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Dedicated to the memory of
Prof. Petre T. FRANGOPOL (1933-2020)

THE ANTIFUNGAL ACTIVITY OF ZEIN NANOPARTICLES LOADED WITH TRANSITION METAL IONS

Rawaa SHAKIR SHNAIN AL-SHAMMARI,[a] Ahmed KAREEM HAMMOOD JABERI,[b] Ana LUNGU,[a] Ioana BREBENEL,[a] Waleed KHALED KADDEM AL-SUDANI,[a] Jasim HAFEDH MOHAMMED AL-SAEDI,[a] Cristian-Emilian POP,[a] Maria MERNEA,[a,*] Gheorghe STOIAN[a] and Dan Florin MIHAILESCU[a]

[a]Faculty of Biology, University of Bucharest, Splaiul Independentei, No 91-95, Bucharest, 050095, Roumania
[b]General Directorate for Education Salah Al, Ministry of Education in Iraq, Tikrit, Iraq

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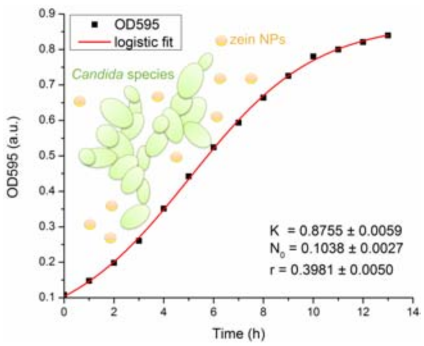
Zein nanoparticles (NPs) are promising drug-delivery agents due to their colloidal stability and biodegradability. Here we investigated the antifungal effect of pristine zein NPs and of zein NPs loaded with transitional metal ions: copper (Cu-zein NPs), zinc (Zn-zein NPs), chromium (Cr-zein NPs), manganese (Mn-zein NPs) and silver (Ag-zein NPs) on three *Candida* species isolated from clinics - *C. albicans*, *C. krusei* and *C. paraspilopsis,* from which *C. krusei* was resistant to fluconazole. Our results showed that zein NPs, Cu-zein and Cr-zein NPs present good inhibitory activities against the three species, while Ag-zein, Mn-zein and Zn-zein NPs present modest or no inhibitory effects. Even more, zein, Cu-zein, Cr-zein and Mn-zein NPs inhibit the growth of the resistant fungal strain. Our study shows that zein NPs are promising antifungal agents, their inhibitory effect being enhanced by loading them with copper or chromium ions.



INTRODUCTION

Candida species are opportunistic pathogenic fungi, responsible for infections in immunocompromised patients. De Oliveira Santos *et al.*[1] summarized that *Candida* species are responsible for vaginitis, candidiasis, candidemia or systemic infections, the last two being highly prevalent in the hospital environment. Several compounds can be used as antifungal agents, including azoles like fluconazole,[2] echinocandins, polyenes, nucleoside analogues, allylamines or thiocarbamates.[3] Since *Candida* species have developed resistance to treatment, especially in the case of azoles, novel therapeutic strategies are required.[1]

Nanoparticle (NP)-based treatments represent a promising approach to overcome microbial resistance.[4] In the case of bacteria and fungi, the formation of biofilms significantly enhances the resistance to therapy.[5] NPs were also proved to inhibit the formation of biofilms, some examples referring to *Candida* species are presented in references.[6-8] Some features of NPs like high surface area, charge, size and shape appear to favor their antimicrobial activity.[4] The mechanisms mediating the antimicrobial activity of NPs are not fully understood, some pathways being the induction of oxidative stress, release of metal ions, cell wall penetration, damaging of DNA and proteins.[4]

[*] Coresponding author: maria.mernea@bio.unibuc.ro

In a previous study we synthesized zein NPs loaded with chromium (Cr), copper (Cu), manganese (Mn), zinc (Zn) and silver (Ag) and proved their antibacterial effects.[9,10] Zein is the major protein in corn kernels. It is not used directly in human diet, but has industrial applications in the production of fibers, adhesives, coatings or biodegradable plastics.[11] Zein can be used to synthesize NPs with increased colloidal stability[12] for drug-delivery applications.[13-15] The zein NPs that we obtained by nanoprecipitation were stabilized by sodium deoxycholate (SD), as described by Gagliardi, *et al.*[13] Different compounds were used as a source of metal ions, as follows: $K_2Cr_2O_7$ as a source of Cr^{3+} for Cr-zein NPs, $MnCl_2$ as a source of Mn^{2+} for Mn-zein NPs, $CuSO_4$ as a source of Cu^{2+} for Cu-zein NPs, $ZnSO_4$ as a source of Zn^{2+} for Zn-zein NPs and $AgNO_3$ as a source of Ag^{+} for Ag-zein NPs.[9,10] Resulting NPs were characterized by energy dispersive X-ray spectroscopy (EDS), revealing that NPs retained a good amount of metal ions.[10] Scanning electron microscopy (SEM) was used to determine the size of NPs that was ~20-50 nm for Cr-zein NPs, ~26-97 nm for Mn-zein NPs, ~22-40 nm for Cu-zein NPs, ~14-85 nm for Zn-zein NPs and ~7.5-10 nm for Ag-zein NPs.[10]

Here we extended our previous study by investigating the effect of pristine zein NPs and zein NPs loaded with transitional metal ions on *Candida* species. The metal ions that we took into account have important antifungal properties. Copper is recognized as an antibacterial, antifungal and antiviral agent; copper-NPs have a potent antifungal activity, being effective even against *Candida* species.[16] Manganese was also selected since ZnO NPs doped with 10% manganese present antibacterial and antifungal activity due to the generation of reactive oxygen species.[17] Even more, it appears that ZnO NPs release Zn^{2+} ions that contribute to their antimicrobial activity.[18] Silver is also an antifungal agent and silver NPs were proved to be effective against *Candida* species.[19] We tested the effect of NPs on four *Candida* species: a reference *C. albicans* strain and three species isolated from clinics, one of which is resistant to fluconazol.

RESULTS AND DISCUSSION

The growth curves of *Candida* species represented by the variation of their optical density at 595 nm in time, in the presence or absence of NPs, were processed as presented in the Materials and Methods section. An example on the logistic fit of growth curves is presented in Figure 1 in the case of the reference *Candida* strain cultured in the absence of NPs (control data). The parameters that we derive are the initial (N_0) and maximum population size (K), as well as their growth rate (r).

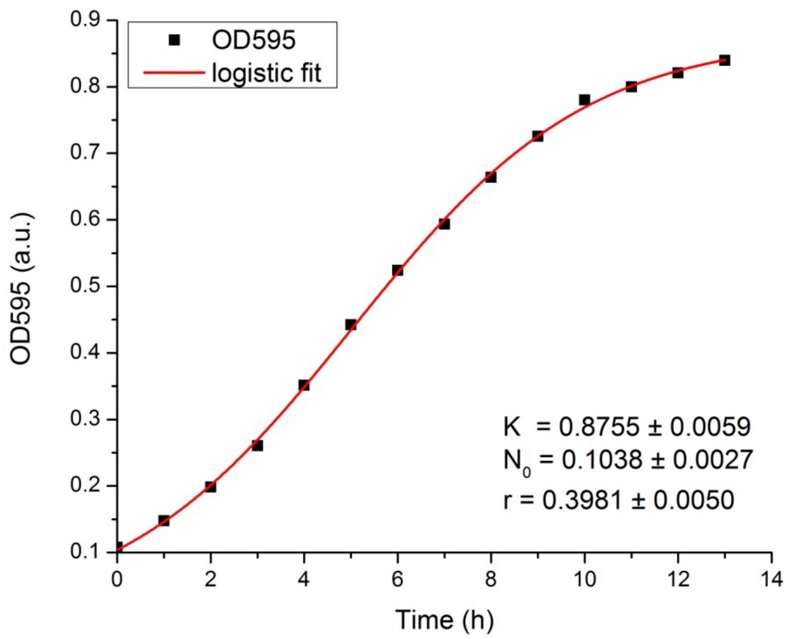


Fig. 1 – Logistic fit (red line) of optical densities measured at 595 nm (OD595) for *C. albicans* ATCC 10231 strain cultured under control conditions (black squares). The resulting parameters (K, N_0 and r) are labeled on the figure.

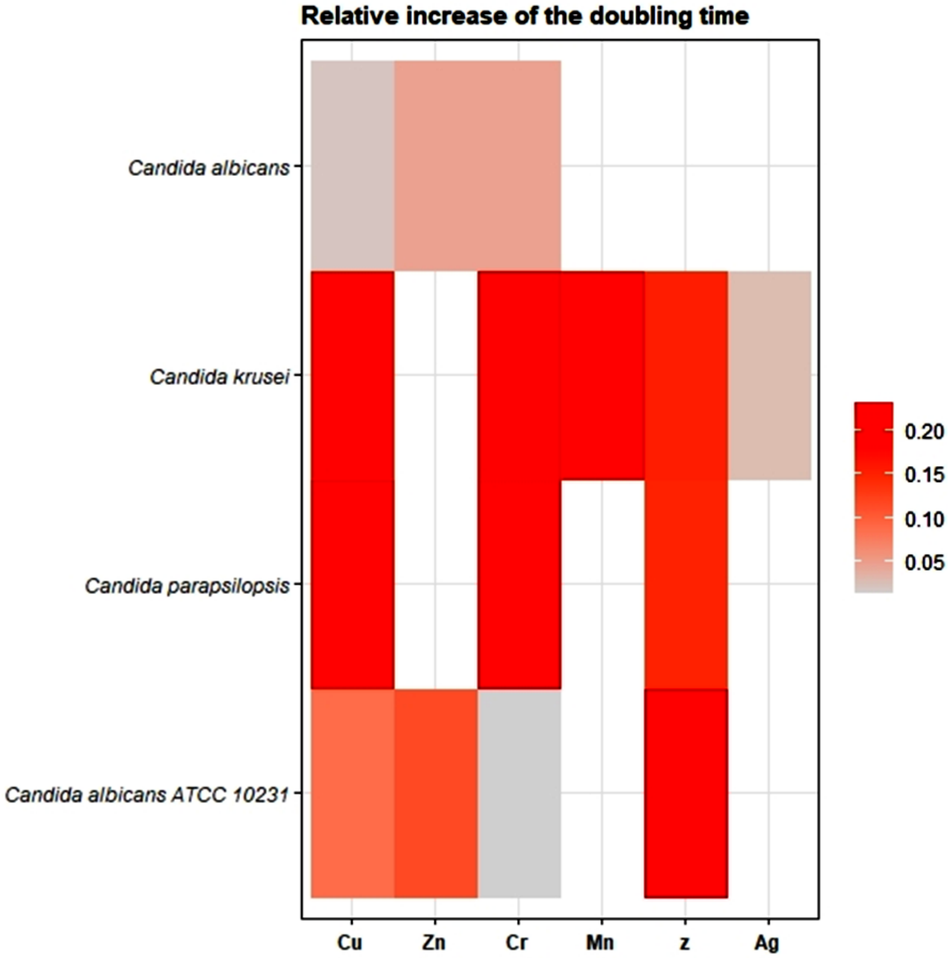


Fig. 2 – Relative increase of the doubling time of *Candida* species in the presence of NPs loaded with copper – Cu, zinc – Zn, chromium – Cr, manganese – Mn, silver – Ag or pristine NPs labeled with z. The plot was generated using reshape2 [21, 22] and ggplot2 [23, 24] packages.

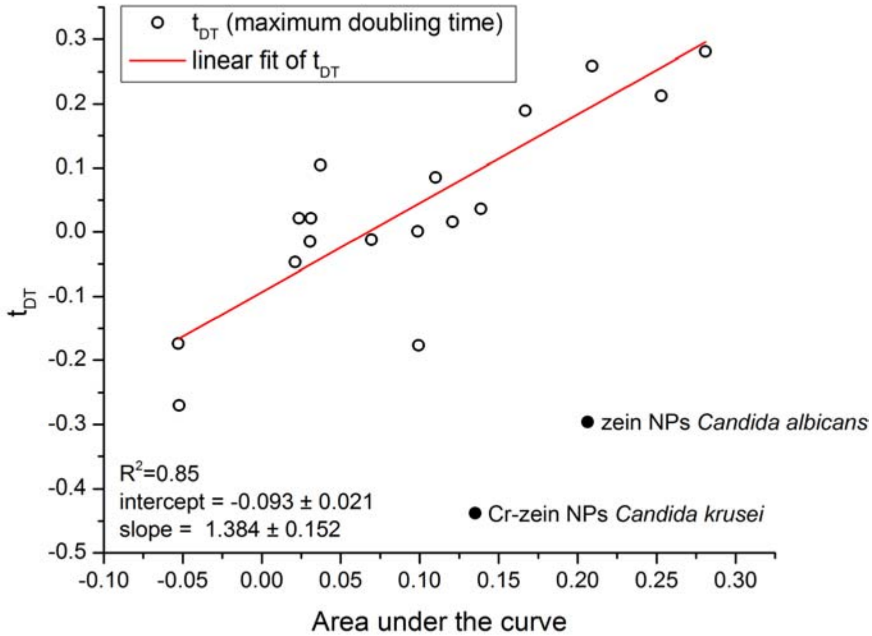


Fig. 3 – The correlation of maximum doubling times (t_{DT} values) with the areas under the logistic growth curves. The values plotted as open circles were fitted with the linear function represented with the red line whose parameters are labeled on the figure. The two values presented as closed circles were excluded from the linear fit.

The fastest doubling time (t_{DT}) was calculated based on r values which can be used to summarize the data in measured growth curves.[20] The relative increase of the doubling time was calculated by subtracting the doubling time of control from the doubling time of samples and reporting the result to control doubling time: (t_{DT}sample – t_{DT}control)/ t_{DT}control. Results are presented in Figure 2. It can be seen that the effect of NPs on *Candida* species varies based on NP type and based on species type. For instance, pristine zein NPs increase the doubling time to a larger extent for all *Candida* species except for *C. albicans*. NPs loaded with copper increase the doubling time for all species, especially for *C. krusei* and *C. parapsilopsis*. The NPs loaded with silver present no effect on inhibiting the doubling time of cultured *Candida* species, presenting only a week effect on *C. krusei*.

In what concerns the sensitivity of species to applied NPs, Figure 2 shows that the doubling time of *C. albicans* is enhanced to a small extent (up to 5%) by Cu-zein, Zn-zein and Cr-zein NPs. *C. krusei* doubling time is slightly enhanced (<5%) by Ag-zein NPs and more significantly (up to 30%) enhanced by Cu-zein, Cr-zein, Mn-zein and pristine zein NPs, while it is insensitive to Zn-zein NPs. *C. parapsilopsis* doubling time is enhanced in a large extent (<30%) by Cu-zein, Cr-zein and pristine zein NPs, while it is insensitive to the other. *C. albicans* ATCC10231 doubling time is slightly enhanced by Cr-zein NPs (<5%), is more enhanced by Cu-zein and Zn-zein NPs (<15%), is enhanced in the largest extent by pristine zein NPs (<30%) and is not altered by Mn-zein or Ag-zein NPs.

For a more detailed description of *Candida* growth inhibition by the NPs synthesized here we analyzed the correlation of calculated maximum doubling times with the area under the logistic growth curves. The area under the logistic curve is an important parameter in describing the growth as it integrates the contribution of other parameters like growth rate, initial population size and carrying capacity.[20] It is also assimilated to the growth potential.[25] Results are presented in Figure 3. We observe a linear variation of the areas under the curves and the maximum doubling times (the linear fit was performed with a R^2=0.85). This suggests that all growth parameters are altered similarly by the treatment with NPs and confirm the inhibitory effects of NPs discussed above.

Two values do not fit in the identified tendency (represented with closed circles in Figure 3), these being associated with the effect of pristine zein NPs on *C. albicans* and of Cr-zein NPs on *C. krusei*. In these cases, we observe that the correlation fails because the areas under the curve have large values, the maximum population sizes being greater than in the case of control. This suggests that Cr-zein NPs acting on *C. krusei* and pristine zein NPs acting on *C. parapsilopsis* present only a short-term inhibitory effect (a few hours) due to their ability to increase the doubling times, but at the end of the incubation time, these cultures are denser than the control.

Overall, pristine zein NPs, Cu-zein and Cr-zein NPs present the most notable effects on analyzed species, while Ag-zein NPs appear the most ineffective. It is interesting to mention that the *C. krusei* strain that we analyzed was insensitive to fluconazole, an antifungic compound used in clinics. Except for Ag-zein and Zn-zein NPs, the NPs that we synthesized appear effective against this strain, Cr-zein NPs being effective only on short term. When considering only the species isolated from clinics, Cu-zein and Cr-zein NPs perform better that pristine zein NPs because they not only inhibit *C. krusei* and *C. parapsilopsis* in a larger extent, but also inhibit *C. albicans* to a smaller extent.

EXPERIMENTAL

Candida species used in the study

Four *Candida* species were used here, a *C. albicans* reference strain (ATCC 10231) and three species isolated from the clinics and provided by a Microbiology Laboratory: *C. albicans*, *C. parapsilopsis* and *C. krusei*. These are sensitive to amphotericin, nystatinum, flucytosine, econazole, ketoconazole, miconazole, fluconazole. Only *C. krusei* was resistant to fluconazole. The microbial cultures were maintained in the refrigerator (4°C) on Sabouraud Dextrose Agar (Sigma Aldrich) slants. Prior to any experiment, each microbial strain was subcultured onto SAB plates and incubated at 37°C for 24 hours.

Growth inhibition assay

The growth of *Candida* species (*C. albicans* ATCC 10231, *C. albicans*, *C. parapsilopsis* and *C. krusei*) in Sabouraud Dextrose Broth medium for 12 hours was determined in the presence of 50 µg/mL NPs (zein-NPs, Cr-zein NPs, Mn-zein NPs, Cu-zein NPs, Zn-zein NPs and Ag-zein NPs). Fungal growth was determined spectrophotometrically by measuring the optical density at 595 nm. The inoculums used for the assay were standardized *Candida* suspensions with an optical density of 0.05 arbitrary units (a.u.). The growth in the presence of NPs was compared to the positive controls represented by untreated *Candida* cultures.

Data analysis

The growth curves of *Candida* cultures in the presence of NPs and control were fitted with a logistic curve:[20]

$$N_t = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e^{-rt}}$$

In the above equation, N_0 is the population size at the beginning of the growth curve, K is the maximum possible population size in a particular environment and r is the intrinsic growth rate that would occur without any restrictions. The equation gives N_t representing the maximum number of cells at time t.[20] The equation is implemented in Growthfinder, a package implemented in R.[26] Additional parameters that we used in analyzing the curves are *auc_l*, the area under the fitted logistic curve from time 0 to t, and t_{DT}, the maximum doubling time, the fastest generation time during the logarithmic growth phase ($t_{DT} = ln2/r$).[20]

CONCLUSIONS

In the present paper we tested the antifungal effect of pristine zein NPs and of zein NPs loaded with transitional metal ions (copper, zinc, chromium, manganese and silver). These NPs were synthesized in our laboratory and their antibacterial properties were determined in a previous study. Here we investigated their effect on four *Candida* species, namely a *C. albicans* reference strain and *C. albicans*, *C. krusei* and *C. parapsilopsis* strains isolated from clinics, from which C. *krusei* was resistant to fluconazole. The growth of fungi in the absence and in the presence of NPs was described by two important parameters, namely the increase of the doubling time relative to control and the area under the growth curve. These parameters were obtained by fitting the growth curves with a logistic growth function. Our results show that the different NPs present different antifungal effects in the cases of analyzed *Candida* species. Taken all together, the most notable inhibitory effects were determined for zein NPs, Cu-zein and Cr-zein NPs, while Ag-zein, Mn-zein and Zn-zein NPs present modest or no inhibitory effects. In the case of effective NPs, we identified two situations when NPs only enhance the doubling times of fungi but do not decrease the areas under the logistic growth functions, these situations being zein NPs acting on *C. albicans* and Cr-zein NPs acting on *C. krusei*. Zein NPs, as well as Cu-zein, Cr-zein and Mn-zein are able to inhibit the growth of the fungal species resistant to fluconazole. Our results show that zein NPs are good candidates as antifungal agents and their spectrum can be broadened by loading them with transitional metal ions like copper or chromium. Also, future studies can take into account loading the zein NPs with antifungal agents represented by natural or synthetic compounds.

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