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Silver Ions Release from Antibacterial Chitosan Films Containing in Situ Generated Silver Nanoparticles

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ABSTRACT: This study aims to develop antimicrobial films consisting of chitosan and silver nanoparticles that are homogeneously distributed throughout the polymer matrix. Nanoparticles were generated in situ during the neutralization of the chitosan acetate film with sodium hydroxide. The temperature of neutralization and the concentration of silver in the film were crucial determinants of the shape and size of the nanoparticles. Neutralized films exhibited antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* in liquid growth media. However, the effectiveness of the films was considerably greater in diluted growth media. Furthermore, no significant differences were found either in the antimicrobial capacities of films incorporating different amounts of silver or in the amount of silver that migrated into the liquid media after 18 h of immersion of the film. Neutralized films maintained their activity after 1 month of immersion in deionized water, which can be attributed to the slow sustained release of silver ions and thus efficacy over time.

KEYWORDS: *synthesis, silver-based nanoparticles, chitosan, antimicrobial activity*

INTRODUCTION

Silver ions have long been recognized as an effective biocide against a broad spectrum of microorganisms, their inhibition mechanism being the subject of considerable research.[1−3] Ionic silver can exert its antimicrobial action in several ways. It has been reported to complex with the thiol groups of enzymes and proteins, altering their structure and function.[4] Silver ions have also been found to bind with DNA and cause structural changes in the cell envelope and cytoplasmic membrane of bacteria.[5,6] Ionic silver has been used as an antimicrobial in the form of salts, and more recently it has been incorporated into inorganic materials such as zeolites, zirconium phosphate, and glass.[7,8] A new approach is the development of silver nanoparticles that can act as nanoreservoirs for the delivery of silver ions, ensuring their availability in the substrate over time. There is a great variety of chemical and physical processes for the synthesis of silver nanoparticles, most of them involving the formation of colloidal nanoparticles or their incorporation in other materials.[9,10] In this regard, increasing attention is being paid to the incorporation of silver nanoparticles in polymer matrices for the development of antimicrobial films and coatings. The antimicrobial capacity of the composites formed will depend on the physical and chemical properties of the nanoparticles and also the silver ion release properties of the carrier matrix. There is also great interest in the use of synthetic and natural hydrophilic polymers and hydrogels as carriers of silver. These materials absorb large amounts of water, owing to the presence in their structure of polar groups (−OH, $-NH_2$, $-CONH_2$, −COOH, ...). Hydrophilic polymers are capable of swelling in a moist environment, thus facilitating the diffusion of the active agent through the polymer matrix and its release to the medium in contact with the polymer.

Chitosan is a biodegradable and biocompatible polymer obtained from biomass and possesses excellent film-forming properties, which have made it of great interest for technological applications in several areas such as pharmacy, medicine, agrochemistry, and packaging. The hydrophilic nature of chitosan has aroused interest in its use as a sustained release carrier when the release of the retained active compound is required in a moist environment. Inclusion of silver-based nanoparticles as an antimicrobial nanofiller in a chitosan matrix could be done directly or by using silver nitrate as a precursor. Chitosan is soluble in aqueous solution and has the ability to bind many metal ions, including silver, via chelation with the amine groups. Chitosan has also been reported to be a mild reducing agent used for reduction of silver ions, and it is frequently employed as an ion capping agent to control the growth of nanoparticles and avoid their aggregation.[11]

The aim of this study, therefore, was to develop films for the slow, sustained release of silver ions, consisting of chitosan polymer as the carrier matrix filled with silver-based nanoparticles synthesized in situ. The resulting films were characterized and the antimicrobial activity was tested in liquid growth media. The ability of the carrier system to exert antimicrobial activity over time was also studied.

MATERIALS AND METHODS

Synthesis of Chitosan/Silver-Based Nanoparticle Films. Low molecular weight chitosan (MW 50−190 kDa, 75−85% deacetylated) from shrimp shells, sodium hydroxide (ACS reagent, ≥97.0%, pellets), and silver nitrate (ACS reagent, ≥99.0%) were obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, USA. A 1.5% (w/w) chitosan solution was prepared in 0.5% (w/w) acetic acid and stirred at 40 °C for 1 h. After the solution had cooled to room temperature, silver nitrate, previously diluted with a small amount of distilled water, was added to the solution and left shaking, protected from light, until complete dissolution. Several chitosan solutions with different silver

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nitrate concentrations were prepared, corresponding to silver concentrations of 0.1, 0.2, 0.5, 1, and 1.5% (g/100 g chitosan). Films were formed by casting on polystyrene plates and dried at 37 °C with a relative humidity of 22% for 48 h. The chitosan acetate films were neutralized with sodium hydroxide to make them insoluble in water at a pH above the pK_a of chitosan. Hydroxyl ions also accelerate the reduction reaction of silver ions and the formation of silver-based nanoparticles by increasing the reducing power of chitosan.

For this purpose, the films were immersed in a solution of 0.1 M sodium hydroxide for 20 h in a thermostatic chamber and protected from light. The effect of the neutralization temperature on the formation of nanoparticles was studied by assaying two temperatures, 22 and 37 °C. After neutralization, the films were washed with deionized water and dried in an oven at 37 °C. Finally, the films were stored in a glass desiccator at 22 °C and 0% relative humidity prior to use.

Characterization of Chitosan/Silver-Based Nanoparticle Films. *Film Color.* The color of neutralized chitosan film was measured using a Konica Minolta CM-3500d spectrophotometer set to D65 illuminant/10° observer. Film specimens were measured against the surface of a standard white plate, and the CIELAB color space was used to obtain the color coordinates L^* (lightness) [black (0) to white (100)], a^* [green (−) to red (+)], and b^* [blue (−) to yellow (+)]. The color was expressed using the polar coordinates $L^*C^*h^\circ$, and ΔE^*, where L^* is the same as above, C^* is chroma, h° is hue angle, and $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Eight measurements were taken of each sample, and three samples of each film were measured.

Thermogravimetric Analysis (TGA). TGA of chitosan films neutralized at 37 °C was carried out using a Mettler Toledo TGA/SDTA/851. Samples of approximately 10 mg were heated from room temperature to 800 °C at 10 °C/min and held at an isotherm for 3 min. Nitrogen was used as the carrier gas. The TGA data were plotted as the weight percentage versus temperature, and the decomposition temperature was obtained from the first derivative of weight loss curve (DTG).

UV−Visible Spectroscopy. The particles generated in chitosan films neutralized at 37 °C were characterized by testing their optical absorption with an Agilent 8453 UV−vis diode array spectrophotometer.

Transmission Electron Microscopy (TEM). The morphology of the nanoparticles generated in chitosan films neutralized at 37 °C was studied using a JEOL-1200 EX transmission electron microscope. The morphology of the nanoparticles generated in films neutralized at 37 °C was studied using a JEOL-1200 EX transmission electron microscope at an acceleration voltage of 100 kV. TEM specimens were cut from films using a Porter−-Blum MT-2B ultramicrotome equipped with a diamond knife to give around 70 nm thick sections and placed on 300 mesh copper grids. Images were obtained with a digital micrograph acquisition software (Gatan, Inc., 2007).

X-ray Diffraction (XRD). The XRD patterns of chitosan films neutralized at 37 °C were recorded using a Bruker AXS D500 spectrometer with a Bragg−Brentano geometry at a wavelength of 1.5406 (corresponding to the peak Cu Kα). X-ray diffractograms were recorded in a diffraction angle (2θ) range of 5−80° using a step size of 0.02° and an exposure time of 2 s.

Antimicrobial Activity of Films Neutralized at 37 °C. *Staphylococcus aureus* ATCC 12600 and *Escherichia coli* ATCC 25922 were obtained from the Spanish Type Culture Collection (Valencia, Spain). Strains were stored in tryptone soy broth (TSB, Scharlab, Barcelona, Spain) with 20% glycerol at −80 °C until needed. For experimental use, the stock cultures were maintained by regular subculture on tryptone soy agar (TSA, Scharlab) slants at 4 °C and transferred monthly. In a first step a loopful of each strain was transferred to 10 mL of TSB and incubated at 37 °C for 18 h to obtain early stationary phase cells. Cell cultures of each microorganism in stationary phase, with an optical density of 0.9 at 600 nm, were diluted in TSB and incubated at 37 °C until an optical density of 0.2 at 600 nm (10^5 CFU/mL) was reached. Tubes with 10 mL of Mueller−Hinton broth (MHB, Scharlab) and 1:125 diluted MHB were inoculated with 100 μL of the exponential phase culture of each microorganism. Only films neutralized at 37 °C were tested. Previously, a set of films were first autoclaved to study the effect of moist heat at >100 °C on their antimicrobial activity. This study was done because some silver ions could remain in the matrix after the formation of nanoparticles and hydrothermal treatment could lead to chemical reduction and possibly modify their antimicrobial activity. Samples weighing 0.25 g were then cut into 1.5 cm^2 pieces and added to each tube. A control film of neutralized chitosan without the incorporation of silver nitrate was used as a blank in each experiment. The tubes were then incubated at 37 °C for 18 h. Depending on the turbidity of the tubes, serial dilutions with peptone water were made and plated in Petri dishes with 15 mL of TSA culture medium. Colonies were counted after incubation at 37 °C for 18 h.

Antimicrobial Activity of Films Neutralized at 37 °C in Liquid Media over Time. Three sets of experiments were carried out with films having 0.2 and/or 1.5% (w/w) of silver and neutralized at 37 °C to study the antimicrobial capacity of the films over time. The first experiment studied how the immersion time of the film in the growth medium prior to inoculation with bacteria affects its antimicrobial activity. For this purpose, 0.25 g of film was immersed in 10 mL of 1:125 diluted MHB for 0, 2, 10, 24, 48, 168, or 360 h, after which bacteria were inoculated in the tubes and the antimicrobial activity was evaluated as described above.

In the second experiment, we determined the antimicrobial activity of the culture medium in which the film was previously immersed. To do this, 0.25 g of film was immersed in 10 mL of 1:125 diluted MHB, the old culture medium being replaced every 24 h with fresh medium. The antimicrobial capacity of the replaced culture medium was assayed at 1, 3, 5, 10, 15, and 30 days. After 30 days, the antimicrobial capacity of the film was also tested in fresh culture medium as described above.

In the third experiment, films were immersed in 200 mL of sterile distilled water for a month. During this period the water was periodically refreshed to avoid microbial contamination. The antimicrobial activity of the films was evaluated at 1 and 30 days; films were put in tubes with 10 mL of 1:125 diluted MHB and inoculated with *S. aureus*. The antimicrobial activity of the films was studied as described previously.

Release of Silver into the Liquid Culture Medium. Migration of silver from films neutralized at 37 °C to the culture growth medium was studied by immersion of 0.25 g of films of different silver concentrations comprising 0.1, 0.2, 0.5, 1, or 1.5% (g/100 g chitosan) in 10 mL of 1:125 diluted MHB for a period of time at 37 °C, incubation conditions resembling those of microorganism growth. After this time, the media were diluted with 0.5% HNO_3 and the concentration of silver in the samples was quantified by graphite furnace atomic absorption spectroscopy (GFAAS) with a longitudinal AC Zeeman (Analyst 600, Perkin-Elmer, Madrid, Spain) equipped with a transversely heated graphite atomizer and a built-in, fully computer-controlled AS-800 autosampler (Perkin-Elmer). The furnace program [temperature (°C)/ramp time (s)/hold time (S)] employed for silver determination was as follows: drying (90 °C/10 s/20 s; 120 °C/10 s/20 s; 130 °C/5 s/40 s; 300 °C/5 s/5 s); pyrolysis (500 °C/10 s/20 s); cooling (20 °C/10 s/20 s); atomization (1400 °C/0 s/5 s); cleaning (2450 °C/1 s/5 s). Twenty microliters of sample with 10 μL of matrix modifier (0.05 mg of Pd and 0.003 mg of $Mg(NO_3)_2$) was injected. Triplicate analyses of three independent samples were performed for each defined time.

RESULTS AND DISCUSSION

In Situ Synthesis of Silver-Based Nanoparticles in a Chitosan Film. Films were successfully developed, based on silver nitrate as a nanoparticle precursor and chitosan acting as a polymer carrier and mild silver ion reducing agent. The amino groups of chitosan serve as ligands to complex silver ions at near-neutral pH. To a lesser extent, this polymer can also form complexes with hydroxyl groups. This makes chitosan a chelating polymer with excellent adsorption capacities for silver ions in the preparation for the formation of silver nanoparticles.

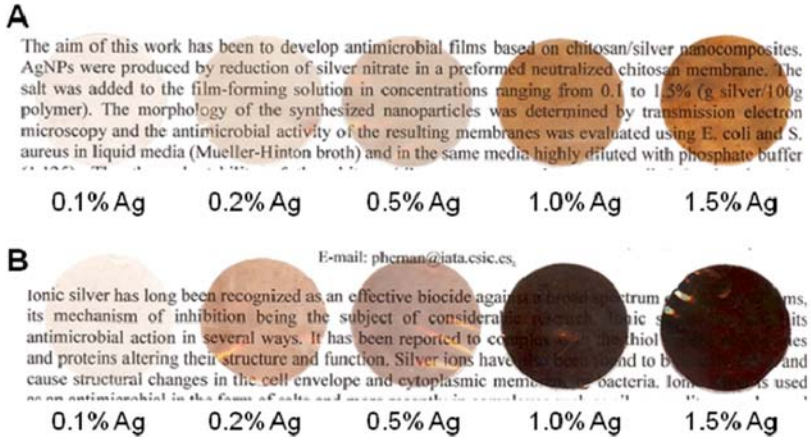

Figure 1. Chitosan/silver-based nanoparticle films with different concentrations of silver: (A) neutralized at 22 °C; (B) neutralized at 37 °C.

Table 1. Color Parameters of Chitosan Films with Different Concentrations of Silver Neutralized at 22 and 37 °C[a]

	Ag (%)	L^*	a^*	b^*	C^*	$h°$	ΔE^*
control	0.0	94.27 ± 0.82 a	−0.61 ± 0.05 a	7.10 ± 0.71 a	7.13 ± 0.71 a	94.91 ± 0.60 a	
	0.1	88.71 ± 0.44 b	−0.62 ± 0.03 a	5.82 ± 0.19 b	5.85 ± 0.18 b	96.11 ± 0.44 b	5.71 ± 0.39 a
	0.2	75.10 ± 1.47 c	2.28 ± 0.84 b	12.57 ± 0.96 c	12.78 ± 1.09 c	79.72 ± 1.03 c	20.14 ± 1.66 b
films neutralized at 22 °C	0.5	70.84 ± 2.11 d	4.40 ± 0.86 c	15.50 ± 1.14 d	16.11 ± 1.22 d	74.15 ± 0.76 d	25.39 ± 2.32 c
	1.0	43.01 ± 1.28 e	7.50 ± 0.09 d	21.00 ± 0.85 e	22.30 ± 0.89 e	70.35 ± 1.14 e	53.73 ± 1.31 d
	1.5	41.43 ± 1.97 e	10.31 ± 1.72 e	26.00 ± 1.73 f	27.97 ± 1.98 f	68.38 ± 0.58 f	57.17 ± 1.42 e
control	0.0	94.27 ± 0.82 a	−0.61 ± 0.05 a	7.10 ± 0.71 a	7.13 ± 0.71 a	94.91 ± 0.60 a	
	0.1	86.06 ± 0.36 b	0.13 ± 0.15 b	8.21 ± 0.81 b	8.21 ± 0.81 b	89.08 ± 1.04 b	8.32 ± 0.43 a
	0.2	64.06 ± 0.83 c	2.75 ± 0.83 c	15.90 ± 0.47 c	16.14 ± 0.49 c	80.19 ± 1.35 c	31.65 ± 0.91 b
films neutralized at 37 °C	0.5	55.37 ± 1.92 d	4.81 ± 0.55 d	14.00 ± 0.69 d	14.80 ± 0.71 d	71.04 ± 2.02 d	39.88 ± 1.85 c
	1.0	31.32 ± 0.92 e	2.00 ± 0.53 e	6.03 ± 0.48 e	6.35 ± 0.51 e	71.64 ± 1.99 d	63.02 ± 0.91 d
	1.5	26.66 ± 0.74 f	1.00 ± 0.12 f	4.18 ± 0.31 f	4.29 ± 0.30 f	76.53 ± 2.01 e	67.69 ± 0.73 e

[a]Values within a column followed by a different lower case letter are significantly different from each other comparing different amounts of silver in the films neutralized at 22 or 37 °C (Tukey's adjusted analysis of variance $P < 0.05$).

This polysaccharide has also been described as a weak reducing agent owing to the presence in their structure of the organic compounds aldehyde, ketone, and alcohol. The presence of sodium hydroxide accelerated the reduction rate of silver ions[12] and, thus, the formation of silver nanoparticles in the film. However, the coexistence of elementary silver and silver oxide nanoparticles is expected, owing to the presence of sodium hydroxide.

Treatment with sodium hydroxide allows neutralization of amino groups in chitosan film, promoting the integrity of the resulting films in aqueous medium at a pH above the pK_a of chitosan. This less toxic method for the synthesis of nanoparticles avoids the employment of commonly used toxic reducing agents.

Chitosan/Silver-Based Nanoparticles Films. Films neutralized at 37 °C were successfully developed with nominal concentrations of silver between 0.1 and 1.5%. Higher concentrations of silver greatly increased the viscosity of the film-forming solution, giving rise to the formation of a gel. The films were homogeneous to the naked eye, and their thickness ranged between 55 and 65 μm. Figure 1 shows a photograph of films with different concentrations of silver, neutralized at 22 or 37 °C. The incorporation of silver nitrate produced films with a different color after the neutralization step. It can be observed that transparency decreased and the films acquired a reddish brown tone as the concentration of silver and the neutralization temperature increased. Color coordinates of the films are shown in Table 1.

Chitosan−silver nitrate films neutralized at 22 °C experienced an increase in chroma (C^*) and color difference (ΔE) and a decrease in hue angle ($h°$) and lightness (L^*) compared to un-neutralized films. As the concentration of silver increased, the films experienced a decrease in C^*, $h°$, lightness, and ΔE. These changes were more acute for films neutralized at 37 °C, indicating a greater conversion of silver nitrate into silver-based nanoparticles. The C^* values of the films neutralized at 37 °C also increased with silver; however, C^* values of 1 and 1.5% silver films were lower than the control and films became opaque.

Because a higher neutralization temperature ensures a greater conversion of silver nitrate into silver-based nanoparticles, studies of the thermal stabilities of the films and their antimicrobial activities, along with characterization of the nanoparticles formed in the films, were undertaken only with those neutralized at 37 °C.

Thermogravimetric Analysis. Figure 2 shows the effect of silver content in 37 °C neutralized chitosan films on the first derivative of weight loss curves (DTG). The initial thermal decomposition of films neutralized at 37 °C happened at a slightly higher temperature than the film prepared without silver. It can be seen in the DTG_{max} curves that the maximum decomposition temperature of chitosan appeared at 288 °C,

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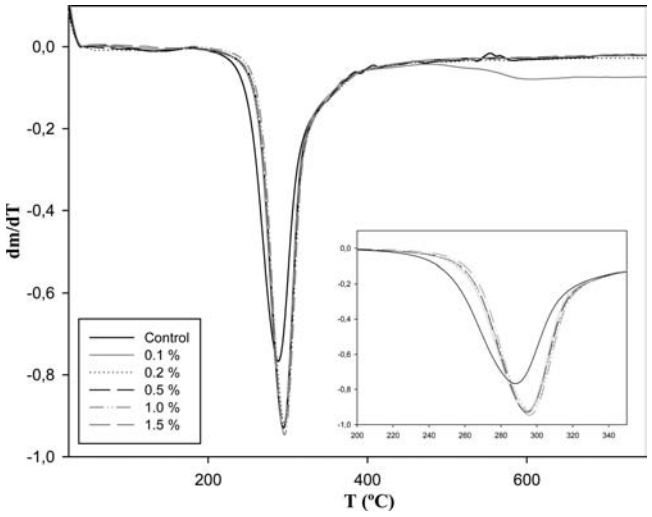

Figure 2. Effect of silver content in 37 °C neutralized chitosan films on the first derivative of weight loss (DTG) curves. (dm/dT = first derivative of weight loss vs time.)

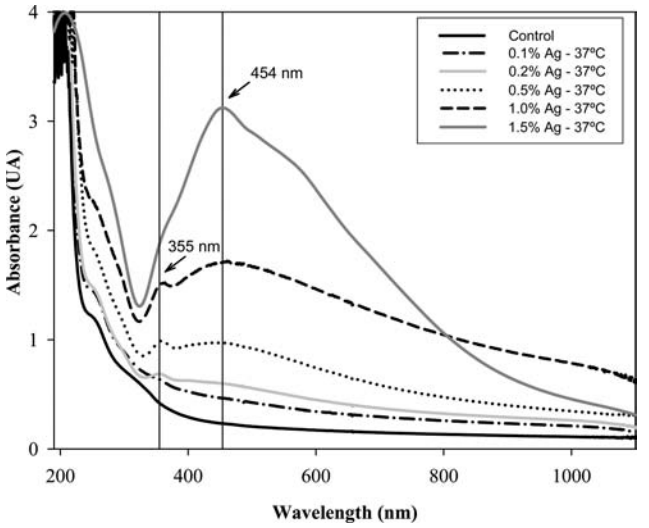

Figure 4. UV−visible spectrum of chitosan films with 0, 0.1, 0.2, 0.5, 1, and 1.5% of silver neutralized at 37 °C.

whereas for film with silver-based nanoparticles the peak shifted to a higher temperature, indicating that silver-based nanoparticles increase the thermal stability of the films.

Transmission Electron Microscopy Studies. TEM was used to study the size and shape of the nanoparticles generated in films with 0.1, 0.2, 0.5, 1, and 1.5% of silver. Panels A and B of Figure 3 show TEM images of neutralized chitosan films with silver concentrations of 0.2 and 1.5%, respectively. The formation of nanoparticles with a spherical morphology and sizes ≤5 nm, distributed homogeneously throughout the polymer matrix, was observed for films having 0.1−0.5% of silver. Films possessing silver concentrations of 1 and 1.5% displayed the formation of spherical nanoparticles of 5−10 nm in size and a second population of round-shaped, anisotropic nanoparticles with diameters ranging from 30 to 50 nm. These larger nanoparticles are probably aggregates formed from smaller ones.

UV−Visible Analysis. Figure 4 shows the UV−visible spectra of chitosan films incorporating various concentrations of silver. The figure depicts two spectral bands at 355 and 454 nm. Under conditions in which one-electron reduction of metal ions occurs predominantly, the subsequent aggregation of the resulting atoms and ions gives more or less complex small clusters and then quasimetallic particles. These species are associated with the presence of bands around 350 nm.[13]

The conduction electrons on the surface of metallic nanoparticles undergo a collective oscillation when stimulated with incident light. This oscillation is known as a surface plasmon resonance (SPR) and results in strong scattering and absorption properties. Silver nanoparticles have a characteristic surface plasmon band around 400 nm in the UV−visible spectrum. SPR of spherical silver nanoparticles is responsible for the band appearing at 454 nm in Figure 4 for films neutralized at 37 °C. It can be observed that this band became stronger, with a large asymmetrical broadening, as the silver concentration in the film increased. This band shape has been associated with the deviation of nanoparticles from a perfect spherical shape and an increase in size distribution, which has been previously observed in TEM studies. The presence of a shoulder at 570 nm can be observed, a phenomenon that has also been reported by other authors. According to Mie's theory, small spherical nanoparticles should exhibit a single surface plasmon band, whereas anisotropic particles should exhibit more than one band, depending on their shape.[14] Spherical metallic nanoparticles give a symmetrical intense band close to 400 nm, whereas the presence of silver oxide in metallic nanoparticles gives broader, less intense bands that shift to red.[15] In this work, the absence of well-defined bands could also be associated with the formation of silver oxide during neutralization with sodium hydroxide. The typical band associated with the SPR of silver nanoparticles is not observed

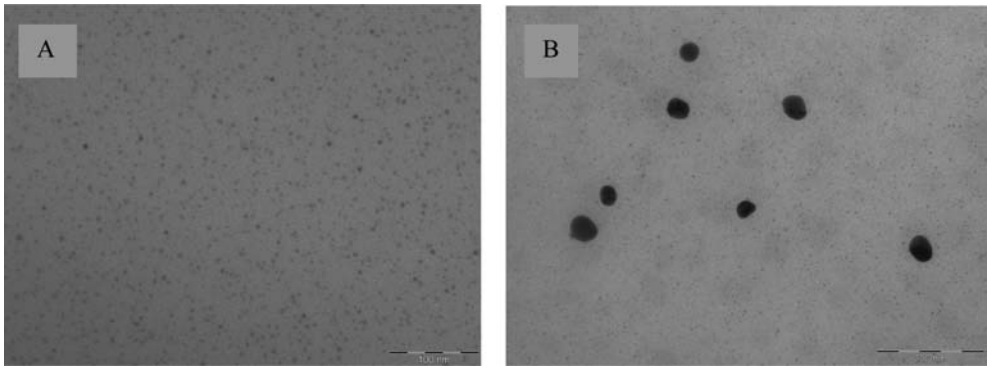

Figure 3. TEM images of films containing (A) 0.2% and (B) 1.5% of silver and neutralized at 37 °C.

in the UV−visible spectra of films with 0.1 and 0.2% of silver, which is probably due to the formation of small nanoparticles greatly dispersed in the matrix.

Structural Characterization. The crystal structure of chitosan films neutralized at 37 °C was determined by XRD. Figure 5 shows the XRD patterns of films incorporating

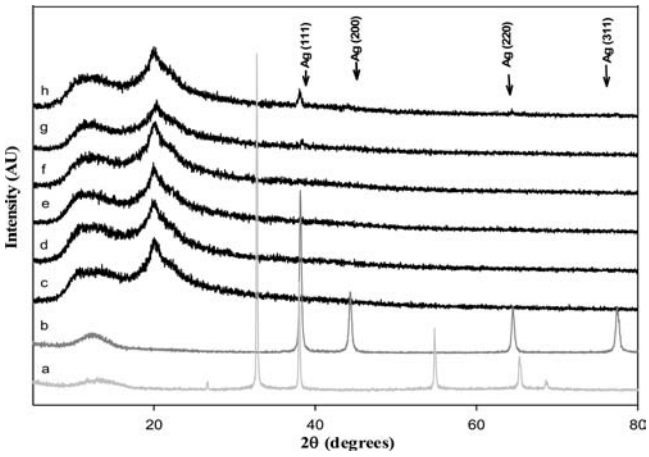

Figure 5. X-ray diffraction spectra: (a) silver oxide; (b) silver; (c) chitosan neutralized at 37 °C; (d−h) chitosan neutralized at 37 °C with 0.1% (d), 0.2% (e), 0.5% (f), 1% (g), and 1.5% (h) of silver.

different amounts of silver and neutralized at 37 °C and also the powder diffraction pattern of metallic silver and silver oxide. The typical XRD pattern of commercial silver nanoparticle powder (diameter < 100 nm) includes four diffraction peaks at 2θ of 38.4°, 44.5°, 64.7°, and 77.6°, corresponding respectively to the [111], [200], [220], and [311] planes of the face-centered cubic (fcc) structure of silver. The powdered silver oxide sample possesses a simple cubic structure with diffraction peaks at 2θ of 32.7°, 38°, 54.7°, and 65.2°, assigned to the reflections from the [111], [200], [220], and [311] planes.

As Figure 5 shows, the XRD pattern of the neutralized chitosan film has a broad peak at 2θ of 20°, indicating low levels of crystallinity, and this peak was also observed in films neutralized at 37 °C. Only one diffraction peak of low intensity, at 2θ = 38.04°, was found in films containing 1.5% of silver. This diffraction peak might be associated with the [111] plane of metallic silver or the [200] plane corresponding to powdered silver oxide, because their positions are very close. However, the greatest intensity peak, at 2θ = 32.7°, corresponding to the [111] reflection plane of powdered silver oxide, was not observed. In fact, faint, diffuse peaks associated with the crystalline structure of metallic silver can hardly be seen. The shape of these peaks suggests the presence of small crystalline silver nanoparticles.

Antimicrobial Activity of Films Neutralized at 37 °C. The antimicrobial capacity of the resulting films neutralized at 37 °C was evaluated against *S. aureus* and *E. coli*. It is well-known that chitosan with deprotonated amino groups does not exhibit antimicrobial activity.[16] In this work, neutralized chitosan films without silver acted as a control. Thus, the antimicrobial properties of the films were expected to be due to the release of silver ions from the nanoparticles embedded in the chitosan matrix, which acted as a support for silver-based nanoparticles.

Table 2 shows the antimicrobial capacity of films neutralized at 37 °C with a silver concentration ranging from 0.1 to 1.5% against the pathogen microorganisms *S. aureus* and *E. coli*, using MHB or 1:125 diluted MHB as culture medium. It is noteworthy that the antimicrobial capacity of the films did not change after autoclaving. Some authors have reported that hydrothermal treatments such as autoclaving can produce nanoparticles from silver ions.[17,18] In our experiment, nanoparticles were supposed to be generated during the neutralization step, without free silver ions remaining in the matrix.

When MHB was used, films containing 0.1% silver produced reductions of 0.62 log for *S. aureus* and 0.88 against *E. coli*, whereas for higher silver concentrations the growth of microorganisms was reduced by about 2 log. The antimicrobial activity of the films increased significantly when microbiological tests were carried out in 1:125 diluted MHB, giving an approximate reduction of 3 log cycles of viable cells for each microorganism tested. The lower antimicrobial capacity of the films in MHB compared with the films in diluted MHB might be due to excess proteins in the culture media chelating the

Table 2. Antimicrobial Activity of Nonautoclaved and Autoclaved (AUTO-) Chitosan Films Neutralized at 37 °C against *S. aureus* and *E. coli* in Mueller−Hinton Broth (MHB) and Diluted MHB (1:125)[a]

		bacterial count (log CFU/mL)			
	Ag (%)	MHB	AUTO-MHB	MHB (1:125)	AUTO-MHB(1:125)
S. aureus	control	8.34 ± 0.37 a	8.34 ± 0.37 a	7.45 ± 0.23 a	7.45 ± 0.23 a
	0.1	7.65 ± 0.52 b	7.49 ± 0.21 b	4.78 ± 0.69 b	4.97 ± 0.03 b
	0.2	5.74 ± 0.40 c	6.52 ± 0.17 c	4.50 ± 0.17 b	4.74 ± 0.10 b
	0.5	6.44 ± 0.24 c	6.32 ± 0.06 c	4,25 ± 0.26 b	4.60 ± 0.12 b
	1.0	5.79 ± 0.40 c	6.35 ± 0.11 c	4,29 ± 0.13 b	4.53 ± 0.29 b
	1.5	6.25 ± 0.19 c	6.20 ± 0.20 c	4,28 ± 0.21 b	4.31 ± 0.45 b
E. coli	control	8.16 ± 0.14 a	8.16 ± 0.14 a	7.31 ± 0.54 a	7.31 ± 0.54 a
	0.1	7.28 ± 0.09 b	7.42 ± 0.14 b	4.90 ± 0.00 b	5.10 ± 0.25 b
	0.2	6.78 ± 0.02 c	6.29 ± 0.03 c	4.32 ± 0.32 b	4.28 ± 0.44 b
	0.5	6.26 ± 0.05 c	5.33 ± 0.06 c	3.57 ± 0.30 b	4.12 ± 0.30 b
	1.0	6.44 ± 0.08 c	5.52 ± 0.04 c	3.79 ± 0.70 b	3.89 ± 0.05 b
	1.5	6.02 ± 0.19 c	6.18 ± 0.36 c	3.89 ± 0.29 b	4.08 ± 0.19 b

[a]Values within a column followed by a different lower case letter are significantly different from each other comparing different amounts of silver in the films against *S. aureus* or *E. coli* (Tukey's adjusted analysis of variance $P < 0.05$).

released silver ions. This could decrease the availability of free silver ions to exert antimicrobial activity.

Although we observed a slight tendency for the antimicrobial activity of the films to increase as their silver concentration increased, no significant differences were found in the antimicrobial capacity of films incorporating different amounts of silver ranging from 0.1 to 1.5% in MHB diluted to 1:125. This behavior might be due to a similar quantity of silver ion migrating to the medium. To confirm this hypothesis, the silver concentration in diluted MHB after 18 h of being in contact with films incorporating different amounts of silver was evaluated by GFAAS. The release of silver proved to be similar in all of the samples. The silver concentration in the medium ranged from 135 to 150 μg/L, and there were no differences ($P \geq 0.05$) in migration values between films of different silver concentrations. Although the films released a similar amount of silver after 18 h of contact with the test environment, the kinetics of silver ion migration may differ between films and thus affect their antimicrobial activity. This might explain slight but nonsignificant differences in the antimicrobial effectiveness of the films.

At present, the mechanism of action of silver is not clear. When silver ions are inside the bacterial cell, this causes condensation of the DNA molecule, which loses its ability to replicate, thus affecting cell viability.[19] The silver ions also interact with thiol groups of proteins, causing bacterial enzyme inactivation.[4] The entry of silver ions through cell walls can cause deposition of proteins in cells.[19] Other authors claim that silver ions affect only the membrane surface, activating a bacterial defense mechanism.[20] The presence of silver ions has an antimicrobial effect, but some authors also suggest that small nanoparticles might be bactericidal.[21−23] The mechanism of action by which silver nanoparticles have an antimicrobial effect is mainly due to their adhesion to the cell membrane, altering its permeability and attacking the respiratory chain,[24] but they can also penetrate inside bacteria and release silver ions, which interact with thiol groups and/or phosphates of compounds such as bacterial DNA or protein. Silver nanoparticles show a clear antimicrobial capacity compared with silver salts because of their high specific surface, which allows a greater area of contact with microorganisms. Studies show that the antibacterial effect of silver nanoparticles depends on their size, with those between 1 and 10 nm presenting a more direct interaction with bacteria.[20]

In this work, we did not expect migration of nanoparticles from the film, given their confinement in the chitosan matrix and the difficulty for them to diffuse through the polymer. However, generation of silver ions on the surface of the nanoparticles embedded in the chitosan matrix is expected. Diffusion of silver ions through the polymer matrix has been probed, this being encouraged by the hydrophilic nature of chitosan. Swelling of chitosan by water facilitates the mobility of the polymer chains and therefore the transport of ions through the matrix to the release media.

Table 2 shows that the films had a slightly higher antimicrobial capacity against *E. coli* than against *S. aureus*. Studies by TEM energy dispersive X-ray showed that the morphological changes that occur in the internal structure of the cell are similar in both types of bacteria, such that it loses its ability to replicate and proteins are inactivated by interaction with silver ions.[19] However, although similar morphological changes were observed in both organisms, they were less marked in *S. aureus*, which is attributed to differences in the cell wall of the two microorganisms. In this case *S. aureus* has a more effective defense system than *E. coli* because Gram-positive cell walls have a thicker peptidoglycan layer than Gram-negative cell walls. It has been reported in the bibliography that the peptidoglycan cell wall has a greater capacity to protect the cell from the penetration of silver ions and small nanoparticles into the cytoplasm.[9]

Antimicrobial Activity of Films Neutralized at 37 °C in Liquid Media over Time. Three studies were carried out to determine the transfer of silver to the media. In the first study, we studied how the immersion time of the film in the growth medium prior to inoculation with bacteria affects its antimicrobial activity. For this purpose, films with a silver concentration of 0.2% were immersed in 1:125 diluted MHB and stored in an incubator at 37 °C for 1, 2, 10, 24, 48, 168, and 360 h prior to inoculation of the microorganism. Table 3 shows the antimicrobial activity of diluted MHB (1:125) against *S. aureus* after being in contact with 0.2% silver films neutralized at 37 °C as a function of the immersion time. As can be seen, the antimicrobial effect of the films did not vary over time. After 24 and 360 h in contact with the culture medium, films showed log reductions of 3.38 and 3.80 log, respectively. According to the migration values obtained for these films, after 10 h of immersion in the medium, the amount of total silver (both ionic and elemental) in the medium increased with time: levels of 58, 60, 57, 135, 170, 256, and 354 μg/L silver were found in the growth medium after 1, 2, 10, 24, 48, 168, and 360 h, respectively. However, the antimicrobial capacities of the films did not change. These results could be explained by the fact that the amount of free silver ions available in the medium would be lower than the amount of total silver. Some of the migrating silver ions might be reduced to elemental silver over time or might not be available because of their interaction with proteins present in the medium.

Table 3. Antimicrobial Activity of Diluted MHB (1:125) That Has Been in Contact with Films Incorporating 0.2% of Silver for Various Times[a]

	S. aureus
time (h)	bacterial count (log CFU/mL)
0	7.02 ± 0.30 a
1	3.63 ± 0.13 b
2	3.70 ± 0.71 b
10	3.22 ± 0.17 b
24	3.50 ± 0.06 b
48	3.50 ± 0.16 b
168	3.36 ± 0.09 b
360	3.19 ± 0.32 b

[a]Values within a column followed by a different lower case letter are significantly different from each other (Tukey's adjusted analysis of variance $P < 0.05$).

In the second study, films with 0.2 and 1.5% silver were immersed in 1:125 diluted MHB, the medium being replaced with fresh medium every 24 h to avoid possible saturation of the system. The medium was collected at 1, 3, 5, 10, 15, and 30 days, and the antimicrobial activity was evaluated against *S. aureus*. After 30 days, the antimicrobial activity of the films was also evaluated. Table 4 shows that the antimicrobial capacity of the culture medium where 0.2 or 1.5% silver films were immersed produced a reduction of 3.5 log, and this activity was maintained throughout the 30 days of the test. Films with 0.2

Table 4. Antimicrobial Activity of Diluted MHB (1:125) That Has Been in Contact with Films Containing 0.2 or 1.5% Silver for 24 h, the Medium Being Replaced with Fresh Medium Every 24 h for a Total Period of 30 Days, and Antimicrobial Activity of These Films after 30 Days[a]

	bacterial count (log CFU/mL)	
time (days)	0.2% Ag	1.5% Ag
control	7.39 ± 0.27 a	
1	4.53 ± 0.31 b	4.28 ± 0.21 b
3	4.84 ± 0.26 b	3.88 ± 0.13 b
5	4.52 ± 0.41 b	3.54 ± 0.65 b
10	4.95 ± 0.95 b	4.14 ± 0.76 b
15	4.62 ± 0.45 b	4.02 ± 0.01 b
30	4.74 ± 0.35 b	4.28 ± 0.33 b
30 film	3.63 ± 0.24 c	2.87 ± 0.04 c

[a]Values within a column followed by a different lower case letter are significantly different from each other (Tukey's adjusted analysis of variance $P < 0.05$).

and 1.5% silver were tested after 30 days and produced an inhibition of 3.8 and 4.5 log, respectively. The antimicrobial activity of the films was slightly higher than that of the liquid. This could be explained by assuming that the films exert antimicrobial activity by the release of silver ions to the medium but also by direct contact of the film surface containing ionic silver with the microorganism. It is worth noting that the antimicrobial activity of 1.5% silver films which were in contact with the medium for 30 days was almost 1 log higher than that of fresh films. This result shows that the release of silver ions from the film had not slowed after 30 days. In addition, the immersion of 1.5% film in liquid medium for 30 days might promote the formation of a large amount of silver ions on the surface of the nanoparticles embedded in the chitosan matrix compared with the fresh films that were tested, giving rise to a greater migration of silver ions.

The third study was conducted to verify the long-term antimicrobial capacity of the films after immersion in liquid medium; 0.2 and 1.5% silver films neutralized at 37 °C were immersed in an excess of sterile distilled water at a temperature of 22 °C. The water was replaced with fresh water every 3 days. The antimicrobial capacity of the films was tested at day 1 and after 1 month, and the results are shown in Table 5. The antimicrobial capacity of the films after 1 month of immersion in water remained constant for films containing 0.2% silver, whereas the films with a higher silver concentration showed a slight increase in activity. These results are similar to those obtained in the experiment described above for the antimicrobial activity of 0.2 and 1.5% silver films after immersion in 1:125 diluted MHB for 30 days. Although the experimental conditions were different, both experiments show that films are capable of releasing silver ions after immersion in liquid medium and maintain their effectiveness over time. It is noteworthy that films with a greater silver concentration slightly increased their antimicrobial activity after 30 days of immersion in water compared with 0.2% silver films. This result might indicate that, although initially the films neutralized at 37 °C tested had similar antimicrobial activity, the films with a greater number of nanoparticles may have a greater number of silver ions available to exert their antimicrobial activity over time. These silver ions would be released after oxidation of silver from the surface of the nanoparticles over time.

Table 5. Antimicrobial Effect of Films Neutralized at 37 °C with 0.2 and 1.5% Silver against *S. aureus* Tested in Diluted MHB (1:125) after Different Immersion Times in Distilled Water[a]

	bacterial count (log CFU/mL)	
time (days)	0.2% Ag	1.5% Ag
control	7.39 ± 0.27 a	
1	4.50 ± 0.17 b	4.28 ± 0.21 b
30	4.46 ± 0.23 b	3.31 ± 0.29 c

[a]Values within a column followed by a different lower case letter are significantly different from each other (Tukey's adjusted analysis of variance $P < 0.05$).

In this work, a methodology using compounds of low toxicity has been developed to obtain silver-based nanoparticles embedded in a chitosan film. Silver nitrate was used as a precursor for the synthesis of nanoparticles, and sodium hydroxide accelerated the reduction rate of silver ions and the formation of nanoparticles during the neutralization step. However, the coexistence of elementary silver and silver oxide is expected, owing to the presence of sodium hydroxide. TEM images showed the formation of spherical nanoparticles in films incorporating 0.1 and 0.2% of silver and neutralized at 37 °C. Films with 0.5 to 1.5% silver presented two populations of nanoparticles, one comprising spherical nanoparticles of 5−10 nm in size and a second population of round-shaped, anisotropic nanoparticles with diameters ranging from 30 to 50 nm. Films neutralized at 37 °C incorporating different amounts of silver ranging from 0.2 to 1.5% released similar amounts of total silver to the culture medium after 18 h of immersion and showed similar antimicrobial activity. The release of silver to the culture medium from films containing 0.2% silver neutralized at 37 °C was monitored for 360 h and showed an increase in the release of silver after the first 10 h of contact. However, the antimicrobial activity did not change. This could be explained by conversion of ionic silver to elemental silver or interaction with the proteins present in the culture medium. It has been shown that chitosan is capable of acting as a carrier of silver nanoparticles, allowing slow, extended release of silver ions in a liquid medium for 30 days and maintaining their antimicrobial activity. Moreover, films with a higher silver concentration can exert their antimicrobial activity for longer as they have a larger reservoir of silver ions in the form of silver-based nanoparticles. The developed films could be used in several fields such as medicine, pharmacy, and food packaging when a long-term antimicrobial effect is desired.

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