Design and Synthesis of Nano-Sized Polymer Stabilized Hybrid Materials Based on Iron Oxide and Silver Nanoparticles and their Antifungal activity

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Abstract
The high level of resistance of yeast species of the genus Candida to antifungal agents used in the practice, defines the scientific interest to investigate the antifungal properties of newly synthesized hybrid materials. The research is directed to find a viable alternative for the treatment of infections caused by the highly resistant Candida species. The tested in the experiments nano-sized bimetallic polymer stabilized hybrid materials based on iron oxide and silver nanoparticles (γFe2O3/AgNps/PVA), were characterized by different techniques and for the first time tested onto six clinical Candida strains with a predetermined resistance to different antifungals. The results showed the presence of very strong antifungal activity.

Keywords
Candida; Antimycotics; Resistance; Magnetic nanoparticles; Silver nanoparticles

Introduction
Species of the genus Candida are the fourth most causative agent of nosocomial bloodstream infections in USA as well as one of the most isolated microorganisms from hospital-acquired infections in Europe [1,2]. Nowadays a tendency for increasing of cases caused by non-albicans Candida species resistant toward one or more antifungal agents was observed. Among them C. glabrata demonstrated a high degree of resistance to fluconazole and voriconazole and enhance resistance to echinocandins [3-7]. C. glabrata is a fungus that unlike other fungi of the genus Candida is polymorphic (i.e., does not form hyphae or pseudo hyphae) and exists only in the form of blastokonidium. In the beginning, this fungus has been classified to Torulopsis species. The tested in the experiments nano-sized bimetallic polymer stabilized hybrid materials based on iron oxide and silver nanoparticles (γFe2O3/AgNps/PVA), were characterized by different techniques and for the first time tested onto six clinical Candida strains with a predetermined resistance to different antifungals. The results showed the presence of very strong antifungal activity.

Materials and Methods
Materials
Polyvinyl alcohol (PVA) (Sigma – Aldrich; 87-88% hydrolyzed, Mw =13000 – 23000 mol \(^{-1}\)); silver nitrate (AcrosOrganics); FeCl\(_3\).6H\(_2\)O (Sigma-Aldrich); Fe(NO\(_3\)).9H\(_2\)O (Sigma-Aldrich) were used as received without further purification.

Clinical yeast strains resistant toward one or more antimycotics were involved in the experiment as follow: C. krusei 112, C. krusei 8-48, C. krusei 8-126, C. glabrata 0-73, C. glabrata 8-122, C. nivariensis.

Methods
- Transmission electron microscopy (TEM) images were recorded on a HR STEM (JEOL JEM 2100). Samples were prepared by placing a drop of the precursor solutions on carbon-coated grids and dried under air at room temperature.
- Dynamic light scattering measurements (DLS) were performed on a NanoBrook 90 plus PALS instrument
reddish-brown. After sedimentation and washing with 2M HNO₃, the supernatant was removed and Fe was established by ICP as 88 mg/L and 5401 mg/L respectively. For control, Fe₂O₃ hydrosol mixed with PVA solution was prepared according to by dissolving 3.25 g FeCl₃.6H₂O and 1.197g FeCl₄. 4H₂O in 70 ml deoxygenated water [22-24]. Then 7 ml 25% NH₄OH was added drop-wise into solution under vigorous stirring with formation of black precipitate. After sedimenting the precipitate with a permanent magnet, the supernatant was collected; the clear, reddish yellow sediment was dispersed by adding demineralized water and stirred. The supernatant was taken; the reddish brown supernatant is a hydrosol of Fe₂O₃ nanoparticles, which was stabilized with AgNps/PVA products were determined by TEM analysis. TEM images of the films were recorded in transmittance mode (Brookhaven Instruments Corporation), equipped with a 35 mW red diode laser (λ ¼ 660 nm) at a scattering angle of 90°.

UV- Vis absorption spectra were recorded at room temperature in the wavelength range from 200 to 800 nm using Perkin Elmer spectrophotometer.

IR spectra of the films were recorded in transmittance mode in the range from 400 to 4000 cm⁻¹ using Perkin Elmer FTIR.

XRD analysis is performed on PANalytical- X-ray diffractometers.

Synthesis of silver nanoparticles via thermal reduction using Polyvinyl alcohol (PVA) as a stabilizer

The synthesis of silver nanoparticles stabilized by PVA was performed as reported by Bryaskova et al. [19]. Briefly, five grams of PVA was dissolved in 95 mL deionized water under stirring at 80°C. Silver nitrate (30 mg) dissolved in 5 mL (water) was added drop-wise under stirring to 95 ml of PVA solution (5%) thus achieving final concentration of silver nitrate in the solution equal to 300 mg/L. The prepared solution was heated for 1 h at 100°C in dark thus leading to the formation of silver nanoparticles (AgNps) stabilized via PVA. The color of the solution was light yellow.

Preparation of γFe₂O₃/AgNps/PVA

First magnetite (Fe₃O₄) nanoparticles in aqueous solution were prepared according to by dissolving 3.25 g FeCl₃.6H₂O and 1.197g FeCl₄. 4H₂O in 70 ml deoxygenated water [22-24]. Then 7 ml 25% NH₄OH was added drop-wise into solution under vigorous stirring with formation of black precipitate. After sedimenting the precipitate with a permanent magnet, the supernatant was removed by decantation and the precipitate was washed with water several times until the neutral pH of solution. Then 20 ml 2M HNO₃ was added to the black sediment and the mixture was stirred for 10 min. The oxidation of magnetite to maghemite was then performed by addition of 30 ml 0.35M Fe(NO₃)₃. The mixture was stirred under reflux for 1h at 100°C. The color of the solution changed from blue-black to clear reddish-brown. After sedimentation and washing with 2M HNO₃, the reddish yellow sediment was dispersed by adding demineralized water and stirred. The supernatant was collected; the clear, reddish brown supernatant is a hydrosol of Fe₃O₄ nanoparticles, which was mixed with solution of AgNps stabilized with PVA. This sample will be referred as P1 (product1) in the text. The concentration of the Ag and Fe was established by ICP as 88 mg/L and 5401 mg/L respectively. Then, the solid was dispersed with certain amount of AgNps solution stabilized with PVA and mixed for 1h. The obtained suspension will be referred as P2 (product 2) in the text. The concentration of the Ag and Fe was established by ICP as 157.5 mg/L and 1989 mg/L respectively. For control, Fe₃O₄ hydrosol mixed with PVA solution was used and this product will be referred as P3 (product 3) in the text. The concentration of the Fe was established by ICP as 6097 mg/L.

Testing the fungicidal properties of the γFe₂O₃/AgNps/PVA hybrid materials

For evaluation of the fungicidal activity of the materials the Minimal Fungicidal Concentration (MFC) was determined. This was achieved by the method of macro dilution in a final volume of 1 ml. Materials P1 and P2 are tested initially from outgoing dilution 1:100 by two-fold serial dilutions up to 1:1600, but the MFC was not achieved. Therefore, the subsequent tests were started from a dilution of 1:1000 and two-fold dilutions are conducted up to 1:32000. The product P3 was tested without initial dilution as the subsequent two-fold dilutions were performed to 1:1024. As a diluent was used injection water. To each dilution of the material, standardized inoculum of the test strain containing 10⁶-10⁸ CFU/ml of the respective species of yeast was added. After incubation at 32.5 ± 2.5°C for 18-24 h, seeding was performed from each dilution by the surface agar method on plates with Sabouraud agar. The results are reported in advance at 24 hours, and final on the 5th day after incubation at 32.5 ± 2.5°C.

Results and Discussion

To prepare yFeO₃/AgNps/PVA bimetallic hybrid materials for targeting delivery and in vivo applications, surface modification of naked iron oxide nanoparticles with AgNps/PVA hybrid materials possessing antimicrobial properties was performed. For this purpose, initially PVA stabilized silver nanoparticles was synthesized by boiling the PVA solution at 100°C for 60 min in the presence of AgNO₃, as a precursor of silver ions [19]. The UV-vis spectroscopy analysis confirms the formation of well-defined silver nanoparticles with appearance of peak at 420 nm. The TEM analysis also demonstrated the formation of spherical and homogenous distributed silver nanoparticles with particles diameter from 5.0 to 6.0 nm (Figure 1 a-b).

Then, Fe₃O₄ nanoparticles were synthesized applying wet-chemical coprecipitation of ferrous and ferric ions in an aqueous solution upon addition of ammonium hydroxide. The stability of thus prepared iron nanoparticles is however critical and oxidation to more stable maghemite yFeO₃ was performed. The main problem of those naked magnetic nanoparticles is their ability to aggregate. To overcome this problem, yFeO₃ nanoparticles were coated with biocompatible and non-toxic PVA with included silver nanoparticles. Uncharged polymers such as PVA or PEO are known to adsorb nonspecifically on oxide surfaces. The interaction with the surface results from hydrogen bonding between polar functional groups of the polymer and hydroxylated and protonated surface sides of the oxide [25].

Two different yFeO₃/AgNps/PVA bimetallic hybrid materials were prepared: 1) hydrosol of yFeO₃ stabilized with AgNps/PVA, referred as P1 and 2) sediment of yFeO₃ stabilized with AgNps/PVA, referred as P2. The morphology and the size of thus prepared yFeO₃/AgNps/PVA products were determined by TEM analysis. TEM images of the first product (P1) showed the presence of individual magnetic/silver nanoparticles with diameter ranging between 5-10 nm (Figure 2a). The same observation was made for the second product (P2), with the presence of individual magnetic/silver nanoparticles, which however tend to form aggregated clusters (Figure 2c). The selected area electron diffraction pattern (SAED) of yFeO₃/AgNps/ PVA materials indicates that they are nanocrystalline. The patterns of SAED are indexed as (200), (313), (317), (426), (513) and (517) reflections, which correspond to tetragonal crystal stucture of yFeO₃, with lattice constant: a=8.346, c=25.034 according to JCPDS (89-5894) (Figure 2 b, d).

The average hydrodynamic diameter (Dh) and particles size distribution of the bimetallic yFeO₃/AgNps/PVA nanoparticles was determined by DLS. The average hydrodynamic diameter was Dh=75 nm with a polydispersity of 0.15 for P1, and Dh=115 nm and polydispersity of 0.17 for P2 respectively. The naked yFeO₃ showed significantly higher hydrodynamic diameter Dh = 720 nm, which indicates that AgNps/PVA coating of magnetic nanoparticles improved the overall stability of iron oxide nanoparticles in aqueous
solution shown by significantly reducing the size of the nanoparticles 
thus preventing to some extent the formation of large aggregates 
(Figure 3).

The observed difference between the hydrodynamic diameter 
and those observed by TEM is due to the formation of small clusters 
between particles in aqueous solution.

FTIR analysis of the synthesized γFe₂O₃/AgNps/PVA product 
(P2) showed the presence of all-characteristic for PVA signals at 
3300 cm⁻¹, which is due to the O-H vibrations of hydroxyl groups 
originated from the main PVA chain. The peaks at 1326 cm⁻¹ are 
characteristic for the C-H deformation vibration in PVA and the 
absorption peak at 1000-1100 cm⁻¹ can be assigned to the C-O 
stretching and O-H bending vibrations arising from the PVA chain. 
The presence of magnetic iron oxide nanoparticles can be seen by two 
strong absorption bands at around 634 and 566 cm⁻¹ (Figure 4).

XRD analysis

For the the γFe₂O₃/PVA/AgNps products (P2), diffraction peaks 
at 30.36 (220), 35.77 (313), 43.35 (400), 53.76 (422), 57.16 (511), and 
62.98 (440) were observed which are ascribed to the normal structure 
of γFe₂O₃, and confirm the results of SEAD analysis [26]. Additionally, 
not very intensive peaks at 38.1° (111), 44.09° (200), 64.36° (220), 77.29° 
(311), were observed as well, which are referred to the standard data 
of Ag. Evidently, no other impurities peaks are detected (Figure 5). 
Moreover, the X-ray fluorescent (XRF) results confirm the existence of 
γ-Fe₂O₃ and presence of Ag with determined mass percentage of Ag - 
1.5% and for Fe – 96.5% respectively (Figure 5).

Fungicidal activity

To test the fungicidal activity of thus prepared γFe₂O₃/AgNps/
PVA bimetallic nanoparticles, six clinical strains with proven
resistance toward one or more antifungotics were involved in the experiment as follow: C. krusei 112, C. krusei 8-48, C. krusei 8-126, C. glabrata 0-73, C. glabrata 8-122, and C. nivariensis. Their antimycotical resistance determined using commercial kit ATB Fungus 3 is shown in Table 1. The minimal fungicidal concentration (MFC) of the tested products towards six clinical Candida strains were determined by surface agar method and the results are shown in Table 2. The presence of fungicidal activity of all synthesized products (P1-P3) onto the tested clinical yeast strains was observed. As expected, taking into account the difference into the type of the yeasts, which belong to various Candida strains, difference in MFC of the products was established.

The MFC for the strain C. krusei 8-126 are significantly higher for the P1 and P2 in comparison to the MFC of other strains Candida. This is indicative for the higher resistance of the strains compared to all tested yeasts (Figure 6 a). C. krusei demonstrated ability to grow in a medium with a lack of vitamins, temperature maximum of 43-45°C, production of phospholipases and proteases, ability to attach to the surfaces of the host and formation of hyphae. It is established that C. krusei is attached easier towards inert substances, than to the epithelial cells of the buccal mucosa [12]. These specific characteristics of the strain probably determined the higher resistance of the tested strain. The lowest values for MFC were observed for C. nivariensis 383, which determined it as the most sensitive strain from all tested Candida strains (Figure 6 b).

The MFC of P1 for strain C. krusei 112 was determined at 0.04 mg/L Ag and 2.71 mg/L Fe concentrations. For strains C. krusei 8-48 and C. glabrata 0-73, the MFC is higher in comparison to the rest of the strains – at concentration of AgNps 0.09 mg/L and 5.401 mg/L of Fe nanoparticles. The lower concentration was determined for C. glabrata 8-122 -0.02 mg/L Ag and 1.4 mg/L Fe concentrations.
Table 1: Antimycotical resistance of the tested clinical yeast strains.

<table>
<thead>
<tr>
<th>No</th>
<th>Clinical strain</th>
<th>Antimycotical resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C. krusei 8-112</td>
<td>FCA, ITR</td>
</tr>
<tr>
<td>2.</td>
<td>C. krusei 8-48</td>
<td>FLU, MCZ</td>
</tr>
<tr>
<td>3.</td>
<td>C. krusei 8-126</td>
<td>ITR</td>
</tr>
<tr>
<td>4.</td>
<td>C. glabrata 0-73</td>
<td>FLU</td>
</tr>
<tr>
<td>5.</td>
<td>C. glabrata 8-122</td>
<td>FCA, ITR</td>
</tr>
<tr>
<td>6.</td>
<td>C. nivariensis 383</td>
<td>FLU, VOR, ITR</td>
</tr>
</tbody>
</table>

Legend: Fluconazole (FCA), Itraconazole (ITR) and Voriconazole (VRC), using commercial kit ATB Fungus 3 *Bio Merieux and Miconazole (MCZ).

Table 2: MFC of the products determined for six clinical fungi strains.

<table>
<thead>
<tr>
<th>Clinical fungi strains</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ag mg/L</td>
<td>Fe mg/L</td>
<td>Ag mg/L</td>
</tr>
<tr>
<td>C. krusei 112</td>
<td>0.04</td>
<td>2.7</td>
<td>0.1</td>
</tr>
<tr>
<td>C. krusei 8-48</td>
<td>0.09</td>
<td>5.4</td>
<td>0.1</td>
</tr>
<tr>
<td>C. krusei 8-126</td>
<td>0.2</td>
<td>13.5</td>
<td>0.2</td>
</tr>
<tr>
<td>C. glabrata 0-73</td>
<td>0.09</td>
<td>5.4</td>
<td>0.04</td>
</tr>
<tr>
<td>C. glabrata 8-122</td>
<td>0.02</td>
<td>1.4</td>
<td>0.04</td>
</tr>
<tr>
<td>C. nivariensis 383</td>
<td>0.01</td>
<td>0.7</td>
<td>0.04</td>
</tr>
</tbody>
</table>

The MFC of P2 for strains C. krusei 112 and C. krusei 8-48 was higher than the strains C. glabrata 0-73 and C. glabrata 8-122: 0.1 mg/L Ag and 1.0 mg/L Fe concentrations. For the strains C. glabrata 0-73, C. glabrata 8-122 and C. nivariensis no growth was observed at 0.04 mg/L Ag and 0.5 mg/L Fe concentrations. The observed deviations in MFC for both the products are a result of the observed difference in their size and this determined their antifungicidal activity.

P3 containing only γFe₂O₃ nanoparticles showed the presence of fungicidal activity for all tested strains but at concentration much higher than those products containing AgNps. MFC for the strain C. krusei 8-48 was 3048 mg/L. For the strains C. krusei 112 and C. glabrata 0-73, MFC was 1524 mg/L. Only for C. krusei 8-126 and C. glabrata 8-122, MFC was lower respectively 762 mg/L (Figure 6 c, d, e, f).

The presence of antimicrobial activity of magnetic nanoparticles based on Fe⁰ or Fe₂O₃ is reported in the literature [27,28]. It is supposed that several oxido-reduction reactions take place that produce very reactive oxygen species because of Fenton reaction or Haber-Weiss cycle, thus iron in magnetite nanoparticles is fully oxidized to maghemite (γ-Fe₂O₃) via a series of reactions [27]. This causes oxidative stress to bacterial cells, and bacterial cell death, respectively. In contrast, fully oxidized maghemite is relatively stable in culture medium and do not generate significant cytotoxicity or genotoxicity in vitro due to the absence of electronic or ionic transfer [29,30].

Conclusion

Bimetallic nanoparticles based on magnetic iron oxide and silver nanoparticles (γFe₂O₃/AgNps) stabilized by PVA were synthesized and characterized. The results indicate a synergistic action at the combination of silver and iron nanoparticles in the hybrid materials when tested onto selected six clinical strains of yeast. The presence of only iron nanoparticles showed a fungicidal activity...
at exceeding concentration compared to their content in hybrid materials containing both silver and magnetic nanoparticles. These results indicate that thus prepared bimetallic γFe₂O₃/AgNps/PVA nanoparticles can find potential application for target delivery under magnetic field in biomedical area.

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References


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