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Dedicated to Dr. Maria Zaharescu
on the occasion of her 80th anniversary

BIOSYNTHESIZED DEXTRAN COATED MAGNETIC NANOPARTICLES WITH ANTIFUNGAL ACTIVITY

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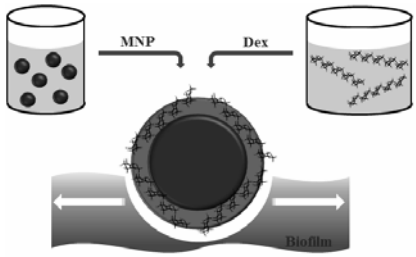
Biofilm formation is a challenging problem of the modern world, the most serious implications being associated with severe microbial infections in humans. The solution can be the use of nanostructures which might interact directly with the microorganism's cell envelope. We engineered dextran coated iron oxide nanoparticles, loaded with propiconazole, in order to test the hypothesis of a combined effect of the polymer and drug, both known for their antifungal activities. Magnetic nanoparticles were coated with biosynthesized dextran (obtained from an exopolysaccharides producing strain) of 1% and 2% concentrations, followed by the embedding of propiconazole onto the dextran shell. FT-IR, EDX, DLS and TEM measurements showed a successful polymer coating, while the 2% formulation nanoparticles revealed an activity on *Candida albicans* strain, breaking 77% of biofilm. The improved version of the system (loaded with propiconazole) showed a maximum antifungal activity on *C. albicans*, in both planktonic and biofilm phase.



INTRODUCTION

In the last decade, biomedical applications became important, nowadays studies focusing on finding nano-materials that can act as new and improved antimicrobial agents, since, generally, nano-systems can interact with microbial cells directly by disrupting/penetrating the cell envelope.[1] In this context, magnetic nanoparticles (MNP) revealed unique properties, which led to their application in different areas, such as environmental[2] and medical applications.[3] It is important to note that by coating MNP with biocompatible surfactants their stability can be enhanced, ensuring the ferrofluid stability. It is also important that the coating agent should have specific functional groups desired for a specific application.[4] Moreover the shell of MNP can be loaded with various antibacterial and antifungal drugs and relevant studies have shown the ability of such nano-structures to be used as drug carriers.

On the other hand, dextran is a good stabilizer for nano-scale magnetite due to its excellent biocompatibility and water-solubility, and has been utilized in laboratory products and some commercial MNP.[5] Cytotoxicity evaluation of the MNP coated with dextran on four types of cells showed that they affected different cells in different manners and, in consequence, it is possible to have different applications (ex. can be

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used as contrast agent or drug carrier for solidary organs).[6,7] Moreover, the dextran coated MNP proved delayed hypersensitivity and no alteration in antioxidant enzyme levels of cellular system and its rapid uptake and internalization through endocytosis by human fibroblasts compared to uncoated nanoparticles.[8] It is well known that biofilm is a community of microorganism adhering to biotic or abiotic surfaces embedded by a self-produced extra-polymeric matrix, facilitating the survival in an adverse environment. Many inconveniences are related to the biofilm formation, comprising the implications in food industry, the role played in many life-threatening infectious diseases, the phenomenon of metal corrosion, and the increased risk of product contamination, the decreased quality of water at water and sewage treatment facilities.[9] Probably, the worst part of biofilms belongs to those affecting the medical and healthcare industries because biofilm-associated organisms are responsible for more than 60% of all microbial infections in humans.[10] Nowadays, many efforts have been made to destroy the yeast biofilm formation by means of synthetic or natural compounds or by nanotechnology.[10]

In this study, we report the preparation of MNP covered with biosynthesized dextran with antifungal properties. By loading the MNP-dextran system with propiconazole, a synergistic effect was obtained and the resulted system being investigated as a drug delivery system. All nanoparticles were characterized by different techniques (FT-IR, EDX, DLS and TEM) and by *in vitro* assays.

RESULTS AND DISCUSSION

Core-shell magnetic nanoparticles (MDex) based on magnetite (as a core) and biosynthesized dextran (as a shell) were obtained by dispersing the MNP (previously synthetized by co-precipitation method[3]) in a dextran solution of a suitable concentration (1% or 2% w/w) and mixture was stirred for two hours at 90°C for two hours. MDex samples will be abbreviated MDex1% and MDex2%, depending on the concentrations of the dextran aqueous solutions used in the synthesis. Considering the antifungal properties of dextran, MDex nanoparticles were tested for antifungal activity against *C. albicans*. Further, MDex nanoparticles were loaded with propiconazole, as an antifungal drug, by simple mixing of MDex with an aqueous solution with suitable concentration of propiconazole for 24 h, obtaining a synergic effect between dextran and propiconazole and, in the same time, the resulted system acting as a drug delivery agent.

Exopolysaccharides (EPS) were obtained from yogurt isolated lactic acid bacteria, identified as *Weissella confusa* by 16sDNA gene sequencing[11]. The dextran in high amount was obtained in MRS (De Man, Rogosa and Sharpe) culture medium improved with sucrose (80 g/L) and dissolved in UHT milk. The obtained EPS were quantified by gravimetric analysis. In this order, after 48h of fermentation, 25.2 g of freeze-dried biopolymer were obtained. Due to the dextran antifungal properties[11], MDex were loaded with an antifungal drug in order to improve their activity against *C. albicans*. The pathogenicity of this microorganism relies on a series of inherent and environmental factors mostly related to the host. During the last two decades, there has been a significant increase in the proportion of severe fungal infections caused by the excessive use of broad-spectrum antibiotics, catheters and the increasing number of immuno-suppressed patients.

A. Characterization of MDex nanoparticles

a) Fourier transform infrared spectroscopy (FTIR)

The biosynthesized dextran structure was confirmed in comparison with standard dextran with a molecular mass of 40.000 Da by FTIR analysis (Fig. 1). FTIR technique was able to highlight the presence of dextran on the surface of the MNP by revealing the characteristic vibrational bands of the functional groups and the internal chemical bond linkages. The band at 3352 cm^{-1} can be assigned to the hydroxyl stretching vibrations of the dextran, while the band region of 2925 cm^{-1} corresponds to the C-H stretching vibration (Fig.1a).[12]

The most relevant signals appear at 1149 cm^{-1} and the region between the 1002-1030 cm^{-1} (shoulder) assigned to the C-O and C-C bond vibration and deformation vibrations of the C-CH and HC-O bonds (Fig. 1a). The band at 1149 cm^{-1} can be assigned also to valent vibrations of C–O–C bond and glycosidic bridge.[13] The presence of the signals at 844 cm^{-1} and 836 cm^{-1} confirm the α-(1-6) respectively α-(1-3) bonds from dextran structure.

FTIR spectra of the MNP coated dextran (MDex) retains most of the dextran features with all values of the bands vibrations shifted to the lower values while

few of them are shifted to higher values (Fig. 1b). Thus, the presence of the vibration bands of C-H bonds can be observed at 2921 cm^{-1}, the presence of the carboxylic groups at 1749 and 1641 cm^{-1} and the high intensity peak of the C–O–C bond and glycosidic bridge at 1147 cm^{-1} shifted to lower values with 2 cm^{-1} (Fig. 1a, b). The almost perfect fit between the two spectra (biosynthesized dextran and MDex), combined with the discreet differences between them, led to the conclusion that MNPs were coated with dextran and were stabilized by physical attractions.

b) Energy dispersive x-ray analysis (EDX)

EDX spectra of MNP coated dextran were presented in Fig. 2. Both types of EPS coated MNP, MDex1% and MDex2%, have important carbon content due to the dextran coated nanoparticles. It can be noticed that for the two samples there are differences in the carbon percentage, derived from dextran, and oxygen content, due to the Fe_3O_4 and dextran contributions.

MDex1% has a lower carbon and oxygen contents than MDex2%. Since MDex1% were prepared by dispersion of the MNP in 1% dextran solution, while MDex2% were prepared by dispersion of the same MNP in 2% dextran solution, it seems that, despite the fact that the two types of the nanoparticles have the same structure, the content of the dextran into the shell of the MNP depends on the concentration of the dextran solution in which the nanoparticles were synthetized.

Table 1

Element percentage of the MNP coated dextran elements

Sample	C, At %	O, At %	Fe, At %
MDex1%	37.86	51.42	07.75
MDex2%	45.91	43.21	10.88

c) Dynamic light scattering (DLS)

DLS has shown very large particle diameters, about 679 nm for MDex1% (Fig. 3a) and 761 nm for MDex2% in water with narrow size distributions (Fig. 3b). Zeta potential have small negative values, -12.5 mV for MDex1% and -5.8 mV for MDex2%, suggesting that the stability of the dispersion is rather low and large aggregates are created in aqueous solution, which explains the large sizes reported before.[3,14]

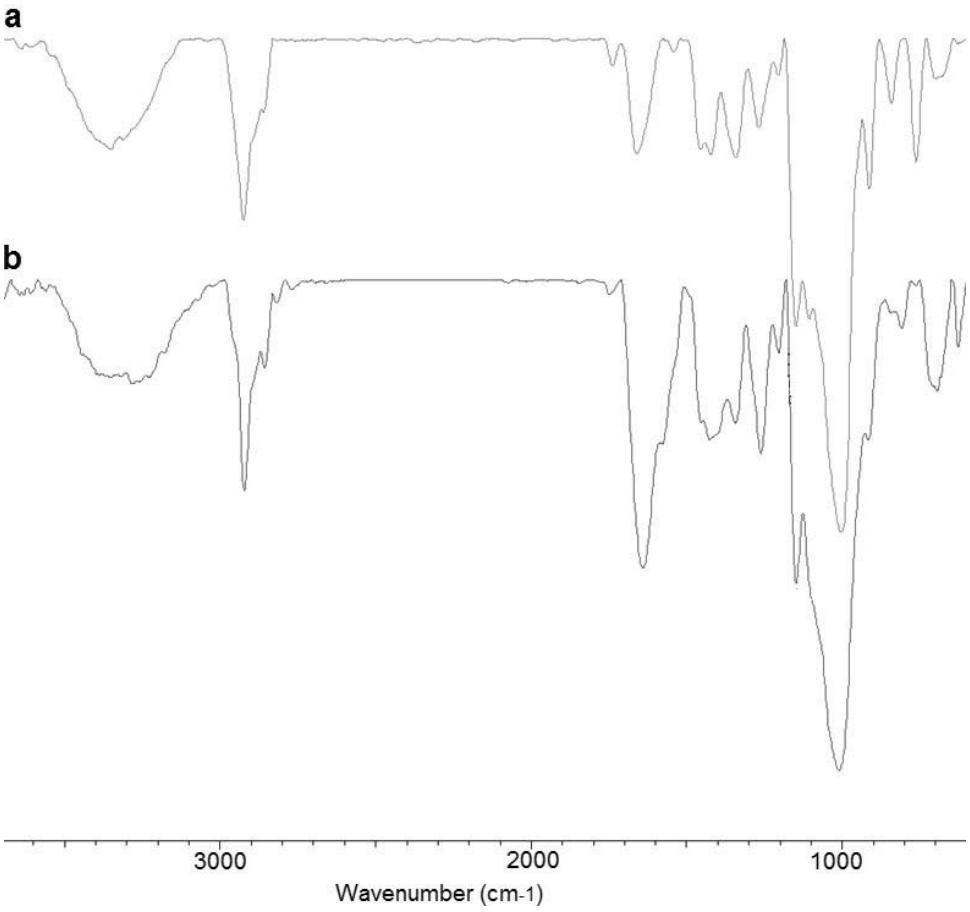

Fig. 1 – FTIR spectra of exopolysaccharides (a) and MNP coated with exopolysaccharides (b).

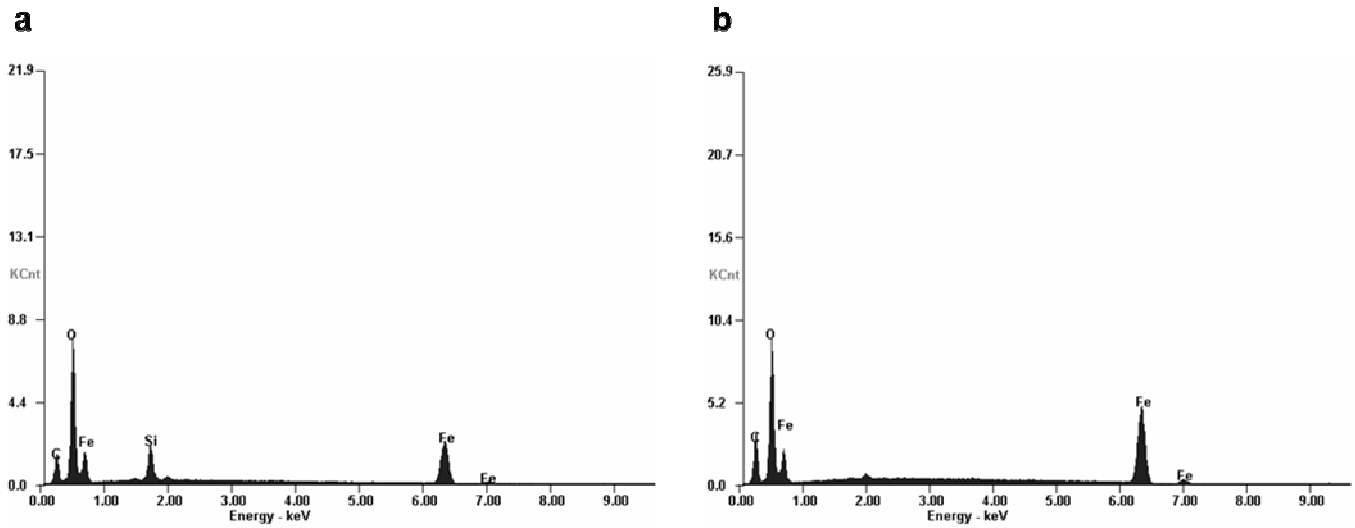

Fig. 2 – EDX spectra and percentage of C, O, Fe of MDex 1% (a) and MDex 2% (b).

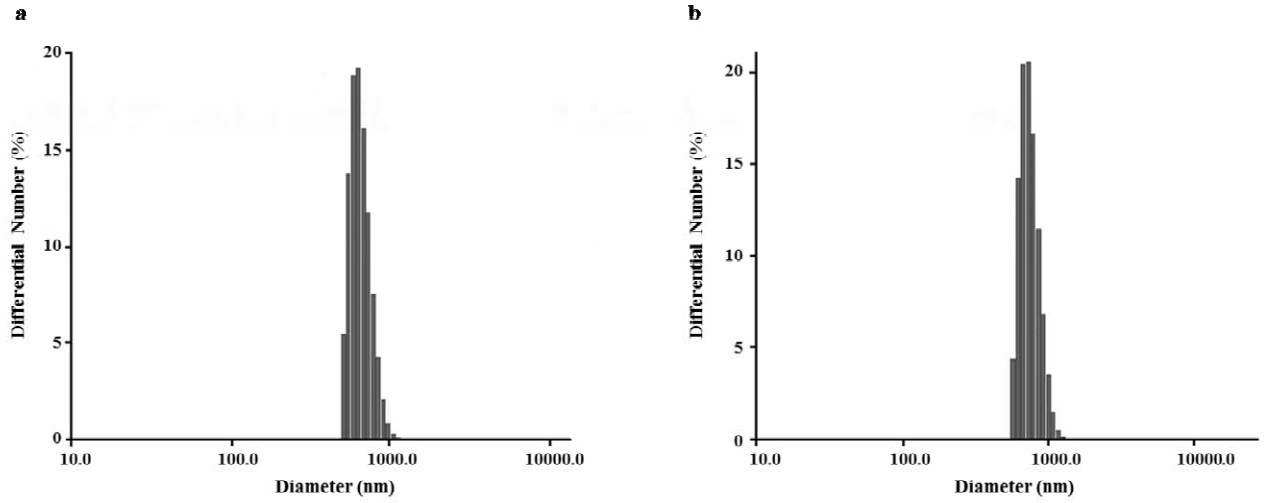

Fig. 3 – DLS data for MDex1% (a) and MDex2% (b).

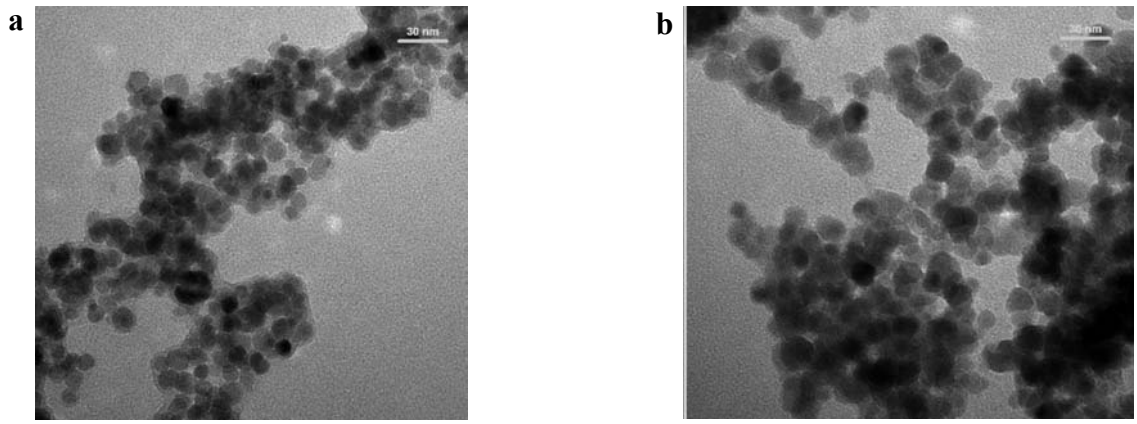

Fig. 4 – TEM image of MDex1% (a) and MDex2% (b).

d) Transmission electron microscopy (TEM)

The particle sizes and shapes were better evaluated with TEM, being possible to visualize individual nanoparticles even in binary oxide material.[15] The electron micrographs of MDex have shown spherical nanoparticles with 15-20 nm in diameter, in large aggregates, confirming the results from DLS measurements (Figure 4a,b).

B. In vitro assessment of antifungal properties

a) In vitro assessment of antifungal properties of MDex1% and MDex2%

First, two possible antifungal agents containing different concentration of dextran and denoted MDex1% and MDex2% were tested. The tested concentration of the active principle was between 0.004–10 mg/L and *C. albicans* ATCC 10231 was used for quality control.

Antifungal susceptibility testing using *C. albicans* strain showed that MDex2% can destroy by up to 77% of the biofilms formed by the pathogenic to minimum inhibitory concentration (MIC) of 10 mg/mL (Fig. 5), results that have not been reported in the literature. Also it seems that a concentration below this one has no activity against the pathogen. On the other side, the compound denoted MDex1% did not showed any relevant activity on *C. albicans* strain (Fig. 5), so in the second phase of the experiments it was used only MDex2% in combination with an antifungal named propiconazole (PP) in order to improve its antifungal properties.

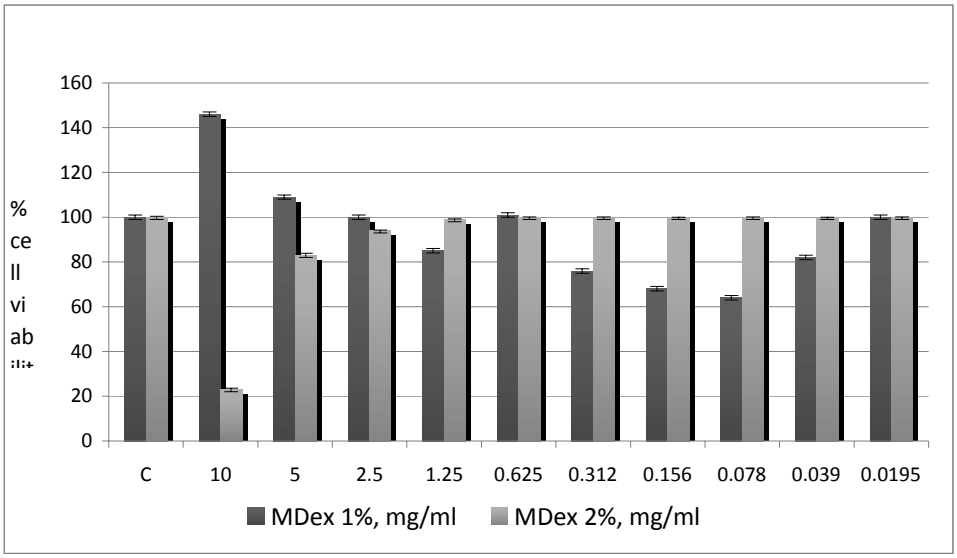

Fig. 5 – MDex1% and MDex2% antifungal susceptibility assay.

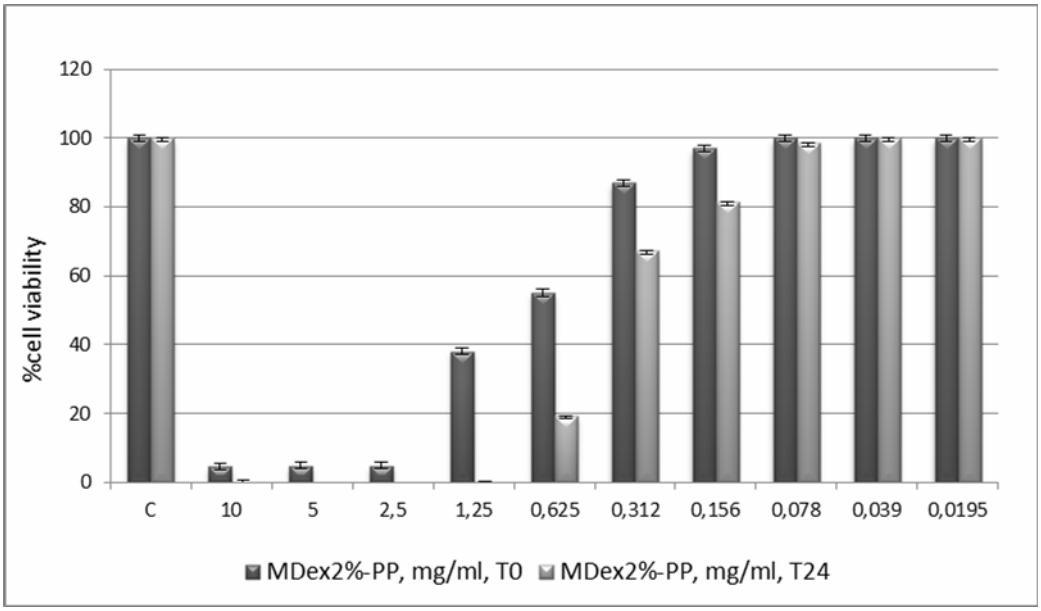

Fig. 6 – MDex2%-PP antifungal susceptibility assay against *C.albicans* in planktonic (T0) and biofilm (T24) stages.

b) In vitro assessment of MDex2%-propiconazole (MDex2%-PP) antifungal properties on C. albicans (in planktonic and biofilm stage)

The MDex nanoparticles were tested *in vitro* by embedding the propiconazole (PP), with antifungal properties, into the dextran shell of the MNP.

Antifungal susceptibility testing using *C. albicans* strain showed that MDex2%–PP can destroy by up to 100% of the biofilms formed at a MIC of 1.25 mg/mL, and it seems that at concentrations below 0.156 mg/mL had no antifungal activity against *C. albicans* (Fig. 6). On the other side, the same compound proved to have relevant activity on *C. albicans* strain in planktonic phase also, but lower than on the biofilm, MIC being 2.5 mg/mL (Fig. 6). No similar data were reported by literature.

EXPERIMENTAL

Exopolysaccharides biosynthesis and characterization. The lactic acid bacteria used for exopolysaccharide biosynthesis was isolated from Romanian commercial yoghurt and identified by 16S rDNA sequence as *Weissella confusa*. The culture medium used had the following composition: MRS (55.3g/L) and sucrose (80g/L) dissolved in commercial UHT milk. The culture medium was sterilized at 110°C for 30 minutes and inoculated with 30% of fresh inoculum (24 hours) with A_{600nm} of 0.5,[16] and incubated at 33°C for 48h. Before EPS extraction and purification, the enzymatic equipment capable of degrading the biopolymer was inactivated by heat at 100°C for 15 min.[17] The cells and proteins were removed by trichloroacetic acid (TCA) 20% precipitation and centrifugation at 10,000 rpm, 10 min at 4°C. EPS was separated by precipitation with three chilled ethanol volumes for 24 hours at 4°C, collected by centrifugation at 12,000 rpm for 15 min at 4°C, and dissolved in double distilled water (DDW) for dry freezing. The EPS amount was quantified by gravimetric analysis, and the dextran structure was confirmed by FTIR analysis.

FTIR analysis of the samples. The FTIR spectra of all samples were recorded in transmission on a Bruker Vertex 70 spectrometer (Bruker Optics, Germany) at a resolution of 2 cm^{-1}, on KBr pellets and for the data processing OPUS 6.5 software (Bruker Optics, Germany) was used. FTIR spectra were processed with a specialized program from the SpectraManager series.

Preparation of MDex nanoparticles and propiconazole loaded nanoparticles (MDex-PP). The magnetic nanoparticles were synthetized by co-precipitation method.[3] Two solutions of $FeCl_2$ and $FeCl_3$ were mixed in a molar ratio Fe^{2+}/Fe^{3+}=0.5. An amount of NH_4OH 27% solution was added under mechanical stirring and inert atmosphere at 70°C in order to determine the precipitation of mixed iron oxides in the form of nanoparticles at pH>9. The black precipitate was washed with water until the pH was about 7.

An amount of nanoparticles obtained in the previous experiment was dispersed in a small amount of water. The dispersion was added under mechanical stirring at 90°C in a proper concentrated aqueous solution of EPS (1% and 2% dextran) in inert atmosphere for 2 hours. The grey precipitate was washed then multiple times with distilled water. The samples were noted as MDex1% and MDex2%.

Further, a suspension of dextran coated magnetite nanoparticles (50 mg/ml, 1ml) was mixed with a solution of propiconazole (50mg/ml, 1ml) and stirred for 20 min. The reaction continued for 24 h at 500 rpm and 20°C, using a shaker. Finally, functionalized magnetite was purified by decantation and washed several times with distilled water.

Energy dispersive x-ray analysis (EDX). An EDX system available on a Quanta 200 Environmental Scanning Electron Microscope (ESEM) was used for qualitative analysis. The EDX studies were performed on samples fixed on aluminium supports at 10 mm WD (working distance), which is the stage eucentric position and the collection point of the EDX detector and 20 KV. The EDX detector used is the Si detector - EDX silicon-drift detector enables rapid determination of elemental compositions.

The dynamic light scattering (DLS). The hydrodynamic diameter and Zeta potential of the nanoparticles were evaluated using Delsa Nano C Submicron Particle Size Analyzer (Beckman Coulter, Inc., Fullerton, CA). This device is equipped with a laser diode operating at 658 nm. Measurements were made at room temperature in flow cell for both size and Zeta potential, after the solution was dispersed by ultrasonication.

Transmission electron microscopy (TEM). TEM investigations were carried out with a Hitachi High-Tech HT7700 Transmission Electron Microscope operated at a 100 kV accelerating voltage in High-Contrast Mode. The samples were prepared on carbon-coated copper grids with 300-mesh size. Microdroplets of the nanoparticles dispersed in water were placed on the grids, and then the solvent was removed under vacuum.

In vitro assessment of MDex1%, MDex2% and MDex2%-PP antifungal properties on C. albicans.

In vitro susceptibility testing was performed following the EUCAST EDef 7.1 guidelines, using as a pathogen *C. albicans* ATCC 10231 in planktonic phase (the antifungal agent was added at the T0 inoculation time) and in biofilm stage (the antifungal agent was added after 24h of incubation-T24, when biofilm was formed). Three compounds were tested: MDex1%, MDex2% and MDex2%-PP. The stock solutions were prepared in RPMI medium as a solvent and the active principle concentration ranges were 0.0195–10 mg/L. The MIC was determined spectrophotometrically at 405 nm, after 24h or 48h of incubation at 37°C using EnVisions plate reader (PerkinElmer).

CONCLUSIONS

Iron magnetic oxide nanoparticles coated with EPS (obtained by bacterial strain biosynthesis) with improved antifungal properties were prepared. The TEM micrographs of MDex nanoparticles presented a spherical shape with an average diameter of around 20 nm. The MDex nanoparticles tend to cluster into aggregates, as also evidenced by DLS and zeta potential values. Furthermore, the loading of an antifungal drug such as propiconazole in the dextran shell of MNP nanoparticles, a synergistic effect has been proven

by destroying up to 100% of the *C. albicans* biofilm at a MIC of 1.25 mg/mL. In conclusion, MDex2%-PP system is a promising drug against *C. albicans* pathogen.

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