Antifungal potential of some collagen-based nanocomposites Against *Candida lusitanae*

Dragomira S. Stoyanova¹, Iliana A. Ivanova^{1*}, Anna Staneva², Madalina Alby-Kaya³ and Todorka G. Vladkova²

¹Department of General and Industrial Microbiology, Sofia University, "St. Kliment Ohridski", Bulgaria

²Department of Polymer Engineering, University of Chemical Technology and Metallurgy, Sofia, Bulgaria

³Collagen Section, INCPI, Bucharest, Romania

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***Corresponding author:** Ivanova IA, PhD, Assoc. Professor, Dept. General and Industrial Microbiology, Faculty of Biology, Sofia University "St. Kliment Ohridski", Bulgaria; Email: ilivanova@abv.bg

Abstract

The possible mechanism of action of different nanomaterials on pathogenic fungi, significant for medical practice is summarized in this article. Furthermore, the antifungal action of different collagen based nanocomposites against *Candida lusitanae* was evaluated and compared. Collagen-based biomaterials with antimicrobial activity are attractive candidates for wound dressing, tissue engineering, components of implantable devices, etc. One of the easiest and most effective ways among the large variety of known approaches to add antimicrobial activity of biomaterials is development of composites including antimicrobial agents. In this study RGO, Ag/RGO, Ag/SiO₂/RGO, ZnTiO₃, ZnTiO₃/SiO₂/RGO and Ag/ZnO/ZnTiO₃ were entrapped in a porous collagen matrix by sol-gel cryogenic drying to preserve the native biological activity of the collagen. No chemical interactions were expected in the Collagen/antimicrobial compounds nanocomposites under these conditions. The nanocomposites were prepared in different ratios of the compounds. The formed sterile zones around the disc samples (diameter of 9.0 mm; thickness of 3 mm) were measured in mm (± 0.5). The representative of eukaryotic organisms *Candida lusitanae* demonstrated high sensitivity with large sterile areas at some of the tested materials.

Key words: Pathogenic fungi; Collagen matrix; Nanoparticles; Zone of inhibition; RGO- Reduced Graphene oxide

Introduction

Nanoscience has been emerged as a powerful tool to develop new approaches in the field of designing new antimicrobial drugs. Antifungal potential of nanoparticles should be considered in two directions connected to the seriousness and relevance of the issues. On the one hand is the clinical significance of fungal strains as *C. albicans*, *T. asahii*, *T. mentagrophytes*, *A. niger*, representing a direct threat to human health, causing skin, eye and other diseases. On the other hand the nanotechnology could be an alternative to deal with massive losses caused by phytopathogenic fungi such as *Phoma destructiva*, *Curvularia lunata*, *Alternaria alternata*,

Fusarium oxysporum etc. In striving to feed the increasing global population [1].

Candida albicans is the most common pathogenic fungus isolated in bloodstream infections in hospitalized patients, and candidiasis represents the fourth most common infection in United States hospitals, mostly due to the increasing numbers of immune- and medically-compromised patients. *C. albicans* has the ability to form biofilms and morphogenetic conversions between oval cells and hyphal morphologies contribute to biofilm development. Moreover, these attached communities of cells are surrounded by a protective exopolymeric matrix that effectively shelters *Candida* against the action of antifungals. Because of dismal outcomes, novel antifungal strategies, and in particular those targeting biofilms are urgently required. As fungi are eukaryotic, research and development of new antifungal agents is difficult due to the limited number of selective targets, also leading to toxicity to macro-organism [2].

Similar to *Candida*, *Trichosporon asahii* is an emerging fungal pathogen and exist in several growth forms in response to environmental conditions [3]. *T. asahii* can invade the human body through implantable catheters, abraded skins, mucosa, or respiratory tract, causing systematic and fatal trichosporosis and also has shown drug resistance [4, 5, 6].

Other important dermatophyte fungus is *Trichophyton* [7, 8]. *Trichophyton rubrum* (*T. rubrum*) is known to account for as many as 69.5% of all dermatophyte infections [9, 10, 11, 12]. The results of antifungal activity reveal that the growth of *T. rubrum* was inhibited at concentration of 10 $\mu\text{g/ml}$ Ag-NPs alone. In combination tests, fluconazole (20 $\mu\text{g/ml}$) together with Ag-NPs (2.5 $\mu\text{g/ml}$) and griseofulvin (0.4 $\mu\text{g/ml}$) with Ag-NPs (2.5 $\mu\text{g/ml}$) demonstrated increasing antifungal effects on *T. rubrum* [13].

The problem with ocular pathogenic fungi, which causes keratomycosis increase because they are responsible for vision

loss in the developing world like China [14]. Clinical studies indicate that keratomycosis constitutes about 46.7% to 61.9% of all cases of suppurative keratitis in patients. Filamentous fungi, mainly *Fusarium spp.* or *Aspergillus spp.*, are the most frequently isolated fungi in keratomycosis and the most common ocular pathogenic fungi. Fluconazole has high bioavailability against *Candida spp.*, but *Fusarium spp.* and *Aspergillus spp.* are resistant to it [14, 15, and 16]. Other antifungal is Natamycin, but it penetrates the cornea and conjunctiva poorly and effective drug levels are not achieved in corneas with intact epithelium. The penetration for amphotericin B was negligible after topical application because it was poorly soluble in water [16]. According to Gao et al [17]. The activity of nano-silver against *Fusarium spp.*, *Aspergillus spp.*, and *Alternaria alternata* was significantly superior to those of natamycin and fluconazole against ocular pathogenic fungi in vitro. Previous studies demonstrate significant antifungal activity of Nano-Ag, in an IC₈₀ range of 1-7 µg/ml against *T. mentagrophytes* and *Candida species* [18].

Candida spp. represent one of the most common fungal pathogens often causing hospital-acquired sepsis with an associated mortality rate of up to 40% [19]. Little is known about the epidemiology of infection with *C. lusitanae* [20, 21]. *Candida lusitanae* was originally isolated from the intestinal contents of warm-blooded animals [22]. In humans, *C. lusitanae* rarely causes opportunistic infections, although 13 cases involving various sites, including the kidneys, peritoneum, and blood stream, have been described [20, 21, 22, 23, and 24]. This species of *Candida* is of special interest because of its innate resistance to amphotericin B and its ability to develop resistance to amphotericin during therapy [23, 25, and 26]. A literature review [24] on *C. lusitanae* infections update and better characterize the illness in the era of azole availability and standardized methodologies for antifungal susceptibility testing. In this review *C. lusitanae* infection occurred in relatively young patients in a 55 cases (median age, 44 years). Fungemia was found in 80% of patients. Other infection syndromes, including peritonitis, meningitis, and urinary tract infection, were much less common [24].

Antimicrobial effect of silver nanoparticles (Ag-NPs) were investigated on many bacteria and yeast more than 60 years already, but the mechanisms of action are still not fully understood [27]. The nanoparticles attack the respiratory chain, cell division and finally lead to cell death [28]. Silver also forms complexes with various DNA bases (adenine, guanine, cytosine and thymine) and is a potent inhibitor of fungal DNAases and in the same time it could form complexes with electron donors containing Sulphur, oxygen or nitrogen (thiols, carboxylates, phosphates, hydroxyl, amines, imidazoles, indoles). The inhibition action of silver ions may include displacing of native metal cations from their usual binding sites in enzymes. The possible mechanism of action of Ag NPs has been suggested according to the morphological and structural changes in microbial cells. The Ag NPs show efficient antimicrobial properties that could be explained with their extremely large surface area, which provides better contact with microorganisms [29]. The antifungal activity of Ag NPs also includes damages of fungal spores in nanocomposites with silica or

organic material as pullulan [30, 31]. Silver nanoparticles may inhibit the growth of *T. asahii* by permeating the fungal cell and damaging the cell wall and cellular components, in concentration of 4 µg/ml. SEM Images have shown the deformed, distorted and shrunk and fractured mycelium, with damaged surface, leading to the outflow of intracellular components and shrinkage of mycelium [31].

Zamperini et al [32] reported a membrane depolarization of *Candida albicans*, an arrest of the fungal cell cycle by flow cytometry, release of intracellular glucose and trehalose, a decrease in plasma membrane fluorescence with increasing concentrations of Ag nanoparticles, pits in the cell wall, and pores in the plasma membrane on the TEM images. Other findings demonstrate that Ag nanoparticles destruct the fungal membrane integrity, dissipating the electrical potential of the membrane and inhibiting the normal budding process [33, 34]. AgNPs inhibited the growth of *A. flavus* by affecting cellular functions which caused deformation in fungal hyphae, reduction in spores number, malformation and hypertrophy, leading to destruction and damage of spores [35].

C. albicans and *C. tropicalis* showed high sensitivity to AgNPs, Kim et al. shows the inhibition of *C. albicans* at 2 µg/ml concentration of Ag-NPs [36]. Other authors reports ten to 400 times higher concentrations, depend on their size and ways to obtain Ag-NPs [18, 19, 37]. The combination between fluconazole and AgNP (32.5 nm average size spherical AgNP obtained by extracellular biosynthesis by the fungus *Alternaria*) showed the maximum inhibition against *C. albicans*, followed by *Phoma glomerata* and *Trichoderma spp.*, whereas no significant enhancement of activity was found against *Pleospora herbarum* and *F. semitectum* [38].

It was also determined that AgNP were more effective in reducing biofilm biomass when applied to adhered cells (2 h) than to pre-formed biofilms (48 h), with the exception of *C. glabrata*, which in both cases showed a reduction of ~90%. AgNP were highly effective on adhered *C. glabrata* and respective biofilms. On *C. albicans* the effect was evident as a reduction in the number of viable biofilm cells. These results suggest that AgNP could be an effective alternative to conventional antifungal agents for therapies in *Candida* associated denture stomatitis [39].

AgNP at ultralow doses of 0.001% was effective against *C. albicans*, *C. glabrata* and *M. sympodialis*, yeast species often found in atopic dermatitis patients. Despite its 3.4-times lower silver content, the AgNP preparation exhibits an antimicrobial activity against the bacteria yeasts and dermatophytes tested, comparable to that of silver sulfadiazine at concentrations of 0.1% [40].

Li et al [41] evaluate the surface morphology change of the native and treated *C. albicans* and *C. tropicalis* with the prepared GO nanoparticles. After treatment with pure GO for 24 h, the yeast cells were covered by a single layer wrinkled GO. After contact with carbon nanoscrolls/AgNPs, the yeast morphology significantly changed, with a major damage in cytoplasmic membrane of *C. albicans*, the content of the yeast cell leaked completely. The

similar phenomena also can be seen in *C. tropicalis* cells, as the ionic silver released, and a distinct concave could be observed on the cell membrane. When the *C. tropicalis* cell was covered by a non-AgNPs content GO nanosheets the cell membrane kept relative integrity, but with GO/AgNPs the membrane of the fungal cells collapsed apparently and the cytoplasm released partially [41].

The antimicrobial activity of zinc oxide nanoparticles (ZnONPs) is mainly due to generation of highly reactive species like OH^- , H_2O_2 , O_2^{2-} . H_2O_2 penetrates the cell and OH^- and O_2^{2-} damages the cell wall and cell membrane from outside [42]. The positive charged ZnONPs were considered to have close physical interaction with fungal cells by direct electrostatic adsorption. As a result, cell membrane damage and cellular internalization were promoted [43]. The penetration of ZnONPs could cause more ZnO-induced oxidative damage intracellular than outside the cells. A lot of studies have shown that ZnONPs induced the generation of ROS which cause oxidative stress [42, 43, 44, 45]. Though excessive ROS-generation imposed unacceptable oxidative stress to cells that result in cell damage, small amount ROS can be tolerated by most cell types [44]. ZnO NPs has a very good antifungal activity against different types of yeast including *Candida spp.*, *Trichosporon spp.*, *Geotrichum spp.* and *S. cerevisiae*. The ZnO NPs were also effective against fluconazole resistant *Candida* isolates [46, 47, and 48]. *Candida lusitanae* is important nosocomial pathogen; it is acquired by indirect contact transmission between patients with transplantations and other immunocompromised patients in medical intensive care units [21, 49].

Sponge-like matrices show significant potential for the regeneration and repair of a broad range of damaged anisotropic tissues. The manipulation of the structure of collagen scaffolds using a freeze-drying technique is an intrinsically biocompatible way of tailoring the inner architecture of the scaffold [50]. The Collagen matrix impregnated with antibacterial agents is attractive candidates for wound dressing, tissue engineering, components of implantable devices, etc [51].

No reports about sensitivity of *Candida lusitanae* to the nanocomposites of ZnO, ZnTiO₃, RGO and Ag in different ratio combinations with Collagen till now, which provoked us to conduct this investigation.

Materials and methods

Preparation and characterization of RGO

The used in this study RGO (with less than 2-3 wt % impurities of graphite materials) was prepared by commonly used chemical exfoliation method starting from purified natural graphite powder (99.9 %, Alfa Aesar Co.) and employing sodium borohydride as a reducing agent, as it was described in [52].

The phase formation and structural transformation were detected by X-ray phase analysis (Bruker D8 Advance, Germany; Cu K α ; Lynx Eye detector).

Preparation of Collagen/ RGO composites

Type I fibril collagen gel with concentration of 2.64 wt. % was

extracted from calf hide using previously described technology [53]. The concentration of the collagen gel was adjusted at 1% and pH at 7.3 (that of the physiological medium) using 1M sodium hydroxide and antimicrobial agent (RGO powder) in 2:1, 2:0.8, 2:0.6, 2:0.4 or 2:0.2 ratios (wt/wt) was added. The in this way prepared collagen/antimicrobial agent composites were cross-linked with 0.5 % glutaraldehyde (to dry collagen) at 4°C for 24 h and then lyophilized at -40°C to obtain porous (sponge) material using a Martin Christ freeze-dryer for 48 hours, as it was previously described [54, 55]. Test samples with diameter of 9 mm were prepared after that from each composite. RGO aggregates with different dimensions are wrapped in the collagen matrix, some of them partially covered by matrix collagen. The compressive modulus at 10 % deformation was estimated of the studied Collagen/RGO composites, expecting that the presence of RGO could influence their mechanical strength [56].

Antifungal activity tests

In these study nanocomposites with compounds as RGO, Ag/RGO, Ag/SiO₂/RGO, ZnTiO₃, ZnTiO₃/SiO₂/RGO and Ag/ZnO/ZnTiO₃ were impregnated in collagen matrix in different ratios and were examined as antifungal agent against *Candida lusitanae*. The test fungal strain was obtained by National Bank of Industrial Microorganisms and Cell Cultures, propagated in YGS at 36°C and 120 rpm. At 12 h [57]. Microbial density of 0.5-0, 8 were determined according to McFarland. The aliquots of 100 μL microbial suspension was randomly spread on solid medium (YGS agar) and discs of investigated material were put on them. The plates were left for 20 h at 4-6°C to afford diffusion of the nanoparticles and after that cultivated for 24 h at 36°C. The formed sterile zones around the disks samples were measured in mm (± 0.5). Minimum two till 4 replicates for every composite were tested and mean values presented in the figures.

Results and discussion

The antifungal activity, as a sterile zone in mm, of the studied antimicrobial agents loaded in collagen sponges is presented in Figures 1 to 6.

The used in this study RGO, consists of multilayer (up to 5) sheets with relatively large area (up to about 10 x 20 μm) that tend to aggregate. They were dispersed in a collagen gel to be formed porous collagen/RGO composite after a cryogen drying, the last one keeping the native biological activity of the collagen. No chemical interactions between RGO and the collagen matrix were expected under these conditions. Aggregates of RGO sheets were wrapped in the collagen matrix, some of them partially coated by matrix collagen, as depicted by SEM images.

Although that the mechanism of the antimicrobial activity of RGO is not fully understood, it is generally accepted that it includes an effect of the direct cell membrane contact with sharp RGO nanosheets [58, 59]. In addition, destructive extraction of large amount phospholipids from *E. coli* cell membrane by graphene nanosheets (due to strong dispersion interactions between the RGO and the lipid molecules) is shown as a reason for the antibacterial activity of the graphene nanosheets [60].

In result of the test the antibacterial effect of RGO on the representative eukaryotic organisms *Candida lusitanae* demonstrated high sensitivity with large sterile areas (data was shown in Figure 1) at the maximum tested concentration of RGO and small ones at the lowest of the tested concentrations. The high sensitivity of fungus is probably due to usage of nutrient medium with antibiotic Chloramphenicol and combination of

Figure 1: Antifungal effect of nanocomposite RGO in different weight ratio with collagen matrix – the size of the sterile zone around the disk. Antimicrobial effect of nanocomposites Ag/RGO in different ratios with collagen porous nanocomposites can be seen at Figure 2. *Candida lusitanae* growth was inhibited of the all four different concentrations. The most pronounced results were obtained for the ratio 2:0.7 and 2:0.8. The antimicrobial effect of silver nanoparticles is well known [2, 7, 8, 13, 18, 30, 31, 33, 34, 35, 62, and 63]. Our result is interesting with the ratio of two compounds – the distribution of the silver nanoparticles was more uniformly at a ratio of Collagen: Ag/RGO = 2:0.7, where the highest activity was obtained. The lower antifungal activity of higher concentration could be due to agglomeration of active nano-Ag.

Figure 2: Antifungal effect of nanocomposite Ag/RGO in different weight ratio with collagen matrix (the size of the disk is not included). In the case of Coll: Ag/SiO₂/RGO for *C. lusitanae* the strongest effect was observed for the highest concentration and three times weaker effect for the lower ratios. Data are shown in Figure 3. As other authors mentioned earlier dual action of nanocomposites combined with antibiotic have stronger antifungal effect [64]. The results of synergistic action of nanoAg, SiO₂ and RGO as compounds of nanocomposite with collagen matrix were summarized and the data can be seen at Figure 3. The highest concentrations demonstrate significant effectiveness compared with other weight ratios.

Figure 3: Antifungal effect of nanocomposite Ag/SiO₂/RGO in different weight ratio with collagen matrix – the size of the sterile zone around the disk. Collagen/ZnTiO₃ nanocomposite has shown antifungal activity at higher tested concentrations against *Candida lusitanae*. Collagen: ZnTiO₃ = 2:1 (Figure 4). It is comparable to antifungal effect of Coll: Ag/SiO₂/RGO, but the effect is more stable and does not reduce so quickly as that of the Coll: Ag/SiO₂/RGO nanocomposite.

Figure 4: Antifungal effect of nanocomposite ZnTiO₃ in different weight ratio with collagen matrix (the size of the disk is not included). In contrast the highest antifungal effect of nanocomposite Coll: Ag/ZnO/ZnTiO₃ was not at highest concentration of the active compounds to the collagen. The results may be due to agglomeration of nanoparticles. The results were illustrated at Figure 5.

Figure 5: Antifungal effect of nanocomposite Ag/ZnO/ZnTiO₃ in different weight ratio with collagen matrix (the size of the disk is not included). Antibacterial effect of nanocomposite ZnTiO₃/SiO₂/RGO on the tested fungal strain was the weakest by all tested nanomaterials as can be seen from Figure 6. The result means possible gentle effect not only to the fungus, but also to the other eukaryotic cells and could be the aim of the further investigation.

Figure 6: Antifungal effect of nanocomposite ZnTiO₃/SiO₂/RGO in different weight ratio with collagen matrix- the size of the sterile zone around the disk. The Coll:ZnTiO₃/SiO₂/RGO nanocomposite had insignificant inhibition effect on test microorganism *Candida lusitaniae*, but the other examined materials have significantly higher antifungal effect. The most pronounced results were obtained for the ratio 2:1 at the most of the tested materials but Coll: Ag/ZnO/ZnTiO₃ and Coll: Ag/RGO show different results. The first is most effective at 2:0.8 and second nanocomposite at the ratio 2:0.7 respectively. If we compare effectiveness of the test nanomaterials, it can be concluded that the most effective against *C. lusitaniae* was Chloramphenicol in the nutrient medium in combination with hold: RGO (2:1) followed by Coll: Ag/SiO₂/RGO (2:1) > Coll: ZnTiO₃ (2:1) > Coll: Ag/ZnO/ZnTiO₃ (2:0.8) > Coll: Ag/RGO (2:0.7). Our results are comparable with these previously reported by other authors [61, 62, 63, 64]. Strongest effect of Coll: Ag/SiO₂/RGO nanocomposite without antibiotic in nutrient medium was possibly due to the role of SiO₂ as dispersing agent (vehiculum). It is important to mention that SiO₂ alone as micro size powder has no antibacterial effect till the concentration of 6 mg/ml (results are not presented). Noble metals have proved their activity to bacteria and fungi [65], but the supported materials are also important for the obtained antimicrobial effects. As [66] reported, the level of susceptibility of *C. lusitaniae* to amphotericin B did not differ much from those of *C. albicans* and *C. glabrata*, so it could be suggest similar antifungal activity of tested nanocomposites to the other fungal pathogens.

both antimicrobial agents: antibiotic and nanosheets. Such kind of interaction is reported by [61, 62, and 63].

Conclusion

As a result of the current study it can be concluded, that the inhibition effects of the studied collagen-based nanocomposites on the fungal growth of *C. lusitaniae* depends on the type of compounds included in the nanocomposites and their concentration. The results from diffusion method in solid medium indicate that some of the studied nanomaterials have strong pronounced effect on *C. lusitaniae* which are a close relative of the resistant to antibiotics *Candida albicans*. Thus we can say, that the studied collagen-based nanocomposites are promising anti-infective biomaterials.

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