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CYTOTOXICITY AND ANTIFUNGAL ACTIVITY OF CMC/SiO_2/AgNps HYBRID MATERIALS AGAINST *SACCHAROMYCES CEREVISIAE* 537

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Abstract

The paper focused on preparation of antimicrobial silica hybrid materials based on tetraethylorthosilicate (TEOS) as SiO_2 precursors, carboxymethyl cellulose (CMC) as an organic compound and silver (AgNps). The quantity of organic substance was 10 wt.% and the silver concentration varied from 0.0 to 1.5 wt.%. The obtained hybrids were analyzed and characterized using AFM analysis and the hydrophilicity of the materials was quantified by determining the water contact angle.

It has been experimentally demonstrated that these silver doped organic-inorganic hybrids have a well pronounced antimicrobial behaviour against *Saccharomyces cerevisiae* 537. The results showed that hybrid materials with 1.5 wt.% Ag resulted in 66.05% cell reduction after 24 h incubation. In addition the hybrid materials bearing AgNps were tested for their toxicity to 3T3 fibroblasts. Increasing the concentration of AgNps to 0.5 wt.% did not cause any decrease in cell survival; even after 48 h good cell proliferation was observed. With further increasing the AgNps to 1.5 wt.% and prolonged incubation (48 h) the cell viability drastically dropped down to 20%.

Key words: carboxymethyl cellulose, *Saccharomyces cerevisiae* 537, silver nanoparticles, sol-gel techniques, cytotoxicity, cell survival, proliferation

1. Introduction. Silver ions (Ag+) and their compounds are highly toxic to microorganisms exhibiting strong biocidal effects on many species of bacteria but have a low toxicity towards animal cells [1–4]. Silver nanoparticles (AgNps) can directly damage bacteria cell membranes through release of silver ions followed (individually or in combination) by increased membrane permeability, loss of the proton motive force, inducing de-energization of the cells and efflux of phosphate, and leakage of cellular content. Silver metal and inorganic silver compounds ionize in the presence of water, body fluids or tissue exudates. Due to the increasing bacterial resistance to classic antibiotics, the investigations on the antibacterial activity of silver nanoparticles have increased [5, 6].

Silver nanoparticles stabilized by polymers and surfactants exhibited high antifungal activity affected by aggregate stability. The cell walls of yeasts were disrupted by surfactant activity, which increased their sensitivity to AgNps [7]. The mechanisms of silver interaction in fungi cells are not clear. A possible mode of action can be the formation of "pits" on the membrane surfaces which finally results in the formation of pores and cell death. AgNps attach to cell membrane and penetrate in the fungi, then produce a site with low molecular mass in the centre of fungi, leading to cell death [8].

CMC is a water soluble polymer derived from natural cellulose. TEOS is used as silica precursor that is brought into contact with water. TEOS is hydrolyzed and its products are involved in polycondensation reactions that yield hydrated SiO_2 [9]. CMC-based hydrogels could be recommended as dressing materials for healing of burn or cut wounds, as well as a tool for transdermal drug delivery [10]. Silver-based medical products, ranging from topical ointments and bandages for wound healing to coated stents, have proven to be effective in retarding and preventing bacterial infections [11]. Improvements in the development of novel AgNps-containing products are continuously sought. In particular, there is an increasing interest toward the exploitation of AgNps technology in the development of bioactive biomaterials, aiming at combining the relevant antibacterial properties of the metal with the peculiar performance of the biomaterial. This is due to the fact that the nanoparticles, immobilized in the gel matrix, can exert their antimicrobial activity by simple contact with the bacterial membrane, while they can not be uptaken and internalized by eukaryotic cells. This novel finding could advantageously contribute to responding to the growing concerns on the toxicity of nanoparticles and facilitate the use of silver-biopolymer composites in the preparation of biomaterials.

In this study we have developed a sol-gel method to produce organic-inorganic hybrid materials based on CMC and TEOS as a precursor solution, containing uniformly distributed AgNps. Their antimicrobial activity was tested against *Saccharomyces cerevisiae* 537 and the citotoxicity of the hybrid materials was proven on 3T3 mouse fibroblasts.

2. Materials and methods. ***2.1. Materials.*** Carboxymethyl cellulose (CMC), tetraethylorthosilicate (TEOS) and silver nitrate ($AgNO_3$) were purchased from Aldrich and nitric acid from Merck. All chemicals were used as received without any further purification. 3T3 murine fibroblast cells were provided by ATCC, USA.

2.2. Preparation of CMC /SiO_2/ silver-doped hybrid materials. The hybrids contained TEOS and 10 wt.% CMC and silver with concentration from 0.5 wt.% Ag to 1.5 wt.% Ag. First TEOS was pre-hydrolized with H_2O and 0.1 N HNO_3 solutions as catalyst (Sol A). Different amounts of $AgNO_3$ were added to this sol under magnetic stirring. After dissolving of CMC (Sol B) in H_2O the obtained sols were mixed to produce Sol C. The obtained sols were kept at 50 °C for gelation and drying.

2.3. Sample characterization. **Atomic force microscopy (AFM)** imaging was performed in tapping mode in air (NanoScopeV system, Bruker Inc.). Silicon cantilevers (Budget Sensors, Innovative solutions Ltd., Bulgaria) with 30 nm thick aluminum reflex coating were used (tip radius $<$ 10 nm). The round glass slides (diameter of 10 mm), covered by CMC hydrogels containing AgNps were fixed to the metal pads and scanned with rate 0.5 Hz. Each sample was examined at 5–10 different locations all over the slide exploring the areas of 15×15 µm, 10×10 µm, 5×5 µm and 1×1 µm. The images (512×512 pixels) were captured in the height and error modes and further analyzed by NanoScope 6.13R1 software in the following order: second order flattening; roughness analysis; morphology characterization (determination of the height and area of the objects).

2.4. Static Contact Angles Measurements. Contact angles were measured by producing a sessile water droplet on the solid substrate. The most common method for measurement involves looking at the profile of the drop and measuring the angle formed between the solid and the drop profile at the three-phase line. A contact-angle goniometer (Ramé-Hart Automated Goniometer Model 290) was used to take the measurements. Typically, 3 µl of ultrapure water (from Purelab Option-Q purification system with specific resistance of 18 MΩ/cm) were placed on the sample surface by microsyringe. The left and right angles were measured at 3–4 different spots of each sample.

2.5. Cytotoxisity test. MTT [12] assay was used to determine cytotoxicity of the CMC materials with different amount of AgNps (from 0.5 to 1.5% Ag). Initially the materials were incubated in cell culture medium for 72 h at 37 °C and 5% CO_2, in 24 well plates. In parallel plates, 3T3 cells (1×10^5 cells/ml) were cultured for preparation of the adherent cell culture. After the incubation period, the cell culture medium in which the cells were grown was replaced with the medium in which the materials were incubated. Then the plates were incubated again for 24 and 48 h and MTT test was performed for determining the degree of cytotoxicity.

2.6. Antimicrobial experiments. In this work the antimicrobial activity of hybrid materials against *Saccharomyces cerevisiae* 537 was studied with several methods like investigation of the growth rate of microorganisms after exposure to the materials and counting of surviving cells on the agar plate after contact with hybrids.

Determination of antimicrobial activity in YEPD broth using turbidity. The antimicrobial effect of obtained hybrid materials was tested against *Saccharomyces cerevisiae* 537. The strain was obtained from Bulgarian National Bank of Industrial Microorganisms and Cell Culture and was cultivated in Erlenmeyer flasks containing 50 ml YEPD liquid medium. The culture strain was incubated on a rotary shaker (120 rpm) at 30 °C for 24 h. The hybrid materials with different amount of Ag (from 0.5 to 1.5% Ag) were prepared and 10 mg were placed in a flask with the investigated strains. The positive control contained yeast and hybrid materials without silver. The cultivation process was performed on a rotary shaker (200 rpm) at 30 °C. Aliquots were taken every hour and optical density was measured at 610 nm.

Antimicrobial activity based on YEPD agar plating. Microbial growth-inhibiting effect of hybrid materials was further confirmed by plating on YEPD agar plates. 200 µl of cells broth suspension after 24 h of strains cultivating in the presence of hybrid materials with different amount of AgNps were seeded in YEPD agar plates. Samples containing yeast and hybrid materials without ApNps were plated as positive controls. The plates were incubated for 24 h at 30 °C followed by counting the number of colonies on the plate.

3. Results and discussion. Hydrophilicity of samples was quantified by determining the contact angle of a water drop. This is a quite important surface characteristic since hydrophilic surfaces favour the attachment of mammalian cells while proteins prefer moderate hydrophobic surfaces. According to Vogler, biomaterials with contact angle greater than 65° are classified as hydrophobic and surfaces with contact angles less than 65° as moderate hydrophilic [13]. The typical shape of water drops on each of the three types of surface is demonstrated in Fig. 1*A*. From this figure and from the values of contact angles presented in Table 1, it is evident that all three studied surfaces can be classified as hydrophilic and should be effective on cell attachment. However, the AgNps-addition increases the water contact angle substantially, irrespectively of the AgNps-concentration.

The topography and the roughness of CMC-films was studied by AFM. Typical AFM micrographs are shown in Fig. 1*B*, where 3D views are presented for greater clarity. The control sample (CMC without AgNps) is characterized by uniform distribution of small rounded particles with similar dimensions (diameter in the range 250–300 nm, area of 40 000–70 000 nm^2, and height 10–16 nm). The incorporation of 0.5% AgNps to the hybrid materials resulted in a slight rise of the mean surface roughness (R) determined from the AFM micrographs (Table 1). The increase of the content of AgNps to 1.5% leads to the formation of numerous and significantly larger aggregates (area of 31 000–237 000 nm^2) with height between 16 and 125 nm (Fig. 1*B*). The surface roughness of the hybrid materials containing 1.5% AgNps increased nearly fourfold (Table 1).

Cell survival experiments (Fig. 2) showed that the basic material CMC have very high biocompatibility as the cell viability was kept in the range between 80 and 100%. A concentration of 0.5% Ag led to minimal decrease of cell viability (around 80%), but the prolonged incubation (48 h) of the material with the cells caused stimulation of cell proliferation (Fig. 2). The viability of cells incubated

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Roughness and contact angle of CMC hybrid materials with AgNps. Average surface roughness (Ra) of the samples, each averaged from five images of 15×15 μm^2 scanned surface. Average water CA of the samples measured at five different places in the sample

Surface AgNps, [%]	Ra, nm	CA, degrees
0% Ag	0.99 (± 0.24)	22.2 (± 2.8)
0.5% Ag	1.50 (± 0.48)	39.3 (± 2.3)
1.5% Ag	4.25 (± 1.63)	39.8 (± 1.6)

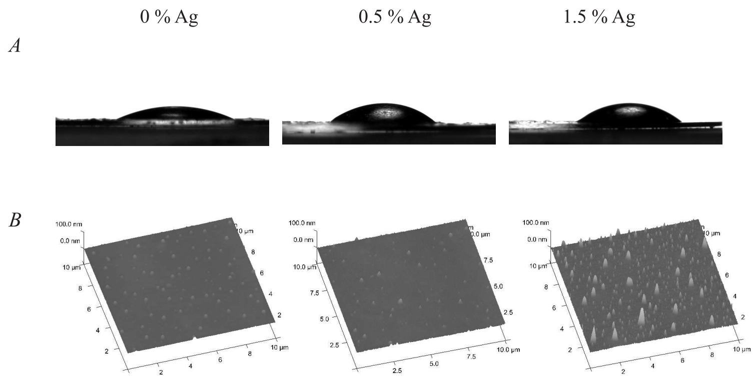

Fig. 1. Photographs of water CA (*A*) and 3D AFM images of CMC hybrid materials with different Ag content (*B*)

with CMC materials containing a higher concentration of Ag (1.5% Ag) for 24 h was around 80%, but after 48 h it drastically decreased and reached approximately 20%. We can explain the decreased cell proliferation after 48 h with increased concentration of AgNps in the cultivation medium, which with prolonged time slows down the cell growth.

The antimicrobial activity of the investigated hybrid materials was tested with *Saccharomyces cerevisiae* 537 by measuring the optical density and CFUs after strains exposure at different Ag concentrations. The microbial growth inhibition of strain is shown in Fig. 3. *Saccharomyces cerevisiae* 537 exposed to 1.5% Ag showed almost a complete inhibition. Materials with 0.5% Ag led to small suppression of growth phases, the lag-phases were prolonged and logarithmic phases were reduced. The growth of microorganisms in the presence of 0.5% Ag is similar to control materials (0% Ag). The lower silver concentrations suppress the initial growth phases of the investigated strain and after 24 h the culture is equalized by development of the control. In this context it can be concluded that yeast in the presence of materials with minimal silver concentration has a similar growth rate like the control. So a small silver content (0.5%) does not influence the growth

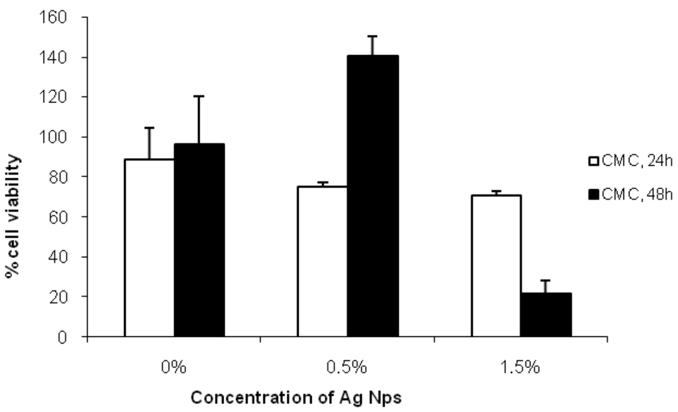

Fig. 2. Cytotoxicity of the CMC materials using 3T3 cells presented in percentage of the control after cell incubation with the materials for 24 (white columns) and 48 h (black columns) ($n = 2$)

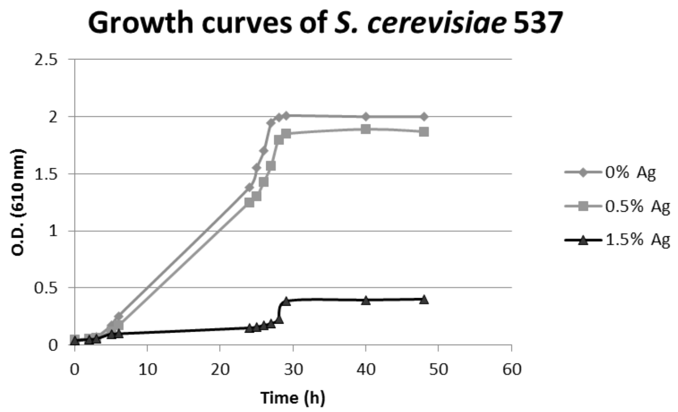

Fig. 3. Growth curves of *Saccharomyces cerevisiae* 537 exposed to pre-coated and dried materials with hybrids derived with different silver content: 0.0 wt.% Ag, 0.5 wt.% Ag, 1.5 wt.% Ag

of culture. A high inhibition of microorganisms is observed when they are in contact with materials with 1.5 wt.% Ag. The delay of microorganism's growth is proportional to the amount of included silver nanoparticles.

In Fig. 4 the test results of *Saccharomyces cerevisiae* 537 cultivated on solid YEPD media after 24 h incubation are shown. It was observed that the numbers of cells were reduced with increasing of silver concentration of the materials.

The percent of inhibited cells after exposure of hybrids with different silver concentrations after 3 h, 5 h and 24 h of cultivation is illustrated in Fig. 5. It was detected that the number of surviving cells was proportional to the silver concentration and also that the time of yeast cultivation in the presence of hybrids influenced the cells growth. The highest inhibition was observed after 24 h

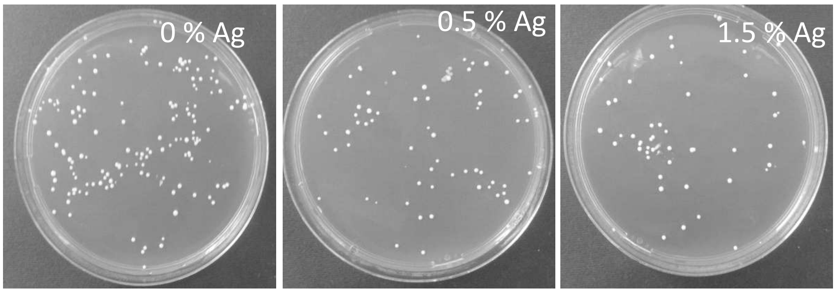

Fig. 4. Test results of *Saccharomyces cerevisiae* 537 cultivated on solid YEPD after 24 h: without Ag, in the presence of 0.5% Ag and 1.5% Ag

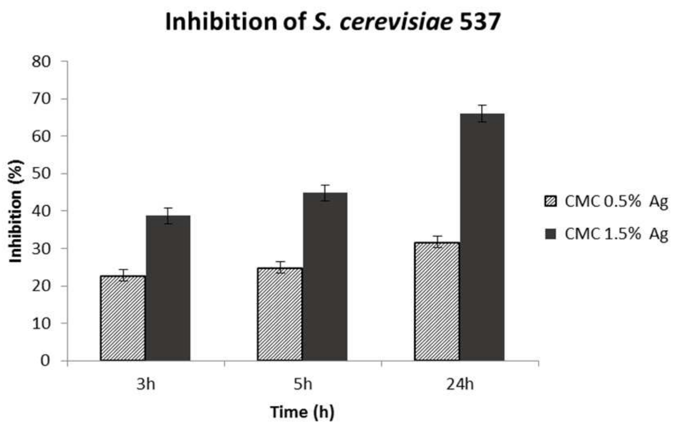

Fig. 5. Percent of inhibited cells in presence of 0 wt.% Ag, 0.5 wt.% Ag and 1.5 wt.% Ag after 24 h cultivation

cultivation by hybrids with 1.5 wt.% Ag – 66.05%, compared to 5 h incubation – 44.8% and 3 h incubation – 38.7%.

In our previous investigations we studied the antimicrobial behaviour of CMC/SiO_2 with different amount of Ag and we found out that the reduction of bacterial growth of *B. subtilis* reached 100% at the presence of 1.5 wt.% Ag, whereas the reduction of cell growth by *E. coli* K12 was 95%. The lowered silver concentration of 0.5 wt.% also leads to reduction of bacterial growth – 75% by *B. subtilis* and 53% by *E. coli* K12 [9]. The results show that bacteria exhibited higher sensitivity to the investigated materials in comparison to the yeast. This could be explained with the different cell structure by prokaryotes compared to eukaryotes.

4. Conclusions. Antimicrobial CMC/SiO_2 silver-doped hybrid materials were prepared by sol-gel method. The microstructure of the hybrid materials investigated by AFM analysis showed increased surface roughness with increasing the AgNps content. All investigated materials exhibited hydrophilic nature but there was a tendency for increasing the water contact angle with increasing the AgNps content. The hybrid materials showed effective bactericidal activities against *Saccharomyces cerevisiae* 537. Results revealed that the antimicrobial activity of the materials depended on the silver concentration. The cytotoxicity effect of the materials on mouse fibroblasts was also time- and AgNps concentration dependent. The obtained results revealed that hybrid materials based on CMC and containing AgNps might be suitable materials for biomedical application since they could combine antifungal activity with good tolerance to fibroblasts.

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