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Broad-Spectrum Antimicrobial Activity of Silver Nanoparticles in Different Types of Chitosan Matrices

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Abstract

Due to the problem of resistance of many infectious agents to the usual treatments, this study addresses the ways of obtaining and using new chitosan-silver nanomaterials as antimicrobial agents. Chitosans of medium (CSM) and high (CSH) molecular weights were used as matrices in the formation of small silver nanoparticles (AgNP) when mixed with $AgNO_3$ under different conditions, such as using the polymer (CS) in acetic acid solutions, as nanoparticles (CS-NP) or in films. The films were prepared by evaporation of the CS-$AgNO_3$ mixtures at different temperatures and in all cases AgNP was formed. When acetic acid solutions of CS were boiled in the presence of $AgNO_3$ CS-NP were formed containing AgNP (Ag@CS-NP). It is evident that the size and form of presentation of CS are not factors that can significantly affect the formation of small AgNP. AgNP inserted in different CS matrices presented significant antimicrobial activity (MIC between 0.6-4 μg/mL) against four Gram-positive and five Gram-negative bacteria, and also against three yeasts. It is interesting to emphasize the highest activities achieved for Ag@CSH-NP against *B. cereus* (0.6 μg/mL) and Ag@CSM-NP against *P. pastoris* (0.7 μg/mL). Although the antimicrobial activity was dependent on the strain assayed, the overall tendency observed was that the nanocomposites made with CSH are more effective than those prepared with CSM.

Keywords

Antimicrobial, Chitosan, Nanoparticle, Silver, TEM, UV-Vis

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1. Introduction

Nowadays, antimicrobial resistance within a wide range of infectious agents is a serious problem which is a concern for governments around the world. It is a public health threat that affects countries and multiple sectors which, in turn, undermine to the achievements of modern medicine [1]. Therefore, the synthesis and biocide effects of novel compounds are intensively studied. One of the most promising strategies for overcoming microbial resistance is the use of nanoparticles [2, 3].

Continuing our work in the search for new compounds with antimicrobial activity [4], we have recently focused our attention on AgNP due to their important antimicrobial properties [5-7] and their possible application in biomaterials such as bandages and stitches [8-10]. We obtained small AgNPusing an eco-friendly and inexpensive procedure with

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CS of low molecular weight and studied their antimicrobial activity [11].

We have selected CS because it is a nontoxic, biodegradable, biocompatible and biorenewable polymer [12] with different applications in many different fields suchas biomedicine, the environment, agriculture and food [13-17]. The chitosan obtained upon deacetylation of chitin from crustacean shells or fungal cell walls has variable composition and molecular weight, depending on the method used to obtain it [18-20]. This diversity in composition and size of CS can be considered as the main reason for the differing reports of the chemical and biological properties of CS derivatives, even leading to completely contradictory results [21, 22]. As a polysaccharide, chitosan is a reducing agent and, as a result, has been used in different chemical processes. One of the major applications of CS in bulk and/or nano dimensions has been to obtain and stabilize metals and metal nanoparticles [23, 24].

However, as previously stated, there have been numerous studies on the synthesis and antimicrobial activity of AgNP and CS, yet there does not seem to be consensus on the influence of the type of CS used nor in the size and type of AgNP obtained, nor in their antimicrobial activity.

Herein we propose the use of chitosan of high and medium molecular weight for the obtained AgNP, in order to have a more complete interpretation of how the interaction between Ag^+ and CS takes place. The synthesis is carried out under different conditions, such as using the polymer in acetic acid solutions, in films and as nanoparticles, using both bulk and CS-NP. Finally, after evaluation of the antimicrobial activity of the different nanohybrided systems, we will try to establish a conclusion about the effect of the CS type and the AgNP size on the antimicrobial activity, if possible.

2. Materials and Method

2.1. Materials

Medium (CSM, 190-310 kDa) and high molecular weight (CSH, 310-375 kDa) chitosan, with 75-85% deacetylation, and also sodium tripolyphosphate (TPP), were purchased from Aldrich. $AgNO_3$ was procured from Scharlau and acetic acid (100%) from Prolabo. Mili-Ω water was used in all cases.

2.2. Preparation of CSM-NP and CSH-NP

Nano-sized chitosan was obtained following a procedure describe in our previous report [11] which briefly consisted in adding drop wise an aqueous solution of TPP 1 mg/mL (4.0 (CSH) or 5.4 (CSM) mL) to a solution of CS (10 mg dissolved in 10 mL acetic acid 0.2%). The solution was stirred at room temperature and after 3 h a white discrete opalescence, with Tyndall effect, was observed.

2.3. Preparation of AgNP inside CS-NP (Ag@CS-NP)

The above solution of CS-NP (CSM-NP or CSH-NP) was heated under reflux and then an aqueous solution of $AgNO_3$ (5-200 mg in about 0.6 mL of water) was added drop wise to it. The boiling solution was stirred for 5-9 h depending on the amount of $AgNO_3$ added and the type of CS used.

2.4. Preparation of AgNP in CS (Ag@CS)

AgNP was synthesized in a solution of CSH or CSM in acetic acid (1 mg/mL) was performed in a similar manner to that explained in section 2.3 above.

2.5. Preparation of Films

CSM and CSH water solutions were prepared by dissolving CS (1 g) in acetic acid (0.2%, 100 mL). To aliquots (10 mL) of this solution was added drop wise, with continuous agitation, the corresponding amount of $AgNO_3$ (5-50 mg/0.6 mL) and was kept for 1.5 h. A change in color was observed depending on the amount of $AgNO_3$ added (from yellowish to brownish). Gelation of the mixture started immediately after the addition of $AgNO_3$. The resulting gel was then transferred to a Petri plate (20 mL) which was introduced in a vacuum stove for 4-100 h at different temperature (40, 55, or 70°C). The films formed were dissolved in acetic acid (2 mL, 99.5%) for their characterization.

2.6. Characterization

2.6.1. Spectroscopy

A Cary 50 Conc (Varian) UV-Vis spectrophotomer was used for the determination of the surface plasmon resonance (SPR) bands of AgNP. High resolution transmission electron microscopy (HRTEM), as well as high angle annular dark field (HAADF) in scanning mode (STEM) was carried out by using a JEOL 2200FS microscope working at 200 kV. Dynamic light scattering (DLS) measurements of obtained CSNP and Ag@CS-NP aqueous colloidal systems was performed using a 90Plus Particle Size Analyzer digital autocorrelator (Brookhaven Instruments Corp.) equipped with 635 nm laser, at $\theta = 90°$ and 25°C. Data were analyzed using CONTIN algorithm.

2.6.2. Determination of the Sizes of the Nanoparticles

The sizes of the synthesized AgNP were determined, in first approximation by UV-Vis spectrophotometry according to the position of the SPR band. The size distribution of the AgNP was obtained from representative HRTEM and

HAADF images by counting statistically useful numbers of particles. The sizes of CS-NP, with AgNP included, were determined by DLS, a technique that gives the mean hydrodynamic diameter of the nanoparticles.

2.6.3. Determination of the Antibacterial and Antifungal Activities

The antimicrobial activity of the samples was estimated using the two fold broth dilution method. The samples were used as prepared and tested at final concentration (of silver) of 8, 6, 4, 2, 1, 0.5, 0.25, and 0.125 µg/mL. Bacteria were grown in Muller-Hinton medium and yeasts cultivated in Saboureaud medium, both from Cultimed. Inocula of $5x10^4$ and $1x10^3$cels/mL were used for bacteria and yeasts, respectively. The tubes were incubated at 35ºC for bacteria, 26ºC for *C. glabrata* and 30ºC for *S. cerevisiae* and *P. pastoris* for 24 h (except in the case of *X. campestris* for which the incubation time was of 48 h). Growth was estimated by the visual turbidity of the tubes noted both before and after incubation. Uninoculated media and positive growth controls were also run at the same time. The minimal inhibitory concentration (MIC, µg/mL) was defined as the lowest concentrations of compound inhibiting the growth of the different strains. The minimal bactericidal concentration (MBC, µg/mL) and the minimal fungicidal concentration (MFC, µg/mL) were determined by subculturing 100 µL of each tube remaining clear in tubes containing 1 mL of fresh medium and growth determined as above described. Assays were done in triplicate.

The bacteria studied were: *Bacillus thuringiensis* (CECT 4497), *Bacillus cereus* (CECT 193), *Staphylococcus aureus* (CECT 4439), *Staphylococcus epidermidis* (CECT 231), *Xanthomonascampestris* (CECT 97), *Escherichia coli* (CECT 101), *Salmonella spp.* (Collection of Industrial Microbiology and Biotechnology Laboratory, University of Vigo), *Klebsiellapneumoniae* (CECT 143), and *Enterococcus faecalis* (CECT 481). Three yeasts were also studied: *Candida glabrata* (CECT 1448), *Pichia pastoris* X33 (Invitrogen), and *Saccharomyces cerevisiae* UV30 (Collection of Industrial Microbiology and Biotechnology Laboratory, University of Vigo).

3. Results and Discussions

3.1. Comparative Formation of AgNP in Bulk CS and CS-NP

In a previous report we focused on the formation of AgNP included inside of CS-NP using CS of low molecular weight and at 100ºC. Under these conditions small AgNP, with sizes between 0.93 and 1.7 nm were synthesized [11]. At that moment we considered that similar results could not be achieved using lower temperatures and CS of higher molecular weights.

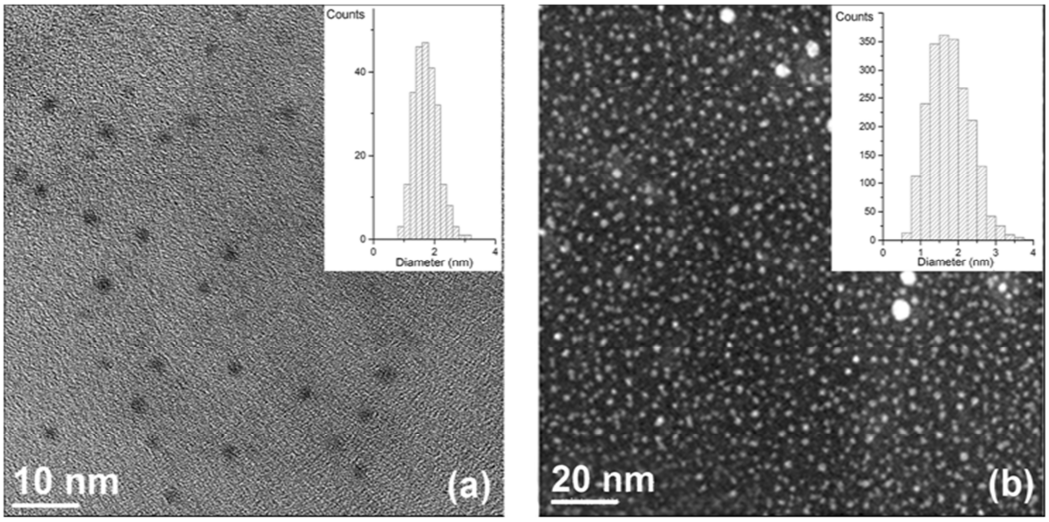

Fig. (1). (a) TEM and (b) HAADF images (inset: size distribution histograms) of two synthesis carried out under different conditions: a) 5 mg of $AgNO_3$ in CSM (d = 1.7 (±0.4)); b) 50 mg of $AgNO_3$ in CSH-NP (d = 1.8 (±0.3)).

However, in this study we decided to carry out a comparative analysis of the formation of small AgNP using bulk CS and CS-NP of higher molecular weights under different conditions. Unexpectedly, small AgNP could also be obtained using bulk CSM and CSH, as determined by TEM analysis. In almost all cases, AgNP with diameters between 1.1 and 1.8 nm were obtained. In Fig.(1)two examples are given, corresponding to the synthesis carried out under the following conditions: a) 5 mg of $AgNO_3$ in CSM (d = 1.7 (±0.4)), and b) 50 mg of $AgNO_3$ in CSH-NP (d = 1.8 (±0.3)). The smallest AgNP, with diameters of 1.1 (±0.5) and 1.5 (±0.3) nm, were obtained using 5 mg of $AgNO_3$ and CSM-NP and CSH-NP, respectively. Only in one case (CSM + 75 mg of $AgNO_3$) larger AgNP were obtained (d = 6.1 (±0.7) nm).

3.2. Formation of CS-NP Without Using a Polyanionas Templating Agent

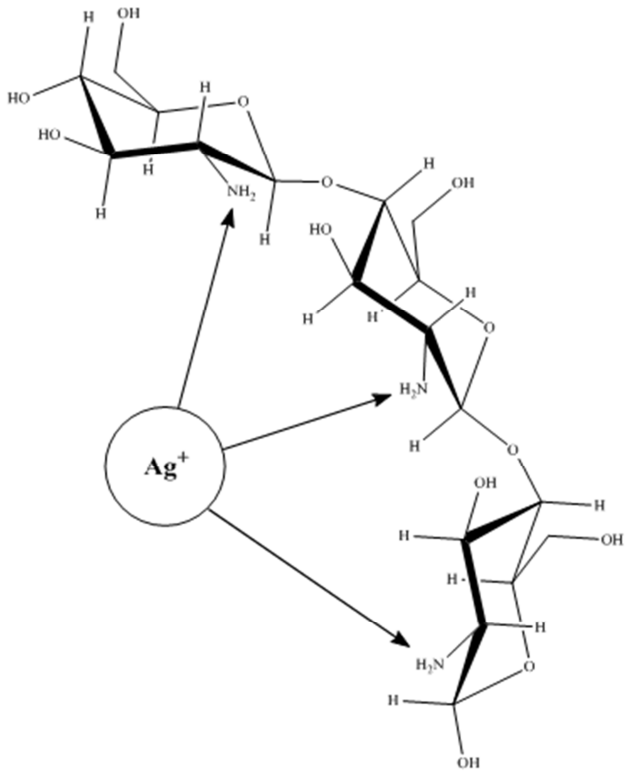

Fig. (2). Representation of the Ag^+ coordination to the amino groups of CS.

In the previous synthesis of CS-NP we used tripolyphosphate (TPP) as a templating agent, modifying earlier reports and considering it the best alternative [11]. We noticed that the obtained CS-NP were larger than when they contained AgNP, a fact that we attributed to the coordination ability of Ag^+ toward the amino groups of CS, but without any other experimental evidence. Only one other report supporting this consideration was found where the authors based their interpretation on FTIR spectra of $AgNO_3$-CS mixtures [25]. Now we decided to synthesize AgNP using bulk CSM (and CSH) as matrices, expecting the formation of the corresponding CS-NP only due to the coordination effect of Ag^+. As expected, the sizes of CSM-NP (245-270 nm) and CSH-NP (250-270 nm) formed during the synthesis of AgNP resulted to be larger than those previously formed with TPP: 270-510 nm and 280-825 nm, respectively. The given diameters actually correspond to average values of CS-NP of two different sizes. In our previous work we obtained CS-NP prepared by the interaction with TPP, inside of which AgNP were formed, with an average diameter of 78 nm (one size). Obviously, the molecular weight of CS strongly influenced the size of the formed CS-NP. Most importantly, from these results it is evident that Ag^+ in a CS matrix can coordinate to the amino groups with the polymer behaving as a chelating agent. Such interaction permits the metallic cations to act as templates and form CS-NP as represented schematically in Fig. (2).

3.3. Synthesis of AgNP in CS Films

The synthesis of AgNP in CS films was considered important by us in order to analyze the possible effects of temperature and, especially, the high viscosity of jellified CS. In the procedure, the amount of $AgNO_3$ which was added varied from 5 to 50 mg mixed with an acetic suspension of bulk CSM (or CSH). CSM-NP and CSH-NP were also used in the formation of the films. The formation of the films was achieved by 4-100 h of evaporation in a vacuum oven at different temperatures (40, 55 and 70ºC).

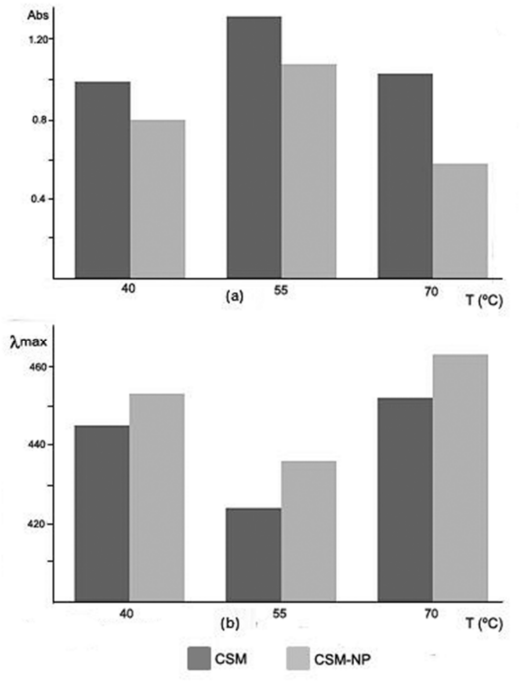

Fig. (3). Bar diagrams representing the average results obtained in the formation of AgNP in CSM and CSM-NP films.

In all cases, the presence of AgNP was observed by spectrophotometric determinations of the surface plasmon resonance (SPR) band (with high intensity) after the film was formed and dissolved in acetic acid solution. In most of the cases, two SPR bands were recorded. The most energetic bands (390-450 nm) corresponded to AgNP obtained with CSM films. When CSM-NP and CSH-NP were used larger AgNP were formed, with maxima of the SPR bands recorded within 430 and 490 nm. The synthesis of the smallest AgNP, with the highest yields, was achieved when using CSM, 10-35 mg of $AgNO_3$, and 55ºC for 4 h (Fig. (3)).

Higher temperatures and small amounts of $AgNO_3$ (5 mg) provoked the formation of AgNP highly dispersed in size, and/or agglomerations. Actually, in most of the cases AgNP with different size distributions obtained as represented in the

TEM images of AgNP (Fig. (4)) formed in a CSM film using 75 mg of $AgNO_3$ and evaporated at 70ºC. Nevertheless, the highest populations of AgNP obtained corresponded to those of diameter lower than 2 nm. The observed agglomeration process should be attributed to the low mobility of the initially formed small AgNP behaving as seeds.

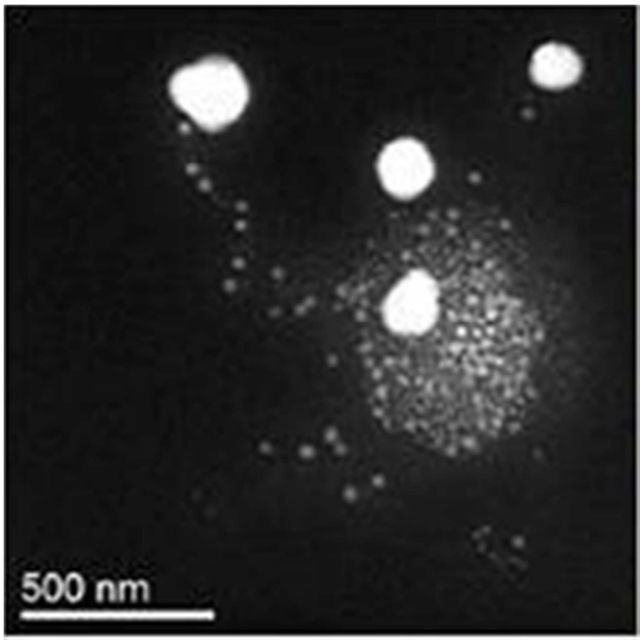

Fig. (4). HAADF image of AgNP formed in the CSM film prepared using 75 mg of AgNO3 and evaporated at 70ºC.

3.4. Antimicrobial Activity

Different AgNP and chitosan-AgNP have been studied mainly as bactericide agents for some bacteria species of clinical importance and, to a lesser extent, as active substances against pathogenic fungi [2]. For this purpose the diverse reports use different semi-quantitative and/or quantitative techniques to determine the biocide activity, which makes it difficult to compare the results obtained.

In our case we applied a standard two fold dilution method which enables not only the Minimal Inhibitory Concentration (MIC), but also the Minimal Microbicide Concentration (MMC) of samples to be established. The antimicrobial activity of the nanocomposites synthesized in this work has been assayed against a wide range of procariotic (Gram-positive and Gram-negative) and eucariotic representative species, involving environmental, foodborne and spoilage microorganisms and also important plant and human pathogens including *Staphylococcus aureus* methicillin-resistant. Firsly, the MICs for the different CS used were established. For CSM, the range was between 6 µg/mL (for *S. epidermidis*) and >200 µg/mL (for *Salmonella* spp.). Meanwhile, for CSH the range varied from 25 µg/mL (*S. epidermidis*) to >200 µg/mL (for *Salmonella* spp.).

The antimicrobial activity of both types of CS-NP was measured as well, oscilating the MICs for the CSM-NP from 12 µg/mL (for *X. campestris*) to >200 µg/mL (for *B. cereus* and yeasts) and for CSH-NP between 50 µg/mL (for *X. campestris* and *E. coli*) and >200 µg/mL (for *K. pneumoniae*, *E. faecalis* and yeasts). Also, the MICs for Ag^+, using $AgNO_3$ aqueous dilutions, was determined and ranged from 6 µ/mL (for *E. coli*) to >24 µg/mL (for *S. cerevisiae*).

The antimicrobial activity of Ag@CSM, Ag@CSH, Ag@CSM-NP and Ag@CSH-NP is shown in Table 1. As can be seen, the antimicrobial activity was dependent on the strain assayed and the nanocomposite used. But in all cases a significant biocide activity was observed with very low MIC values, particularly when compared to other silver nanocomposites [26], in no case higher than 4 µg/mL.

Taking *E. coli* (one of the most assayed species) as example, the tests of susceptibility using different AgNP in previous studies revealed that MICs ranged from 2-75 µg/mL [27, 28]. The values of the MIC and MBC for the strain of *E. coli* used in this study ranged from 0.75-1.5 and from 1.25-3 µg/mL, respectively. It is interesting to emphasize the highest activities achieved for Ag@CSH-NP against *B. cereus* (0.6 µg/mL) and Ag@CSM-NP against *P. pastoris* (0.7 µg /mL).

We assumed that the observed antimicrobial activity is a consequence of a synergistic effect of chitosan and silver. It has been shown that CS and CS-NP have multiple antimicrobial mechanisms [2]. Of key importance, between them, are their ability to enhance cell permeability and to inhibit enzyme reactions by chelating traza elements and nutrients, or binding microbial DNA. On the other hand, AgNP also exert their antimicrobial activity by different mechanisms [2], between them the effects of the released Ag^+ anions that include the formation of holes in the membrane. Once inside the cell they exert many other antimicrobial mechanisms like cytochromes inhibition, binding and damage DNA, denaturing ribosomal subunits or generating free radicals. The fact that the nanomaterials can exert their antimicrobial activity by different mechanisms makes it very difficult for the appearance of microbial resistances which make them attractive antimicrobial drugs.

Previous studies reported that smaller AgNP have a higher antimicrobial activity because they interact more efficiently with the cellular surface, and thus penetrate the cell wall better [27]. In our study, regarding the molecular weight of the chitosan used, the overall tendency observed was that nanocomposites made with CSH are more effective than those prepared with CSM, although the effectiveness was dependent on the strain and the nanocomposite used.

This result could be explained because AgNP are included either in a chitosan matrix or in a chitosan nanoparticle. It has

been shown that chitosan, as a polycation, could interact with anionic groups present in the cell surface increasing its permeability [2]making less important the nanoparticle size. Moreover, silver nanoparticles of larger size than the ones obtained in this work, and also synthetized in high molecular weight chitosan, showed an antimicrobial activity against *S. aureus* comparable to our nanocomposites [29].

Table 1. Biocide activity of new compounds expressed as MIC (MFC/MBC) in µg/mL.

Microorganism	Ag@CSH-NP	Ag@CSH	Ag@CSM-NP	Ag@CSM
Gram-positive				
B. thuringiensis	0.62±0.37 (6±2)	1±0 (4±2)	2.5±1.5 (7±1)	2.5±1.5 (6±0)
B. cereus	0.62±0.37 (1.25±0.75)	1±0 (4±2)	3± 1 (5±1)	1±0 (3±1)
S. aureus	3±1 (5±1)	1±0 (3±1)	3±1 (4±0)	3±1 (4±0)
S. epidermidis	1.25±0.75 (4±2)	0.6±0.3 (3±1)	2.5±1.5 (4±2)	1.25±0.75 (5±1)
Gram-negative				
X. campestris	1.5± 0.5 (3±1)	1.5±0.5 (2±0)	1.5±0.5 (3±1)	1± 0 (2±0)
E. coli	1.5±0.5 (2.5±1.5)	0.75±0.25 (1.25±0.75)	2.5±1.5 (3±1)	1.5±0.5 (3±1)
Salmonella spp.	2± 0 (5±1)	1.25±0.75(2.5±1.5)	3±1 (5±1)	3± 1 (5±1)
K. pneumoniae	1.25±0.75 (1.5±0.5)	1.12±0.87(1.25±0.75)	3±1 (4±2)	2± 0 (4±0)
E. faecalis	1.5± 0.5 (3±1)	1.25±0.75(1.5±0.5)	3±1 (5±1)	2± 0 (4±0)
Yeasts				
C. glabrata	3±1(4±0)	4±0 (4±0)	3±1 (5±1)	4± 0 (5±1)
*P. pastoris*X33	1±0 (3±1)	1.5± 1 (3±1)	0.75±0.25 (4±0)	1± 0 (2±0)
S. cerevisiae	3±1 (4±0)	2±0 (4±0)	3±1 (4±0)	0.75±0.25 (1.5±0.5)

The general tendency observed was that the Gram-negative bacteria resulted to be the most susceptible to the antimicrobial activity of AgNPs, especially considering the MBC. AgNP have been reported to be in general more effective for Gram-negative than for Gram-positive bacteria. This lower susceptibility of Gram-positive bacteria has been attributed to the structure of the cell wall. Gram-positive cell walls contain multiple layers of peptidoglycan when compared to Gram-negative cell walls. Peptidoglycan is a complex molecule that may contain teichoic or lipoteichoic acids which have a strong negative charge which may contribute in capturing the free Ag^+ ions. As a consequence, cell walls of Gram-positive bacteria may allow less Ag^+ ions to reach the plasmatic membrane than Gram-negative bacteria [26] resulting in turn less susceptible to the AgNPs. However, using nanocomposites synthetized in this work, differences in susceptibility between Gram-positive and Gram-negative bacteria are not so apparent, seeming more related to the combination strain/nanocomposited used.

4. Conclusion

Here we have proven that the formation of small AgNP in different types of CS matrices of medium and high molecular weights (in acetic acid solutions, as nanoparticles or and films) is possible under the most diverse conditions, including low temperature and high viscosity. The synthesis of AgNP in CS matrices should be attributed to both the coordinating ability of Ag(I) toward the amino groups of the biopolymer and its reduction capacity. The coordination of Ag(I) to the biopolymer was also expressed in the direct formation of CS-NP without the need of a templating agent. With these results it is evident that the size and form of presentation of CS are not factors that can significantly affect the formation of small AgNP.

The new nanocomposites exhibited a significant antimicrobial activity against a broad range of representative microorganisms (Gram-positive, Gram-negative bacteria and yeasts), including strains resistant to other common drugs. Although the antimicrobial activity was dependent on the strain assayed the overall tendency observed was that the nanocomposites made with CSH are more effective than those prepared with CSM. New silver-based materials that effectively eliminate bacteria and fungi at relatively low concentrations not toxic for humans are of great interest in clinical applications and for food processing. The great inhibition of microbial growth showed by the new nanocomposites studied in this work make them promising compounds with potential to be used in the treatment of important pathogens resistant to routine antimicrobial agents and especially, because of its broad spectrum, as an antimicrobial additive in different materials.

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