti-*Candida* molecules including in biofilm-free surfaces, this being an important trait since colonization of medical devices is a critical factor underlying the success of *Candida* as pathogen.

Keywords: *Candida* ● Ag(I)-camphorimine complexes ● Functionalized coatings

Introduction

Candida are eukaryotic, unicellular microorganisms, members of the Fungi kingdom and can be found in the normal microbiota of an individual's mucosal oral cavity, gastrointestinal and genitourinary tracts and skin. Species found in the human microbiota are considered commensal in healthy humans, which means that they live and thrive thanks to the resources of the host, while the host does not benefit nor is harmed in this association [1]. However, when mucosal barriers are disrupted, the immune system is compromised or when changes occur in the host microbiota, the commensal *Candida* populations transit to a pathogenic phase, invading the bloodstream and causing potentially lethal infections with the overall mortality ranging from 30% to 60% [2]. Factors such as treatment with antibiotics, diabetes, cancer, extreme age, immunosuppression, intravenous catheters or long-term hospitalization pose a higher risk of getting a fungal infection [3]. Around 17 different *Candida* species are known to cause infections, however the majority of invasive infections are attributable to *C. albicans*, followed by *C. tropicalis, C. parapsilosis, C. glabrata* and *C. krusei* [4].

There are four drug classes available for the treatment of *Candida* infections: azoles, polyenes, echinocandins and the pyrimidine analogue flucytosine. Susceptibility testing demonstrated a significant decrease on the rates of susceptibility to azoles among *Candida* species, from 65.2% in 2011 to 60.6% in 2015 [5]. The variation in the mechanisms by which *Candida* species can outweigh drug effects pose a significant challenge, not only in determining the efficacy of the therapy, but also in the early diagnosis of the infection [6]. Besides this, it has been well shown that systemic infections can result from colonization by commensal strains of inert medical devices such as prosthetics, pacemakers and catheters [7]. Strategies to obtain devices with antifungal activity are of utmost importance. This can be achieved by the impregnation of the material with antimicrobial agents or coating with such molecules. This strategy was followed by Balne et al. (2018), that developed a drug-free alternative strategy to coat catheter surfaces using pyrogallol and metal ions of Mg^{2+} and Ag^{+}. Silver has proven to be an effective antimicrobial component and modern silver-containing antimicrobial wound dressings are now widely used for the care of infected wounds and for the prevention of wound infections [9]. An example is Silvercel, that combines the potent broad-spectrum antimicrobial action of a silver-coated nylon fibre with the enhanced exudate management properties of alginate fibres [10].

Several new approaches are nowadays being considered as alternatives to the more traditional treatments for candidiasis: use of natural compounds, probiotics, vaccination, RNAi, small-molecules, antimicrobial peptides, nanoparticles and chemical synthesis. In this last approach, there is great interest in the use of metal complexes [11]. Organometallic chemistry is the study of compounds containing at least one bond between a carbon atom of an organic moiety and a metal, guaranteeing a high stability of complexes in physiological conditions and, therefore, enhancing its pharmacological properties [12]. In the coordination of an organic scaffold to a metal ion, the activity of the compound can be enhanced due to the stabilization provided by the metal center. In parallel, the toxicity of the metal may be reduced due to the shielding effect provided by the organic ligand that can limit the metal interaction with the surrounding biomolecules or targeted it towards the desired effects [13]. As such, this combination gives rise to compounds with peculiar attributes, such as the ability to interact with DNA, enzymes and protein targets or redox features that can enable redox reactions, leading to the production of reactive species resulting in oxidative stress for the cells [14]. Most of the metals that have been exploited are transition metals, of which silver is the most prominent, followed by copper and zinc, well-known for their biocompatibility, with important role in immunity and with demonstrated antimicrobial properties [15]. Silver complexes seems to be effective fighting *Candida* biofilms, by interfering with the extracellular matrix composition of mature biofilms, proteins, carbohydrates and DNA [16]. Savić et al. (2018) synthetized mononuclear silver(I) complexes with 1,7-phenanthroline ligands, that effectively inhibited growth of *C. albicans, C. parapsilosis, C. glabrata* and *C. krusei* with MICs ranging from 1.2 and 11.3 μM. Copper is an essential trace element in most living organisms and is present mostly in proteins and enzymes, where it serves as an electron donor/acceptor by alternating between the redox states Cu(I) and Cu(II), giving rise to ROS in chemical reactions in which it participates [17]. Similar to copper, zinc is an essential element for proper functions of human body, can be readily excreted by natural biological processes and is considered non-toxic to human cells. In addition, these two metals have the advantage of being easily accessible and cheaper than silver [18].

In the practice of design new antimicrobial drugs, is essential the creativity, improvement of synthetic methodologies and focus on the development of novel reactions. The improvement of the synthetic tools will depend on the identification of possible targets in the fight against pathogens that will allow to increase the panoply of possible antimicrobial candidates either as general antifungal drugs or for target delivery in medical devices coatings Thus, the focus of this work will be the synthesis, characterization and quantification of the antifungal activity against *C. albicans* and *C. glabrata* species of new organometallic-like compounds using not only silver precursors other than silver nitrate, but as well copper and zinc salts, and subsequent functionalization of coatings with the most efficient compounds.

Materials and Methods

Synthesis and chemical characterization of the camphorimine complexes

- **Materials**

Camphor and the appropriate amines were purchase from Sigma-Aldrich and Alfa Aesar, respectively. The precursors of coordination complexes were from Sigma-Aldrich and Acros Organic. The solvents (PA grade) were from Carlo Erba and Sigma-Aldrich.

- **Synthesis of Ligands and Complexes**

$C_{32}H_{36}N_2O_2$ (L1): (3,3)-3,3'-([1,1'-biphenyl]-4,4'-diylbis(azanylylidene))bis(1,7,7-trimethylbicyclo [2.2.1] heptan-2-one). Camphorquinone (330 mg; 2.0 mmol) was stirred in ethanol (6 mL) acidified with acetic acid (0.2 mL) at RT for 1 hour. Benzidine (184 mg; 1.0 mmol) was then added and the yellow mixture was stirred at 50ºC for 21 hours. The solvent was removed under vacuum and the yellow solid was washed with diethyl ether and n-pentane. Yield 89%.

$C_{26}H_{32}N_2O_2$ (L2): (3,3)-3,3'-(1,4-phenylenebis(azanylylidene))bis(1,7,7 trimethylbicyclo[2.2.1] heptan-2-one). Camphorquinone (330 mg; 2.0 mmol) was stirred in ethanol (5 mL) acidified with acetic acid (0.17 mL) at RT for 20 minutes. *p*-phenylenediamine (106 mg; 1.0 mmol) was then added and the orange mixture was stirred at 50ºC for 19 hours. After cooling, the solvent was removed under vacuum and the yellow solid was washed with n-pentane (3 x 5mL). Yield 53%.

$C_{26}H_{32}N_2O_2$ L3: (3,3)-3,3'-(1,3-phenylenebis(azanylylidene))bis(1,7,7-trimethylbicyclo[2.2.1] heptan-2-one). Camphorquinone (310 mg; 1.9 mmol) was stirred in ethanol (5 mL) acidified with acetic acid (0.5 mL) at RT for 1 hour. *m*-Phenylenediamine (100 mg; 0.93 mmol) was then added and the mixture was stirred under nitrogen atmosphere at 50ºC for 20 hours. By solvent removal, followed by addition of H_2O (4 mL) and extraction with $CHCl_3$ (3 x 5 mL) a solution was obtained that was dried over $MgSO_4$ for 2 hours. Upon filtration to separate the drying agent, the solvent was evaporated affording a yellow oil. Yield 66%.

The camphor derivatives and Cu(II) complexes were synthesized under air using conventional techniques and the Cu(I) complexes and Zn(II) complexes were synthesized under inert gas atmosphere using vacuum/Schlenk techniques.

[($CuCl_2$)(L1)]: (A)

$CuCl_2{\cdot}2H_2O$ (100 mg; 0.58 mmol) and ligand L1 (258 mg; 0.56 mmol) were mixed and THF (6 mL) was added and the solution was stirred for 1 hour at 40ºC. The solvent was removed under vacuum and the brown solid dried. Yield 84%.

[($CuCl_2$)(L3)]: (C)

$CuCl_2{\cdot}2H_2O$ (140 mg; 0.58 mmol) and ligand L3 (308 mg; 0.64 mmol) were mixed and THF (4 mL) was added. The solution was stirred for 1 hour at 40ºC. The solvent was partially evaporated, and a green solid precipitated that was filtered and dried. Yield 54%.

[($Cu(NO_3)_2$)(L1)]: (F)

$CU(NO_3)_2{\cdot}3H_2O$ (70 mg; 0.29 mmol) and ligand L1 (156 mg; 0.32 mmol) were mixed and THF (4 mL) was added. The solution was stirred for 1 hour at 40ºC. The solvent was partially evaporated and a green solid precipitated that was filtered, washed with ether (4 mL) and dried. Yield 81%.

[($Cu(NO_3)_2$)(L3)]: (G)

$CU(NO_3)_2{\cdot}3H_2O$ (70 mg; 0.29 mmol) and ligand L3 (156 mg; 0.32 mmol) were mixed and THF (4 mL) was added. The solution was stirred for 1 hour at 40ºC. The solvent was partially evaporated until a green solid precipitated that was washed with ether (4 mL) and dried. Yield 97%.

[($(CuCl)_2$)(L1)]: (I)

$CuCl_2{\cdot}2H_2O$ (79 mg; 0.79 mmol) and ligand L3 (190 mg; 0.40 mmol) were mixed and stayed under vacuum for 1h30. The solvent THF (3 mL) was added and the solution was stirred for 30 minutes at RT. The solvent was evaporated and the brown solid precipitated, that was washed with THF (4 mL) and dried under vacuum. The synthesis was made under inert gas atmosphere using vacuum/Schlenk techniques. Yield 57%.

[($(CuCl)_2$)(L2)]: (J)

$CuCl_2{\cdot}2H_2O$ (100 mg; 1.01 mmol) and ligand L2 (204 mg; 0.51 mmol) were mixed and stayed under vaccum for 1h30. The solvent THF (5 mL) was added and the solution was stirred for 30 minutes at RT. The solvent was evaporated and the brown solid precipitated, that was washed with THF (4 mL) and dried under vacuum. The synthesis was made under inert gas atmosphere using vacuum/Schlenk techniques. Yield 62%.

[$(ZnCl_2)$(THF)(L1)] : (L)

$ZnCl_2$ (80 mg; 0.59 mmol) and ligand L1 (160 mg; 0.30 mmol) were mixed and THF (5 mL) was added and the solution was stirred for 3 hours at RT. The volume of the solvent was reduced and the orange solution was placed in the freezer overnight. The crystals obtained were filtered and dried. To the resulting solution it was added ether (1 mL). A precipitate formed that was discarded and the solution was placed in the freezer overnight. Crystals formed that were filtered and the red solid was dried under vacuum. Yield 32%

- **Chemical characterization**

FTIR spectra were obtained from KBr pellets using a JASCO FT/IR 4100 spectrometer. NMR spectra (^{1}H, ^{13}C, DEPT) were obtained from acetonitrile-d3 (CD3CN) or chloroform-d ($CDCl_3$) solutions using Bruker Avance II^+ (400 MHz) spectrometer. NMR chemical shifts are referred to TMS (δ = 0 ppm). Elemental analyses (C, N, H, S) were performed by Laboratório de Análises do Instituto Superior Técnico.

Strains and growth media used for anti-*Candida* assays

A cohort of *C. albicans* and *C. glabrata* clinical isolates previously described to be resistant to azoles was used. [5,6] Besides these clinical isolates, the reference strains *C. albicans* SC5314 and *C. glabrata* CBS138 and environmental strain *C. glabrata* UTAD68 isolated from wine were used. The different strains were maintained at -80ºC in YPD medium supplemented with 30% glycerol (v/v) (Merck). *Candida* cells were batch-cultured at 30ºC, with orbital stirring (250 rpm) in rich growth medium Yeast Peptone Dextrose (YPD) or RPMI (Roswell Park Memorial Institute Medium). YPD contains, per liter, 20 g glucose (Merck Millipore), 10 g yeast extract (HiMedia Laboratories, Mumbai, India) and 20 g Peptone (HiMedia Laboratories). RPMI, contains, per liter 20.8 g RPMI-1640 synthetic medium (Sigma), 36 g glucose (Merck Millipore), 0.3 g of L-glutamine (Sigma) and 0.165 mol/L of MOPS (3-(N-morpholino) propanesulfonic acid, Sigma). All media were prepared in deionized water. YPD medium was sterilized by autoclave for 15 minutes at 121ºC and 1 atm. RPMI medium was filtered with a 0.22 µm pore size filter and preserved at 4ºC until further use. Unless otherwise specified the pH of the RPMI growth medium was adjusted to 7.0 using NaOH as the alkalinizing agent.

Assessment of antifungal potential of camphorimine complexes

The ability of the camphor-derived complexes to inhibit the growth of *Candida* species was assessed using the standardized microdilution method recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) to determine the minimum inhibitory concentration (MIC) values, considered to be the concentration of drug that reduced yeast growth by more than 50% the growth registered in drug-free medium. Briefly, cells of the different species were cultivated at 30ºC and with 250 rpm orbital agitation for 18h in YPD growth medium and then diluted in fresh RPMI growth medium (Sigma) to obtain a cell suspension having an OD_{600nm} of 0.05. From these cell suspensions, 100 µL aliquots were mixed in the 96-multiwell polystyrene plates with 100 µL of fresh RPMI medium (control) or with 100 µL of this same medium supplemented with 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125.0, 250.0, 500.0 µg/mL of the different compounds. The stock solutions of Ag(I)-derived compounds were prepared from the powder, using DMSO (Dimethyl sulfoxide, Sigma) as the solvent. As a control was also examined the inhibitory effect of precursors and free ligands. After inoculation, the 96-multiwell plates were incubated without agitation at 37ºC for 24h. After that time, cells were resuspended and the OD_{530nm} of the cultures was measured in a microplate reader. The MIC value was taken as being the highest concentration tested at which the growth of the strains was 50% of the value registered in the control lane.

Coating functionalization with Ag(I) camphorimine complexes

The polycaprolactone (PCL) solutions were prepared at concentrations of 8% and 4% (w/v) using TFA (Trifluoroacetic acid, Sigma), chloroform (Carl Roth) or dichloromethane (Carl Roth) as solvents. PCL pellets (Sigma) were dissolved for 3 hours in magnetic stirrer at room temperature. For the PCL coating preparation, 316L stainless steel (SS) plates were previously polished with grit P600 and P1200 to ensure a homogeneous surface pattern and then submerged in acetone (Sigma) prior to dip-coating process to wash out any impurity. Dip-coating was done on PCL solutions without and with compounds P or Q. The stock solutions of Ag(I)-derived compounds were prepared from the powder, using DMSO (Dimethyl sulfoxide, Sigma) as the solvent. PCL solutions were

prepared with compound P at concentrations of 0.156 µg/mL and 1.56 µg/mL (P10 and P100, respectively) and with compound Q at concentrations 0.313 µg/ml and 3.13 µg/ml (Q10 and Q100, respectively). The dipping process was made by using Siemens Logo 12/24 RC and consisted of the complete dip of the SS plates, which were removed from the dip-coating solution at a speed of 0.3 $cm.s^{-1}$. After 1 minute drying a second dip was made using the same conditions, after which the plates dried overnight at RT.

Physical-chemical characterization of the surfaces

The morphological and chemical characterization of the isolated compounds and of the newly synthesized coatings were analyzed by scanning electron microscopy (SEM) using a JEOL-JSM7001F or Hitachi S2400 apparatus and the elemental chemical composition by the respective X-ray energy dispersion spectrometer (EDS). To increase the conductivity of the samples, a conductive thin layer of gold and palladium was applied with a Polaron E-5100.

Assessment of the effect of PCL coatings on the growth of *C. albicans*

To assess the ability of *C. albicans* to form biofilms on bare or coated surfaces, the reference strain SC5314 was. Cells were cultivated at 30ºC with orbital agitation in rich YPD growth medium until mid-exponential phase (OD_{600nm}=1±0.1) and then re-inoculated (at an initial OD_{600nm} of 0.1±0.01) in a 25 mL capacity beaker containing 4.5 mL of fresh RPMI growth medium (Sigma) at pH 7. After inoculation, bare or coated plates were submerged in the prepared cell suspension. The cultures were grown at 37 ºC with gentle shaking (30 rpm) for 48 h. After this time, bare or coated plates were removed and washed twice with distilled water to eliminate non-adherent cells. The number of viable cells adhered to the surfaces was quantified after washing the plates with distilled water and scratching the surface to remove the adhered cells. The scratched material was diluted in 1 mL of sterile distilled water and serial dilutions were subsequently performed (in a range of 10^{-1} to 10^{-4}). Fifty microliters of each cell suspension were plated on solid YPD growth medium and the number of colony forming units (CFUs) formed 48 hours after incubation, at 30ºC, was quantified. The number of CFUs present in the supernatant was also determined. For this, 1 mL of each supernatant was diluted (1:10) in sterile water after which serial dilutions (in a range of 10^{-1} to 10^{-5}) were subsequently prepared and fifty microliters of each cell suspension were plated on solid YPD growth medium. For the observation of the biofilms formed on the surface of bare or coated surfaces, plates were washed with distilled water, and immersed in 70 % (v/v) ethanol for 10 min, with 95 % (v/v) ethanol for 10 min and finally with absolute ethanol for 20 min to fix cells to the surface. After complete air-drying, the plates were coated with a conductive thin layer of gold and palladium applied with a Polaron E-5100 and analyzed by SEM (JEOL-JSM7001F or Hitachi S2400).

Results and Discussion

Synthesis of ligands and of Ag/Zn/Cu- based complexes and assessment of their anti-*Candida* potential

To synthesize the metal complexes, it was first necessary to prepare several camphorimine derivatives that were used as ligands. (Fig. 1) The ligands selected were chosen based on previous results that showed that these were convenient ligands for biologically complexes, since these have suitable structural and electronic characteristics [16].

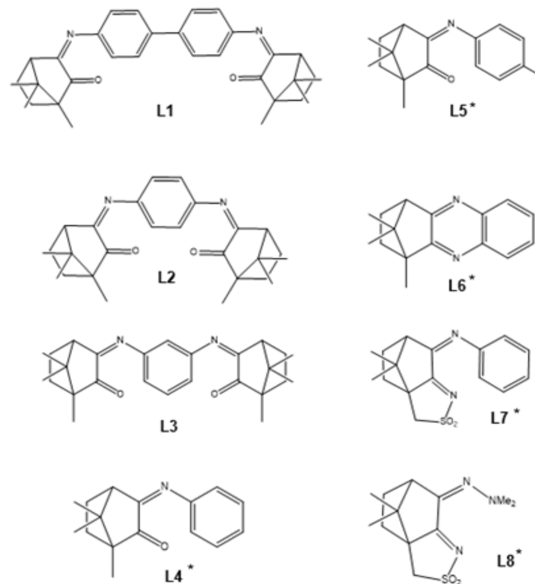

Figure 1 - Camphorimine derivatives used as ligands. Ligands marked with * were synthetized by someone else.

One of the goals of this work was to somehow redesign the use of Ag(I)-camphorimine based complexes to improve their anti-*Candida* potential (extending it to *C. albicans*), while minimizing their cytotoxic potential. To do so, silver nitrate (that was used as a precursor before) [19] was changed for silver chloride

(AgCl) and silver hydroxide [Ag(OAc)]. In an attempt to design new, cheaper and effective antifungal compounds, camphorimine ligands were used to coordinate copper and zinc to generate Cu(I), Cu(II) and Zn(II) coordination complexes. The precursors of Cu(II) coordination complexes used were $CuCl_2.2H_2O$ and $CU(NO_3)_2.3H_2O$, while for Cu(I) coordination complexes it was used CuCl and for Zn(II) coordination complexes, $ZnCl_2$. The anti-*Candida* potential of the precursors and the synthesized complexes was determined based on the MIC against the reference species *C. albicans* SC5314 and *C. glabrata* CBS138. It was verified that none of the ligands and the precursors AgCl, Ag(OAc) and $Cu(NO_3)_2$ had activity against the two *Candida* strains, while the silver complexes were the most effective. Nevertheless, the most promising results were found for the coordination complexes with Ag(OAc) as precursor, whose MIC values were the lowest, corresponding to the highest antifungal potential.

Therefore, one of the initial purposes of this study was reached, since it was possible to identify an alternative molecule to Ag(I)-camphorimine complexes previously reported able to sensitize *C. albicans* [19]. In the work of Cardoso (2017*) et al.* the ability of this *Candida* strain to form silver nanoparticles on the cell's surface was reported and this characteristic is likely to be one of the causes for the lack of activity of complexes of this nature against *C. albicans*. Here, using a different precursor in the synthesis of these complexes it was possible to obtain new compounds with higher antifungal potential and greater spectrum of antifungal action. Regarding *C. glabrata*, the MIC values obtained were like the ones reported by Cardoso (2017) *et al.* in the range of 15.6 to 125 µg/mL. The most promising compounds were complexes P and Q. The MIC value calculated for compound P was 15.6 µg/mL either for *C. albicans* and *C. glabrata*, and for compound Q was 31.3 µg/mL Regarding the cooper complexes, none were effective against *C. albicans*, except compound (K). Cu(II) complexes have been shown to have some activity against *C. glabrata* and Cu(I) complexes appear to be more effective. Finally, zinc complexes were shown to be ineffective against both strains of *Candida*. When comparing the silver complexes with the copper and zinc complexes, the firsts were undoubtedly the most promising. It should be noted that the complex with the highest activity was P, whose ligand was L1. For the copper complexes it was also found that the most effective were coordinated with L1, namely complexes A, F and I. This evidence indicates that this ligand presents characteristics that allow the complex to interact in some way with *Candida* cells and inhibit their growth. These results confirm that when following the chemical synthesis approach to obtain antifungals, namely the synthesis of organometallic-like compounds, the concern should not be only the metal present, but as well the ligand, which is fundamental for the molecule structure. Different ligands coordinated to the same metal gave rise to compounds with different geometries that might determine the distinct specificity of these compounds.

Ability of Ag(I)-based camphorimine complexes to sensitize azole-resistant *Candida* strains and its synergistic effect with fluconazole

Based on the results obtained it was decided to further proceed with the study of compounds P and Q that exhibited the highest efficacy against the reference strains. More drug resistant microorganisms are being identified and it is urgent to concentrate efforts to find new molecules that sensitize these strains. Therefore, these compounds were tested against resistant clinical isolates of *C. albicans* and *C. glabrata*. In order to do this, the MIC level of a previously identified cohort of *C. albicans* (2 strains) and *C. glabrata* (10 strains) azole-resistant strains was determined (Fig.2). In the assay performed was

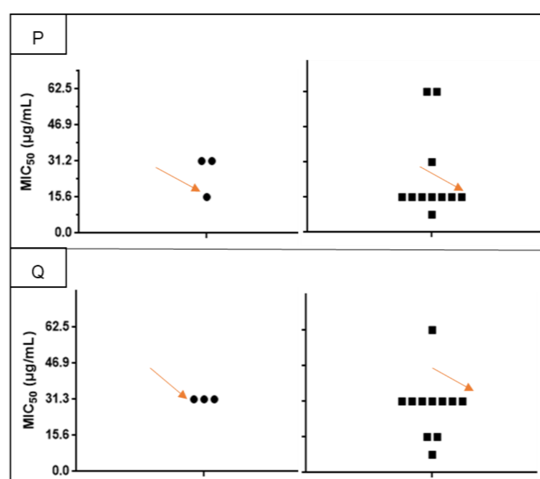

Figure 2 - MIC values (µg/mL) of the complexes P and Q, obtained for the reference *C. albicans* (SC5314) strains and respective isolates, represented by ●, and for the reference *C. glabrata* (CBS138) strains and respective resistant isolates, represented by ■. The reference strains are represented by an arrow.

possible to verify if the compounds P and Q sensitize azole-resistant strains *of C. albicans*

and *C. glabrata*. For this to happen, it was necessary that the MIC values of the resistant strains were equal to or less than the MIC value obtained for the respective reference strain. In Fig. 2 it is possible to verify that complex P was able to sensitize most of the strains of *C. glabrata* and did not had this effect in the strains of *C. albicans*, whereas the compound Q was able to sensitize all resistant isolates of both strains (with the exception of one *C. glabrata* isolate). The fact that these compounds sensitized most of the azole-resistant strains may indicate that the mechanism of action of these molecules is different from that of the azoles, however more studies are necessary to clarify this hypothesis.

Using Ag(I)-based camphorimine complexes in the design new coatings for functionalizing medically relevant surfaces

Considering the results obtained for the antifungal action of P and Q metal complexes, the next goal was the application of these molecules to medically relevant surfaces. For that purpose, a deeper physicochemical analysis of these compounds was performed by scanning electron microscopy (SEM) and elemental chemical composition by X-ray energy dispersive spectrometry (EDS).

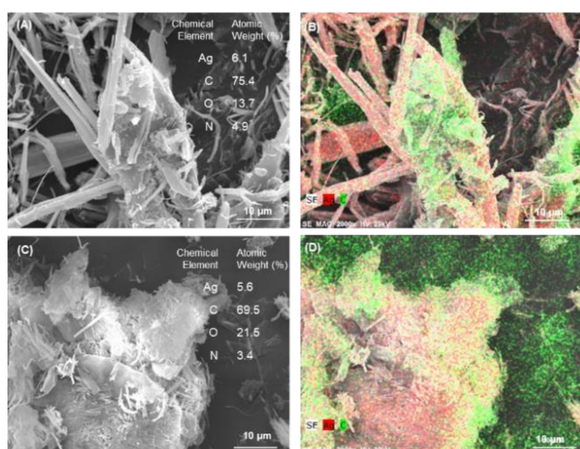

Figure 4 - Scanning electron microscopy (SEM) micrographs for (A, C) and corresponding X-ray energy dispersive spectrometer (EDS) maps (B, D) for compound P (A, B) and compound Q (C,D); In EDS maps silver (Ag) is marked in red and carbon (C) in green.

Despite the different morphology, the presence of silver was confirmed in both compounds. Compound P was composed by needles, where silver was concentrated, and blocks where this element was not detected, as shown in Figure 3 (A, B). In the compound Q silver was detected in different structures, being present in large aggregates (C, D). The strategy used to immobilize these compounds (P and Q) on the surface of biomedical devices was through their incorporation in biodegradable polymeric coatings for 316L stainless steel sheets, used in medical implants and prostheses [20]. For that purpose, polycaprolactone (PCL), a polyester, was used [21]. The most appropriate solvent was selected based on the solubility of both compounds (P and Q) that match with those for PCL solubilization as trifluoroacetic acid (TFA), chloroform and dichloromethane. Initially, PCL coatings were formulated using TFA as a solvent and an 8% (w/v) polymer concentration. After drying, the coating broke off from the stainless steel (SS) surface. Following this result and since TFA releases toxic gases that can cause severe skin burns [22], it was decided to use chloroform and 8% (w/v) PCL. Once again, the coating broke and was detached from the SS surface after drying. Given that the coating broke in the previous tests, PCL concentration was reduce to 4% (w/v). The dried coatings using both chloroform or dichloromethane as solvents remained intact. As reported by the elemental chemical composition from EDS, the contents of C and O were about 85% and 15% for both coatings (Atomic Weight %). At this point, it was possible to obtain two PCL coatings with different solvents that represented two alternatives for the functionalization with compounds P or Q. Each solvent was tested (chloroform or dichloromethane) with P or Q using concentrations of x10 MIC values, hereinafter referred as P10 and Q10. For the functionalization, each compounds was previously dissolved in DMSO and added to a solution of 4% (w/v) of PCL. Once the coatings were synthesized, *C. albicans* SC5314 cells were cultured on bare and coated plates to assess if

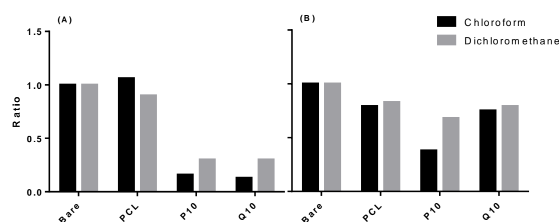

Figure 3 - Graphical representation of ratios between colony forming units per milliliter (CFUs/mL) values on each coated plate with the bare plate for the supernatant (A) and biofilm (B) when using chloroform (black) or dichloromethane (grey). Plates tested were: bare plate, PCL coated plate, PCL plate and compound P at concentration of x10 MIC values (µg/mL) and PCL plate and compound Q at concentration of x10 MIC values (µg/mL).

the compounds kept their antifungal activity. To assess the antifungal potential of the coating functionalized with P or Q, upon immersion, the viability of the planktonic cell in the supernatant

was evaluated, whereas the antibiofilm potential was evaluated on the cells that adhered to the materials' surface. To determine the number of viable cells in each assay, 48 hours after incubating *C. albicans* cells with the bare or the coated plates, colony forming units (CFU) in the supernatant and biofilm were determined. For an easier comparison among assays, CFUs/mL were normalized as ratios between the values obtained in each coated plate to the value for the corresponding bare plate. (Fig.3). As represented in Fig. 3, PCL coating using chloroform or dichloromethane did not significantly affect the growth of *C. albicans* cells in the supernatant (A) nor the ability of cells to adhere to the surface (B), since viable cells were collected upon washing and scratching of the surface in both bare or PCL coated plates. Viable cell in the supernatant (A) or in the biofilm (B) seemed to have been affected in all plates coated with PCL functionalized with compounds P or Q. When comparing the solvents used, chloroform stands out, because this solvent resulted in the smallest ratios, suggesting that the different solvents used during the formulation of these coatings were somehow modifying the chemical environment of the compounds. As major result, both compounds P or Q proved to keep their anti-*Candida* efficiency when embedded in the polymer matrix and the chloroform stood out as a solvent. Therefore, the assay that yields the most efficient results was the one using PCL with a concentration of 4%, a compound concentration of x10 MIC value and chloroform as a solvent. Once the best experimental conditions were selected, the surfaces of the coatings using chloroform as solvent and compounds P or Q were physicochemical characterized (Fig. 4). In

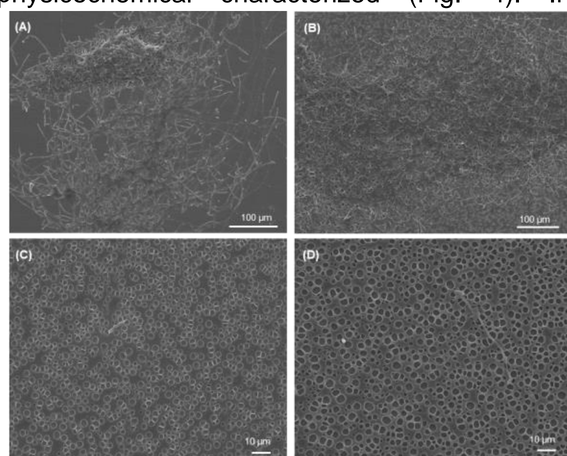

Figure 4 - Colonization of bare and coated steel surfaces by *Candida albicans* SC5314 incubated under simulated physiological conditions and corresponding micrographs from SEM: Bare plate (A); PCL coated plate (B); PCL plate and compound P (C) or compound Q (D) at x10 MIC values (µg/mL).

coatings where compounds P or Q were present, the morphology appears different and it was possible to distinguish different structures on the surfaces, marked by points X and Y, in coatings with P10 and Q10, respectively. According to EDS analysis, were detected 14% of silver at point X and 4% at point Y (Atomic Weight %). These observations suggest that it is in these distinct structures that silver was concentrated on the coatings. Furthermore, the biofilm formation on the surface of bare or coated plates was investigated using SEM after incubating *C. albicans* cells on bare and coated steel plates (Fig. 5).

As depicted in Fig. 5, cells were able to efficiently adhere to the surface of bare (A) or PCL (B)

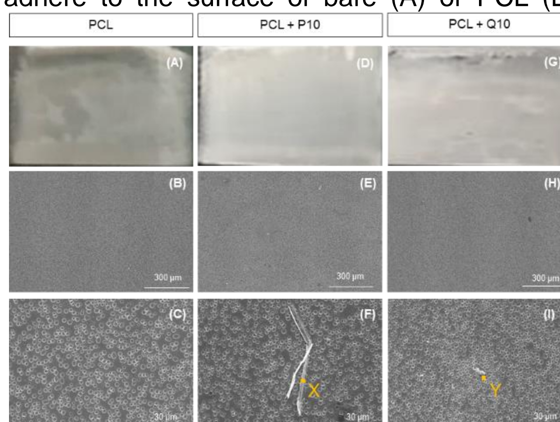

Figure 5 - Photographs of PCL 4% coatings using chloroform as solvent, without compounds (A) and with compound P (D) or Q (G) at concentrations of x10 MIC values (µg/mL). Scanning electron microscopy (SEM) micrographs of PCL 4% coatings using chloroform as solvent, without compounds (B,C) and with compound P (E,F) or Q (H,I) at concentrations of x10 MIC values (µg/mL). Points X and Y mark structures on the coating in which silver was detected.

coated plates, this being further confirmed by SEM analyses. Coating with PCL did not affect biofilm formation on the surface of the material, as had already been verified by the previously obtained CFUs/mL values (Fig. 3). Regarding PCL coating with compounds P or Q (C, D), it was confirmed that the presence of these molecules in the coating made it difficult for *C. albicans* cells to form a biofilm on the surface, as the presence of cells in this surface is significantly lower when compared to the colonization observed in bare and PCL coated plates. Both compounds appear to have an identical influence on colonization deterrent, however it is apparent that PCL coatings differ in morphology.

As the tests with chloroform yielded more effective results, it was decided to extend the assay for compounds concentrations of x100 MIC value using this solvent (Fig.6). Contrary to the initially thought, an increase in the compounds concentration did not result in a more pronounced anti-*Candida* effect of the coating. The biofilm formation capacity and cells growth was higher than in the assay with P10 and Q10, resulting in a less efficient coating when the compounds concentration is increased to 100-fold as can be seen in Fig.6 (C). The chemical environment of the compounds may have been changed by the increased concentration, which could result in distinct compound-polymer interactions, as for instance, compounds preferential aggregation over the solubilization of the polymer.

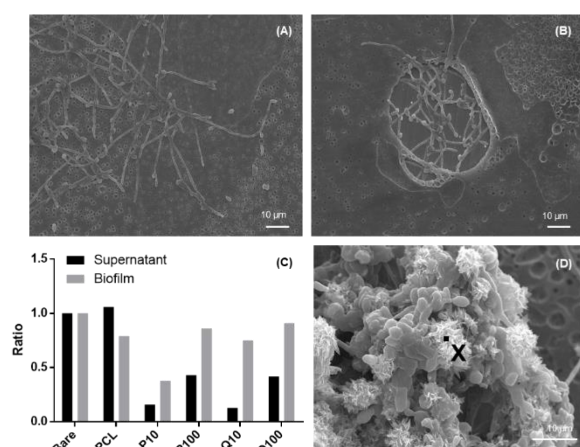

Figure 6 - Colonization of bare and coated steel surfaces *by C. albicans* SC5314 incubated under simulated physiological conditions and corresponding micrographs from SEM: PCL coated plate and compound P (A) or compound Q (B, D) at x100 MIC_{50} values (µg/mL). (C) graphical representation of ratios between colony forming units per milliliter (CFUs/mL) values on each plate and CFUs/mL values on bare plate for the supernatant and biofilm using chloroform as solvent. (D) SEM micrographs of large structures formed by cells and silver particles dispersed on the surface of Q100 coating .

According to Fig. 6 (A), in the P100 assay there was a greater surface colonization and it is possible to distinguish different morphologies in the coating, suggesting that the 100-fold concentration alters the chemical bonds, resulting in regions with different morphologies that can be distinguished on the surface of the coating. Similarly, this 100-fold increase in the concentration of compound Q (B) compromises the integrity of the coating and holes are formed revealing the underneath substrate and *C. albicans* were preferentially colonizing this surface. These observations confirm the obtained ratio values (C), where the ratios were found to be higher in both the supernatant and biofilm when applying compounds P or Q at a 100-fold higher concentration. The observations in Fig.6 (A, B) really demonstrate the threat these microorganisms pose and the ease with which they attach, grow and adapt to adverse situations. *C. albicans* cells were able to detect the areas where the plate was exposed, and holes existed and settled there. Nevertheless, an interesting observation was obtained in the Q100 assay. It was possible to find large structures scattered on the surface formed by cells and particles with silver (100%, Atomic Weight %) in its composition, detected by EDS in the point X of Fig.6 (D). The appearance of these particles was different from the morphology of compound Q, shown in Fig.2 (C).

These *C. albicans* cells are suspected of having the ability to break down the chemical bonds in compound Q, which by being no longer soluble in the coating polymer was exposed to the cells when it is in high concentration. This cleavage facilitates the passage of silver to the zero-oxidation-state and eventually precipitates into nanoparticles. This phenomenon has been described before, in the work of Cardoso (2017) *et al.* where this strain of *Candida* demonstrates the ability to form silver nanoparticles on the cell's surface. Another goal of this work was achieved, which was the functionalization of medical relevant coatings with the newly synthesized Ag(I)-based camphorimine complexes. The assay that yields the most efficient results, that is, which results in lower CFUs/mL values compared to bare plates, was the assay using PCL with a concentration of 4%, chloroform as a solvent and a compound concentration of x10 MIC value.

Conclusions and Future Perspectives

In this work it was possible, on the one hand, to synthesize new silver compounds from silver chloride (AgCl) and silver hydroxide [Ag(OAc)] precursors, capable of acting against *C. albicans* and, on the other hand, to develop functionalized coatings with the compounds with higher antifungal efficacy on medically relevant surfaces. It was possible to establish and optimize experimental conditions that allowed to make coatings with a biodegradable polymer and Ag (I)-derived compounds, with potential to prevent *Candida albicans* surface colonization. Overall, the design of such a complex system required several optimization steps (solvent, polymer concentration, compound concentration, etc.), which will then influence other parameters

than the anti-*Candida* activity or surface properties, as the effective amount of compound retained in the coating, release profile as well as the coating thickness and adherence to the substrate, all important physicochemical properties that should be assessed in the near future. Furthermore, at this moment, there is no knowledge of the spatial orientation of the molecules in the polymeric matrix. As another future perspective, and in attempt to maximize the optimization the design of these coatings, computational modulation can be used to determine which is the most energy-friendly spatial arrangement is attained in the polymeric matrix used. Another key aspect for this line of research is the study of human cells response to these coatings, as the deleterious effects of the functionalized coating should prevails on fungi and not on human cells. This complex, and yet interesting work is just the tip of the iceberg.

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