Development and Characterization of Silver-Based Antimicrobial Ethylene−Vinyl Alcohol Copolymer (EVOH) Films for Food-Packaging Applications

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ABSTRACT: The use of silver as an antimicrobial in the food area has raised wide interest in recent years. In the present work, 0.001−10 wt % silver ions was satisfactorily incorporated into an ethylene−vinyl alcohol (EVOH) copolymer matrix by a solvent casting technique. The antibacterial efficacy of the composite was evaluated under laboratory conditions and in contact with some foods. The ionic compound did not affect the crystallinity or the water-induced plasticization of the materials and was homogeneously distributed across the surface and thickness of the films. When immersed in water, sorption-induced release of 50−100% of the silver ions took place in <30 min. In the bacterial minimal growth medium M9, the minimal inhibitory concentration (MIC) of the film was in the range of 0.01−0.1 ppm. High protein content food samples displayed low susceptibility to the films (<1 log reduction in any case), whereas low protein content food samples exhibited no detectable bacterial counts for films with 1 and 10 wt % silver and about 2 log reduction for films with 0.1 wt % silver. These results represent a step forward in the understanding of silver antimicrobial efficacy and its possible application in the food-packaging industry, most likely as food coatings.

KEYWORDS: *active packaging, antimicrobial silver, ethylene−vinyl alcohol (EVOH)*

■ INTRODUCTION

Market trends toward minimally processed, easily prepared, and ready-to-eat "fresh" food products involve the use of alternative technologies such as lower thermal, high-pressure, UV irradiation, or electric pulse treatments, which might allow survival and proliferation of pathogenic bacteria.[1−4] Recent foodborne microbial outbreaks, globalization of food trade, and distribution from centralized processing are driving a search for innovative ways to inhibit microbial growth in the foods while maintaining quality, freshness, and safety.[5] The combination of these emerging technologies with antimicrobial packaging technologies could allow extension of the shelf life of foods and the prevention of recontamination with pathogens.

In antimicrobial packaging, a substance with biocide properties is included in a sachet, coated, adsorbed, or immobilized on the surface or directly incorporated in the polymer during its processing. As microbial contamination occurs primarily at the surface, the incorporation of the biocide in a film or as a coating has many advantages due to the high exposure areas for the biocide.[6,7]

Various antimicrobials have been incorporated in polymers for food-packaging applications such as organic acids[8,9] or triclosan.[10] Recently, natural antimicrobials such as enzymes,[11] bacteriocins,[12−14] essential oils,[15] chitosan,[16] and others have attracted much attention due to consumer demand trends (see refs 5, 17, and 18 for reviews). Antimicrobial silver has emerged as a new effective technology to prevent microbial proliferation on food contact surfaces in the food industry.

The antimicrobial efficacy of silver has been recognized since ancient times.[19,20] In the past few years, the use of silver or

silver salts as key components to control microbial proliferation has become more and more popular. Much of the research on this compound is still focused on medical applications, such as wound[21] and burn[22] treatments, dentistry, catheters,[23−25] or orthopedics.[26] However, new applications have emerged, so that silver is currently being incorporated in a wide variety of materials used in daily life, ranging from textile clothing,[27,28] coatings in washing machines, refrigerators, and furniture handles,[29−32] home water treatment units, food-contact materials,[5,33] deodorants,[34] and tooth brushes[35] (see refs 36−38 for reviews).

Due to their unspecific mechanism of action, silver ions are active against a very broad spectrum of bacteria, yeasts, fungi, and viruses[39,40] and are not toxic to human cells.[19,20] In the United States, the Center for Food Safety and Applied Nutrition in the Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled waters, and in the European Union, silver is accepted under directive 94/36/EC as a coloring agent (E-174) with no restrictions. Additionally, the European Food Safety Authority (EFSA) has provided positive opinions for silver zeolites, silver zirconium phosphates, and silver-containing glasses in its provisional list of additives for food contact materials with a general restriction of ≤0.05 mg/kg food for the whole group. Regardless of the stringent regulations, silver remains the most

widely used antimicrobial polymer additive in food applications.[5,18]

The approach to the use of silver in the food industry has been mostly bound to silver zeolites[41−44] and silver−zirconium ion exchange resins, which are subsequently incorporated as a coating on predominantly stainless steel surfaces. These systems rely on the sustained release of silver ions via a moisture-dependent ion exchange mechanism. However, the very low migration rates of the silver ions from these materials imply the need for silver filler contents of 2−5%. This high silver content could limit the application of these systems in antimicrobial packaging, due to possible permeability and dispersion problems and a negative environmental impact. For the correct development and final application of silver in the food industry, it is crucial to elucidate the threshold of biocide action and optimize the silver system so that tiny contents are required and the potential is fully realized. A feasible approach to this challenge inside the range of food-packaging polymers might be the use of ethylene−vinyl alcohol copolymers (EVOH).

EVOH copolymers are a family of semicrystalline random copolymers widely used in the food-packaging sector due to their outstanding gas barrier properties, chemical resistance, and high transparency. Upon contact with moisture, EVOH severely plasticizes, leading to increased permeability and up to 9 wt % water uptake,[45] which would enable the sorption-induced release of its content. This might allow the incorporation of much lower quantities of silver salts, which could be activated upon direct contact with the moisture of the food and be released in its entirety during the shelf life of the packaging. The combination of silver and plastics such as EVOH would accordingly reduce the load of silver in the packaging and its environmental impact.

In this work, we have focused on the incorporation of silver nitrate into an EVOH copolymer. The materials were characterized to determine possible property and structural changes due to the incorporation of the biocide compound. Due to the instability of active silver ions in the presence of several ligands, such as sulfur groups, the antimicrobial potential of these materials was evaluated under laboratory conditions and in contact with food samples of different compositions in comparison with pure silver nitrate. The aim of the study was to elucidate how the silver-based system actuates under these various conditions as a means to gain further understanding about silver antimicrobial efficacy and its possible application in active food packaging.

■ MATERIALS AND METHODS

Sample Preparation. Ethylene−vinyl alcohol copolymer with 32% (mol %) ethylene content (EVOH32) supplied by Nippon Gohsei Corp., Japan, was used for preparation of the cast films. Polymer beads were dissolved in 2-propanol/water (70:30 w/w) in the ratio 8:92 (w/w) at 100 °C under reflux. The dissolved polymer was cooled to approximately 60 °C, and a suitable amount of silver nitrate (Sigma-Aldrich) was added to the solution to achieve EVOH films with 0.00001−10% silver nitrate weight in dry conditions. The solution was cast in a glass Petri dish with an adhesive PTFE sheet to prevent sticking to the bottom. The solvent was allowed to dry at 60 °C for 3 h. The thickness of the dry films as measured with a micrometer was 70 ± 15 μm. Films were stored for 20−24 h in 0% relative humidity (RH) desiccators protected from light with aluminum wrapping before undergoing testing.

Elemental Microanalysis (SEM Measurements). The distribution of silver in the cast films was examined by energy-dispersive X-ray

microanalysis (EDS) using a Si (Li) detector (EDAX, Mahwah, NJ, USA) with a superultrathin Be window. Three spectra were collected from each surface employing an area scan mode under 20 kV accelerating voltage, 10 μA beam current at 2000× magnification, 1000−1500 counts/s, dead time of 30%, and 500 s acquisition time. The study of the distribution along the fracture line of the polymer was performed with the line scan mode microanalysis at 1200× magnification and a dwelling time of 400 ms. The SEM microphotographs (S4100, Hitachi, Osaka, Japan) were taken with the same accelerating voltage of 20 keV on the sample surface just before the elemental analysis was performed.

Water Sorption. The vapor water sorption capacities of EVOH with 0, 0.1, and 10 wt % silver at different relative humidities were obtained by storing 100 ± 10 mg of each sample in 26, 53, and 100% RH desiccators and following water uptake gravimetrically with an analytical balance model Voyager V11140 (precision of 0.01 mg) until equilibrium was reached. Samples were measured three times in triplicate.

FT-IR Analysis. Transmission FT-IR experiments were recorded within a N_2 purged environment using Bruker model Tensor 37 equipment (Darmstadt, Germany) with a resolution of 1 cm^{-1}, 20 scan runs, and a typical acquisition time of 60 s.

Differential Scanning Calorimetry (DSC). Thermal properties were studied by DSC using a Perkin-Elmer DSC-7 calorimeter (Perkin−Elmer Cetus Instruments, Norwalk, CT, USA). The rate of both heating and cooling runs was 10 °C/min, where a typical sample weight was around 4 mg. The values of glass transition temperature (T_g), melting point (T_m), specific heat (ΔC_p), and melting enthalpy (ΔH_m) were taken from the second heating run. Calibration was performed using an indium sample. All tests were carried out in duplicate.

Release Study. A voltammetric method was used to determine the release of free silver ions (FSI) from the films to a slightly acidic aqueous environment. With this purpose, 1 g of the cast films with 0.1 wt % silver content was immersed in 100 mL of slightly acidified (1 mM HNO_3 to stabilize silver in its ionic form) distilled water at 5, 25, and 50 °C for 24 h without stirring except before each measurement. For each measurement, 1 mL from the samples was collected, and the amount removed was replaced with fresh water, applying a correction factor (*) as follows:

$$\text{correction factor} = \left(\frac{100}{100-1}\right)^{n-1} \tag{1}$$

where n is the sequential sample number. The FSI content for each measurement was determined by differential pulse anodic stripping voltammetry (ASV) with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin 207/2e "Analysis of silver by stripping voltammetry". The FSI working range was 0.004−0.4 ppm, and a calibration curve was prepared daily for each set of measurements. All experiments were carried out in duplicate.

Bacterial Strains and Growth Conditions. Bacterial strains *Listeria monocytogenes* CECT 5672 and *Salmonella* spp. CECT 554 (Spanish Type Culture Collection, Valencia, Spain) were selected as food-related Gram-positive and Gram-negative model bacteria. These strains were grown overnight in tryptic soy broth (TSB) (Conda Laboratories, Madrid, Spain), and an aliquot was again transferred to TSB and grown at 37 °C to the midexponential phase of growth, having absorbance values of 0.20 for *Salmonella* spp. and 0.15 for *L. monocytogenes* as determined by optical density at 600 nm by ultraviolet−visible (UV) spectroscopy using an SP-2000 UV spectrometer (Spectrum Instruments, Shanghai, China). These cultures were centrifuged at 4 °C and 1888*g* for 20 min. The pellet was resuspended in a solution containing 10% TSB and 10% glycerol. This suspension was transferred to cryotubes and stored at −85 °C as stock cultures until needed. Prior to each study, cryotubes were thawed and diluted in 0.1% buffered peptone water to achieve the suitable concentration for inoculation. Previous studies indicated no significant differences in cell viability could be found between the
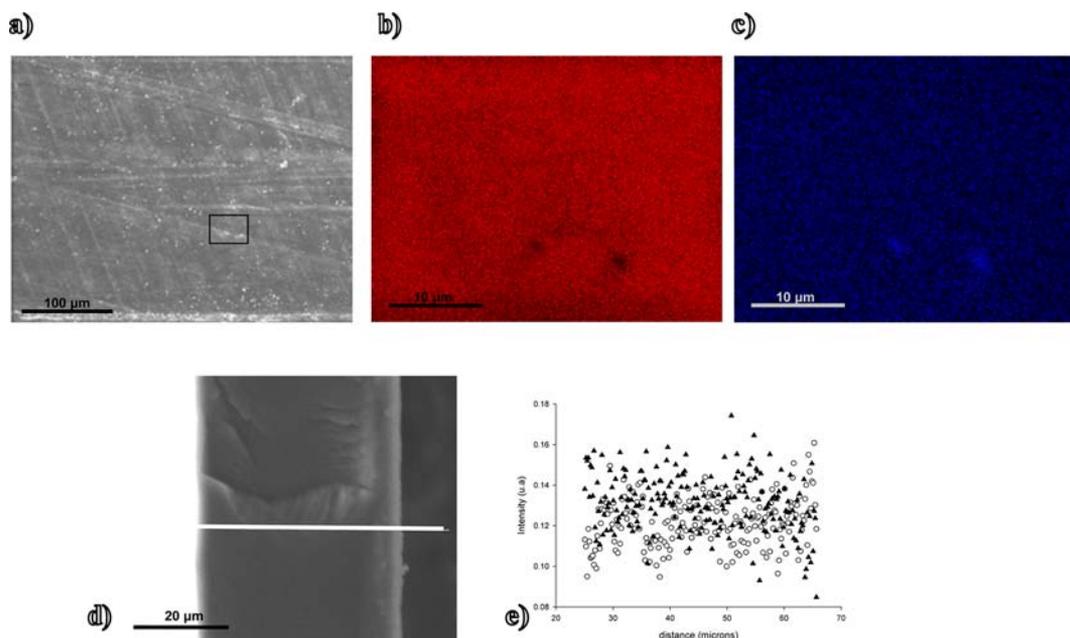
Figure 1. Distribution of silver in EVOH films with 10% silver: (a) typical SEM image of the sample; (b) carbon mapping analysis of the magnified area indicated in panel a; (c) silver mapping analysis of the same area; (d) SEM image of the cryofracture; (e) intensity values (counts/s) of silver/carbon (open circles) and oxygen/carbon (soild triangles) along the fracture line.

ultrafrozen and thawed bacterial stock cultures and the original fresh ones.

Antimicrobial Assays in Laboratory Conditions. For antimicrobial assays against *L. monocytogenes* and *Salmonella* spp., M9 minimal medium supplemented with 1 mM methionine (M9-Met) was used as liquid broth medium. M9 is a minimal medium without any protein sources or components and glucose as a sole carbon source. Although the medium is not suitable for the growth of Gram-positive bacteria, previous findings indicated that *L. monocytogenes* CECT 5672 can grow well if only methionine is supplemented. Susceptibility tests were performed employing the macrodilution method M26-A described by the Clinical and Laboratory Standards Institute (CLSI) with modification. The effectiveness of the antimicrobial EVOH−Ag films was assessed by introducing 100 mg strips of approximately the same size and thickness into tubes with 10 mL of M9-Met. A bacterial suspension in midlog phase was then inoculated in each test tube to achieve an initial inoculum size of approximately 5×10^5 CFU/mL and incubated at 37 °C for 24 h. Then, 0.1 mL of each M9 sample was subcultivated on TSA plates for viable count after incubation at 37 °C for 24 h. These results were compared with EVOH32 samples without silver and samples containing different concentrations of aqueous silver nitrate. Each of these experiments was performed in triplicate.

Challenge Tests. For antimicrobial challenge tests, food samples were differentiated into two groups according to their protein contents. Samples were cut in pieces of 2 × 2 cm, and 25 μL of a *L. monocytogenes* bacterial suspension in M9 medium was spread on the sample to achieve bacterial concentrations of about 10^5 CFU/cm^2 for food samples with high protein content (chicken with and without skin, marinated pork loin, and cheese slices) and 10^8 CFU/cm^2 for food samples with low protein content (lettuces, apple peels, and eggshells). After inoculation, samples were held for 10 min to allow sorption of the microorganisms tested. Then, film pieces of 2 × 2 cm with 0.1, 1, and 10% silver were put on the surface of the food samples, and the set was incubated at 12 °C for at least 48 h. To follow the antimicrobial activity of the films, samples were removed every 12 h and homogenized in a stomacher (Pulsifier, Microgen, U.K.) with 50 mL of peptone water (PW) for 2 min. Serial dilutions in 0.1% PW were made, and the microorganism suspensions were plated on Oxford selective agar (Conda Laboratories) for viable count after incubation at 37 °C for 24 h.

Release Study upon Contact with Food Samples. Additionally, and to ascertain release under more realistic conditions, the amount of silver released from EVOH films with 1 wt % silver to food samples was determined voltammetrically by immersing the films in ultrapure water slightly acidified (1 mM HNO$_3$ to stabilize silver in its ionic form) after having been incubated at 12 °C for 24 h in contact with apple peels at ambient RH and 100% RH as above. All experiments were carried out in triplicate.

Color Analysis of Treated Samples. The change in color of the films after 24 h of contact with the food matrix was determined using a hand-held Minolta Chromameter CR300 (Minolta Camera Co., Ltd., Osaka, Japan) set to D65 illuminant/10° observer. Film specimens were placed on a white standard plate, and the CIELAB color space was used to determine the parameters L^*, a^*, and b^*. L^* values range from 0 (black) to 100 (white); a^* values range from −80 (green) to 100 (red); and b^* values range from −80 (blue) to 70 (yellow). Samples were evaluated per triplicate, and three measurements were taken at random locations on each of the studied films. ΔE^* was calculated as a global parameter (eq 2) using films stored for the same period but without silver and without contact with food as the reference samples.

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad (2)$$

Statistical Analysis. The statistical significance of differences in molecular organization, thermal properties, color changes, and challenge tests with high-protein food samples was determined on the ranks with a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests. In all cases, a value of $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Distribution of the Silver Ions in the Samples. As the antimicrobial effect occurs via a sorption-induced release upon contact with the water medium, it is crucial that the biocide is homogeneously distributed in the film. Elemental analysis offers the possibility of semiquantitatively determining the composition of the surface of the matrix and additionally mapping the presence and distribution of each element in the same area. In the SEM micrographs of samples with 10 wt % silver,

homogeneously distributed particles of sizes between 1 and 20 μm are seen (Figure 1). These may correspond to precipitated silver salt and/or reduced silver particles resulting from the beam exposure. During beam exposure the samples became black and hence the silver reduction argument. When the carbon and oxygen images are compared to the respective elemental mapping analyses for silver, it is revealed that these particles are mostly silver agglomerates consisting of a small fraction of the added silver, whereas most of it remains homogeneously distributed, covering the whole mapping area. This indicates that silver ions were well dispersed on the surface matrix. The silver/carbon intensity ratio plotted by scanning a line in the cryofractured polymer reveals that the distribution is also homogeneous along the thickness of the matrix. Accordingly, it is demonstrated that the incorporation of aqueous solutions of silver nitrate to EVOH by the casting technique enables the silver ions to be well dispersed along and across the films.

Water Uptake. As the release of the silver ions is dependent on the water uptake capacity of the material, it is important to ascertain if these capacities are affected by the incorporation of the biocide. Figure 2 shows water uptake capacities of EVOH
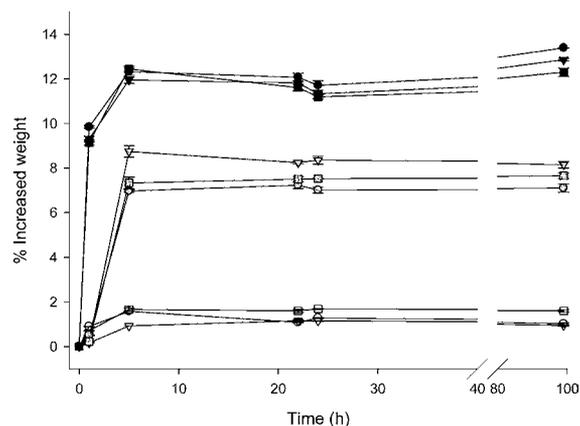
Figure 2. Weight uptake of EVOH32 loaded with 0 wt % (circles), 0.1 wt % (triangles), and 10 wt % (squares) silver at relative humidities of 26% (white), 53% (gray), and 100% (black).

copolymers loaded with 0.1 and 10 wt % silver compared to an unloaded control for relative humidities of 26, 53, and 100%. The sensitivity of the polymer to moisture is denoted with a drastic increase in weight, namely, 1−2% increase for 26% RH, 7−8% for 53% RH, and up to approximately 12% weight uptake for 100% RH. Equilibrium is reached in all cases within the first 5 h. For materials with different silver contents in the same moisture conditions, weight differences of up to approximately 1% can be observed. However, higher sorption values do not seem to be related with the silver content and may be more likely related to material variations in the casting process. Accordingly, EVOH copolymers might undergo a severe plasticization in a moist environment, which does not seem to be affected even if very high concentrations of silver are incorporated in the material.

FT-IR Analysis. Infrared spectra of the EVOH samples with different silver contents were analyzed to evaluate possible changes in molecular organization due to the incorporation of silver. In particular, differences in the crystalline content were investigated by comparing the intensities for the bands at 1410 and 1092 cm^{-1}. The band at 1410 cm^{-1} arises from all-trans

conformation crystallizable chain segments, the vast majority of which are presumed to exist within a crystalline environment, whereas the broad envelope at 1092 cm^{-1} arises from the contribution of at least one amorphous band at 1115 cm^{-1}.[46] No significant differences were found in the ratio of these bands among samples with silver contents up to 10% (Table 1). This indicates that the amount of crystalline fraction in the polymer may not be altered even if high concentrations of silver are incorporated.

Table 1. Intensities Ratio of the FT-IR Bands at 1410 and 1092 cm^{-1} for Samples with Different Silver Contents

Ag$^+$ content (%)	absorbance ratio[a]
0	0.933 A
0.01	0.939 A
0.1	0.936 A
1	0.943 A
10	0.943 A

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests.

DSC. Thermal properties during the second heating run of samples with increasing silver content as measured by DSC are shown in Table 2. The values of T_g were taken at the midpoint

Table 2. Thermal Properties of the Cast Films with Different Silver Contents As Measured by DSC

Ag$^+$ (wt %)	thermal property[a]			
	T_m ($^\circ$C)	ΔC_p (J/g $^\circ$C)	T_m ($^\circ$C)	ΔH_m (J/g)
0	58.16 A	0.040 A	183.82 A	46.24 A
0.001	58.55 A	0.041 A	183.70 A	46.25 A
0.01	58.78 A	0.039 A	183.78 A	45.83 A
0.1	58.64 B	0.040 B	181.42 A	41.66 A
1	61.66 B	0.048 B	179.37 A	30.58 B
10	63.73 B	0.089 B	152.50 B	16.08 B

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests.

of a stepwise increase in specific heat associated with the glass transition, whereas the ΔC_p was calculated as the jump in specific heat. The melting point and fusion enthalpies were calculated from the maximum temperature and peak area, respectively, of the peak associated with the melting process. No significant differences are observed for samples with silver contents of ≤0.1 wt %. When the silver content increases from 0.1 to 10 wt %, a significant increase in the amorphous phase fraction and its stiffness can be deduced from the increase in ΔC_p and T_g, respectively. Additionally, an inhibition of the crystallization process occurs for samples with 1 and 10% silver, as evidenced by the significant decreases in T_m and ΔH_m. These changes do not correlate with the results from the infrared analysis or the water uptake capacities, where no changes were observed between unloaded and highly loaded samples, and could possibly be attributed to the reduction of silver ions to elemental silver particles during the heating process in a N$_2$ purged environment. The appearance of an intense yellow color in the treated food samples, characteristic of the plasmon

resonance of tiny silver particles, would be an indication of this reduction.[47]

Release Study. As a means to preliminarily assess the release kinetics of silver from the films, samples were immersed in slightly acidified bidistilled water to prevent possible reduction of silver by aging or other ambient conditions. The water sorption-induced release of the silver ions from the materials was monitored by ASV for temperatures of 5, 25, and 50 °C to simulate release at refrigeration, average room, or extremely hot temperature conditions. The silver content selected for the release study was 0.1 wt % as it was the lowest concentration for which sensitivity and reproducibility were feasible considering the threshold values of the technique under the tested conditions. Once the material is immersed in the aqueous environment, the release takes place within 30 min, all samples reaching equilibrium before the first hour (Figure 3).
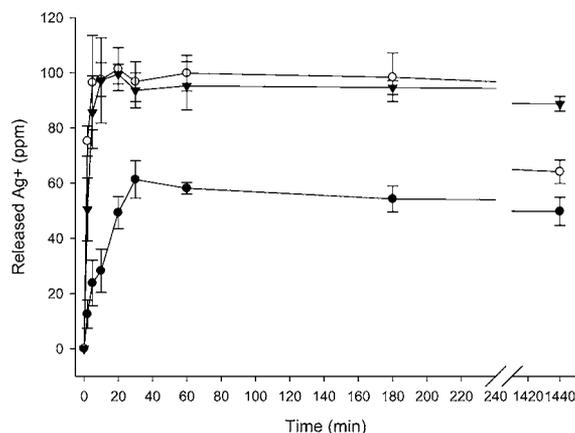
Figure 3. Sorption-induced release of free silver ions from EVOH copolymer loaded with 0.1 wt % silver at 5 °C (black circles), 25 °C (triangles), and 50 °C (white circles).

This could indicate that plasticization of the polymer occurs instantaneously after the polymer is immersed. Consequently, it swells, sorbing water and allowing the silver ions to migrate to the aqueous solution.[48,49] Samples at 25 and 50 °C release 100% of their content in 10 and 5 min, respectively, whereas in refrigerated samples it takes about 30 min to only release 50% of their silver content. The increase in the release capacities at higher temperatures is most probably related to this process being thermally activated and having higher diffusion coefficients, as reported in ref 50. The reason only 50% of the silver content is released in refrigerated samples must be related to the polymer reducing strongly its molecular motions. In fact, it has been determined that for a fully plasticized sample, the T_g of the polymer goes slightly below but not far from 5 °C. Once equilibrium is achieved, the silver ions remain stable in solution for at least 24 h except in samples at 50 °C, where the FSI concentration gradually decreases, dropping to 62% after 24 h. This decrease in FSI could be attributed to the heat-induced formation of elemental silver particles.

Antimicrobial Assays in Laboratory Conditions. Silver is known to be easily inactivated by complexes such as the sulfur groups in proteins.[51,52] To reduce inactivation of the active silver ions and, therefore, assess the potential of the silver-loaded materials under favorable conditions, M9 minimal medium was selected for the susceptibility assays in nutrient broth. Logarithmic increases of silver nitrate concentrations were tested in the form of aqueous solutions and in the form of

silver-loaded EVOH, assuming a 100% release from the polymers, against *L. monocytogenes* (Figure 4a) and *Salmonella*
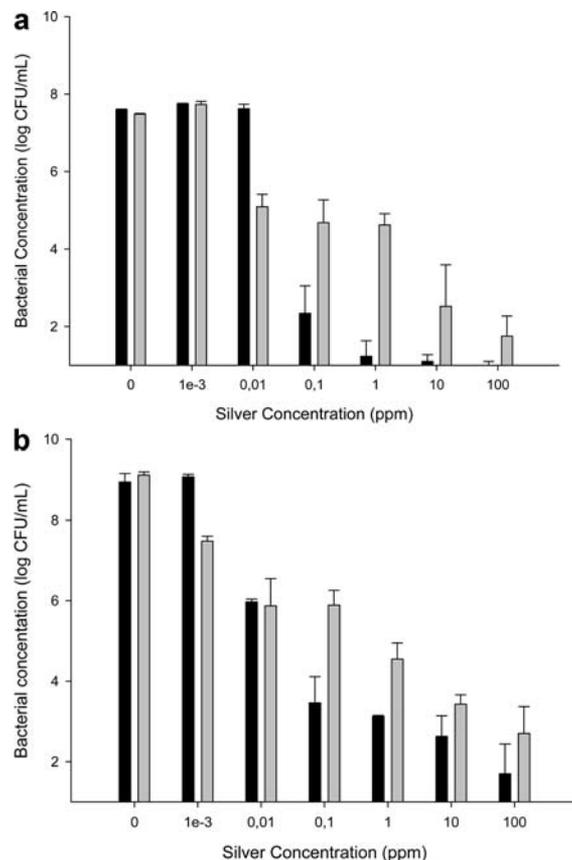
Figure 4. (a) Susceptibility assays in M9-Met minimal medium of *L. monocytogenes* to increasing concentrations of silver aqueous solutions (black bars) and silver-loaded EVOH copolymers (gray bars) assuming a 100% release. (b) Susceptibility assays in M9-Met minimal medium of *Salmonella* spp. to increasing concentrations of silver aqueous solutions (black bars) and EVOH/silver copolymer films (gray bars) assuming a 100% release.

spp. (Figure 4b). The graph shows how bacterial concentration decreases with increasing silver content in both forms, whereas EVOH without silver does not exhibit any antimicrobial effect as compared to the control. The minimal inhibitory concentration (MIC) is defined as the amount of biocide that inhibits culture growth during 24 h, the bacterial counts remaining approximately equal to the initial inoculum size. For *L. monocytogenes*, this effect is achieved with silver concentrations of 0.1 and 0.01 ppm for silver added as aqueous solution and as loaded EVOH, respectively. For *Salmonella* spp., the same effect is achieved with silver concentrations of 0.01 ppm in both forms of release. However, a decrease in the proliferation of *Salmonella* spp. is noted for concentrations as low as 0.001 ppm only in samples in which the silver is released from the copolymer. This points out that for both Gram-positive and Gram-negative bacteria the effectiveness of antimicrobial silver is enhanced when incorporated in the copolymer as compared to pure silver nitrate, indicating the potential of antimicrobial silver is being more efficiently exploited. This phenomenon could be associated with the inactivation of silver by the nutrient broth or by the microorganisms themselves. In solution, the entirety of the silver ions would be potentially capable of being instanta-

neously inactivated, whereas in the polymer, the release extends along 0.5 h. This would increase the time of contact between the bacteria and the active silver ions, accordingly preventing proliferation more efficiently.

However, this tendency is inverted when the effect of higher concentrations of silver is examined. For *L. monocytogenes*, a bactericidal effect (defined as a decrease of 3 magnitudes in the bacterial load) (MBC) is achieved with silver concentrations in the range of 0.1–1 and 10–100 ppm for silver added as aqueous solution or as EVOH films, respectively. For *Salmonella* spp., the bactericidal effect is reached with 10–100 ppm if aqueous silver nitrate is added to the broth, whereas this effect is not yet reached with 100 ppm silver when released by EVOH copolymer. This indicates about 100 times more silver is necessary to exert a bactericidal effect with the polymer compared to pure silver nitrate. It also evidence that the difference between MIC and MBC is extremely high, more so for *Salmonella* spp. than for *L. monocytogenes*. The wide range of concentrations in the survival curves where bacteria remain viable but are not able to proliferate could be related to the mechanism of action of silver and/or explained in terms of solubility. Silver damages bacteria by unspecific binding to membrane and respiratory enzymes.[23,53,54] This unspecific binding could, in minute concentrations, result in sublethal damage making bacteria unable to proliferate but still viable, until a certain concentration is reached when the damage would overtake their repair mechanisms and cause irreversible damage and cell death.[55] In addition, the possible formation of insoluble silver chloride (observed as a visible white precipitate when 10 wt % silver is incorporated) might also reduce the availability of free silver ions to exert the antibacterial effect. The solubility constant (log K) of silver chloride in water is, according to ref 52, about 9.8. This would imply a soluble concentration of 6.3 ppb silver chloride, above which saturation is reached and the equilibrium is gradually shifted toward silver chloride complexes, a very low percentage of silver ions remaining active in solution, according to mathematical modeling reported previously.[56] As a result, the bactericidal concentration would be above the solubility constant, and much higher quantities would be needed to increase the fraction of free silver ions.

Either inside the polymer or in aqueous solutions, the amount of silver necessary to affect bacterial growth as compared to the control is in the range of 0.001–0.1 ppm. Other authors found bactericidal effects for silver ions or silver nanoparticle concentrations in the range of 0.01–1 ppm.[57–59] However, these studies were made in water or salt buffers, which do not support bacterial proliferation. Studies performed on nutrient growth media, such as TSB, Luria–Bertani, or Müller–Hinton broth, gave MBC values of 10–500 ppm.[60–64] In the present study, M9 medium is put forth as a growth medium that supports bacterial growth while fully exploiting the antimicrobial potential of silver ions (minimizing inactivation). Accordingly, this medium could be used as a suitable substrate of reference to assess the full potential of silver-based antimicrobial systems.

In addition, a few studies recently published have dealt with the incorporation of silver as antimicrobial for food-packaging applications. One approach has been the inclusion of the silver ions in inorganic mineral substrates such as montmorillonites as carrier in a food-packaging material such as poly(lactic acid) (PLA).[65] The same polymer has been used for incorporation of silver nanoparticles as antibacterial filler.[66] In these cases,

however, filler contents were in the range of 1–10%. Kubakka et al. reported the use of EVOH loaded with TiO_2–Ag nanoparticles.[67] Silver is known to enhance the UV-induced antibacterial effect of TiO_2 by electron transfer to TiO_2.[68] However, the susceptibility tests were again performed on liquid media, and the antibacterial effect of silver alone was not considered. In the present work, EVOH copolymer was selected due to its exceptional capacity of undergoing plasticization to enhance the release capacities of the material and minimize silver content in the polymer. In this study optimization of experimental conditions led to an antimicrobial effect with filler content of ≤0.01%. With this low filler content, the very stringent restriction limits applied by the EFSA could be fulfilled without the need for other additional filler or carrier for the active silver species.

Challenge Tests. Challenge tests on different food types were carried out to ascertain the antimicrobial effectiveness on real food samples. As inactivation of silver is favored by the presence of proteins and other biomolecules, two sets of experiments were carried out, one involving samples with a high protein content, such as chicken, pork loin, or cheese, and one in which the interaction with proteins would be minimized such as with lettuces, apple peels, and eggshells. The extent of inactivation and consequent loss in antimicrobial efficacy was approached with preliminary studies so concentrations could be set to values for which difference in bacterial counts might be noticed. Viable counts of *L. monocytogenes* after 24 and 72 h of incubation at 12 °C on food samples with high protein content and EVOH with 1 and 10% silver content are displayed in Table 3. Controls of chicken wings, chicken breasts, and pork loin are able to increase their number by about 1.5 and 3–3.5 log after 24 and 72 h of incubation, respectively. Controls of cheese slices, however, were not able to grow properly, and

Table 3. Viable Counts of *L. monocytogenes* in Food Samples with High Protein Content after Incubation at 12 °C

food sample	silver amount	*L. monocytogenes*[a] (log CFU/cm^2)	
		after 24 h	after 72 h
chicken wings	control	6.75 ± 0.07 A	8.68 ± 0.06 A
	10% AgNO$_3$ (aq)	5.90 ± 0.12 B	7.74 ± 0.09 B
	EVOH–Ag$^+$ 1%	6.33 ± 0.25 A	8.84 ± 0.12 s
	EVOH–Ag$^+$ 10%	6.22 ± 0.04 A	7.80 ± 0.04 AB
chicken breasts	control	6.37 ± 0.54 A	8.06 ± 0.15 A
	10% AgNO$_3$ (aq)	5.54 ± 0.41 B	6.92 ± 0.04 C
	EVOH–Ag$^+$ 1%	6.10 ± 0.07 A	7.57 ± 0.06 AB
	EVOH–Ag$^+$ 10%	5.85 ± 0.21 AB	7.01 ± 0.12 B
marinated pork loin	control	6.48 ± 0.14 A	8.31 ± 0.04 A
	10% AgNO$_3$ (aq)	5.53 ± 0.12 B	7.24 ± 0.11 B
	EVOH–Ag$^+$ 1%	6.27 ± 0.16 A	7.96 ± 0.06 A
	EVOH–Ag$^+$ 10%	6.21 ± 0.23 A	7.74 ± 0.05 A
cheese slices	control	5.72 ± 0.24 A	5.34 ± 0.02 A
	10% AgNO$_3$ (aq)	5.12 ± 0.31 A	5.04 ± 0.21 A
	EVOH–Ag$^+$ 1%	5.58 ± 0.07 A	5.41 ± 0.13 A
	EVOH–Ag$^+$ 10%	5.34 ± 0.20 A	5.12 ± 0.02 A

[a]Mean values with different letters in the same food sample and same incubation time represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests.

viable counts decreased with time, indicating this substrate is not suitable for the growth of *L. monocytogenes*. Samples with high amounts of silver added to the food either with the polymer or in aqueous solution exhibited count values up to 1 log lower than the controls without silver, the aqueous solution having slightly more effect than equivalent silver quantities in the polymer.

When the challenge tests are performed on food matrices with low protein content (Figure 5), viable counts remained stationary during the whole experiment except for samples on eggshells, for which bacterial counts decreased with time. Aqueous solutions of either 1 or 10% silver produced a decrease
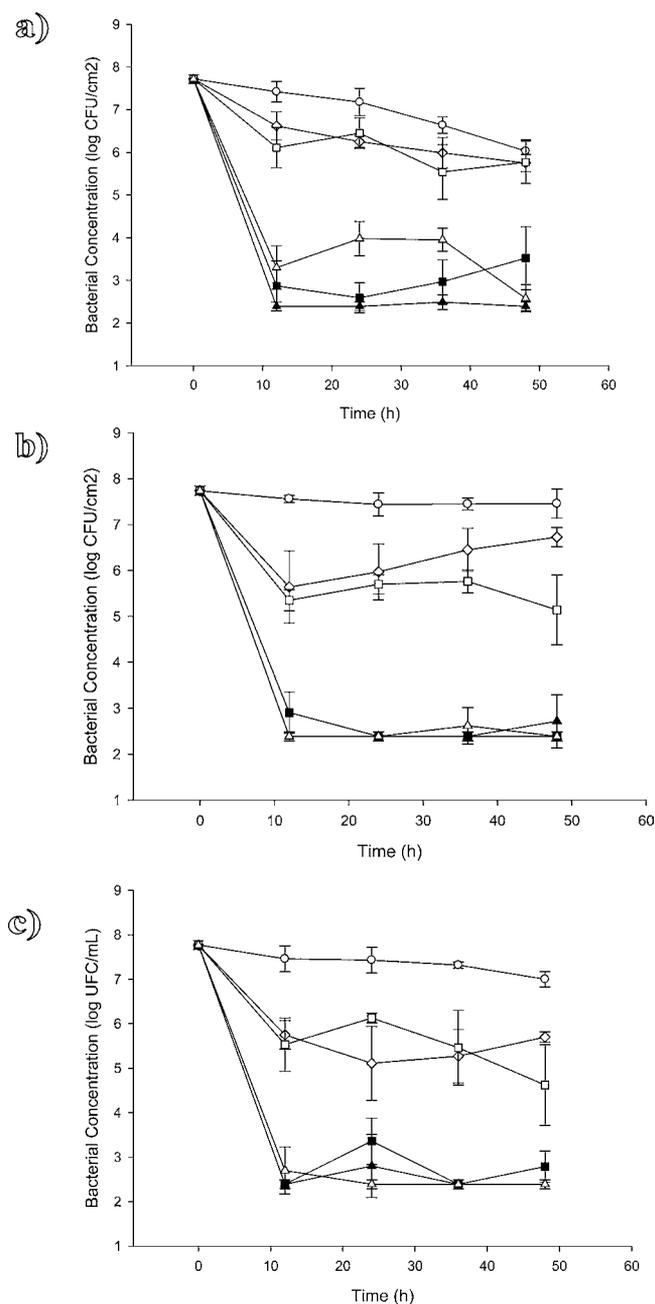
Figure 5. Viable counts in the challenge test on (a) eggshells, (b) lettuces, and (c) apple peels of *L. monocytogenes* versus silver aqueous solutions (solid) and silver-loaded EVOH (open) with 0 wt % (circles), 0.1 wt % (diamonds), 1 wt % (squares), and 10 wt % (triangles) silver content.

in microbial population of 4−5 log. The contact with silver-loaded EVOH films exerted similar efficacy only with the highest content (10%). Polymers loaded with 0.1 or 1% silver did not achieve a bactericidal effect, producing a decrease of about 2 log after 24 h of contact.

This indicates antimicrobial behavior of silver on food samples, which has drastically decreased compared to its efficiency in the liquid medium. This enormous decrease is noted both in the aqueous silver solutions and in the silver-based polymers. Therefore, this decrease can be not only due to release issues but mostly attributed to inactivation of the active silver ions. In addition, silver ions readily react with sulfur groups in proteins, forming very stable complexes.[52] Also, silver ions are easily reduced in the presence of weak reducing environments to elemental silver, which does not exert an antimicrobial effect except in the nanoscale, or by gradually reoxidizing to ions.[47] Both chemical processes could explain the low antimicrobial effect for high protein content samples, where only 1 log reduction is achieved for any aqueous or film samples. In food samples with low protein content, inactivation of the silver ions might occur to a lesser extent, so high bactericidal effect is achieved for 10% EVOH films or 1% aqueous solution, and about 2 log reduction is reached for films with 0.1% silver. However, even when low protein samples are selected for the assays, a 0.1% silver content in the films would still imply surpassing the restriction limits recommended by the EFSA. To the best of our knowledge, successful application on food matrices of EVOH−silver ion releasing technologies has not yet been reported in the literature. In this study, extreme differences in antibacterial efficacy depending on the experimental conditions are evidenced, putting forth the need for standardization of silver biocide tests. Therefore, the present work represents a step forward in the application of silver-based antimicrobial systems, still mostly bound to clinical applications, to the food industry.

Release Study upon Contact of Films with 0.1% Silver with Food Samples. The release of silver ions from the polymer upon contact with food samples can be calculated from the remaining quantity of silver in the polymer after incubation. Apple peels were selected to minimize loss of free silver ions due to complexation and simulate relatively dry food samples at ambient humidity conditions. Therefore, the equivalent samples were stored at 100% RH to investigate the influence of moisture on the release. The fraction of silver released to the food sample was indirectly calculated by subtracting the fraction remaining inside the films from the total (100%). Values after 20 min and 24 h of contact under the stated conditions are shown in Table 4. Control samples not in contact with food give a release fraction of 2%, which is inside the standard deviation of the measurement. After 20 min in contact with the food matrix, about 20% of the silver content

Table 4. Fraction of the Silver Content Released upon Contact of Polymer Samples of 1% Silver with Apple Peels at Ambient Relative Humidity (RH) and 100% RH after 20 min and 24 h

sample	% silver ions released	
	after 20 min	after 24 h
control	2.4 ± 4.0	1.2 ± 3.1
apple peel at ambient RH	19.4 ± 9.7	24.3 ± 8.6
apple peel at 100% RH	19.4 ± 3.6	45.6 ± 3.5

has been released from the polymer regardless of relative humidity. Twenty-four hours of contact results in the release of approximately 25% of the silver content for samples at room ambient RH, whereas 45% of the total silver is released in samples at 100% RH. This suggests the existence of two mechanisms in the release of silver ions. The first one would rapidly take place as the film surface comes in contact with the moisturized food surface, producing an immediate sorption and burst release of a certain fraction of the silver content within 20 min. During this mechanism, the fraction released would depend on the moisture of the food sample. The second would imply the plasticization of the whole polymer and the slow migration of the ions into the foods. This process would be governed by the humidity conditions in the environment. As seen in Figure 2, water-induced plasticization of the films at 100% RH reaches equilibrium after 3 h. Consequently, after 24 h of contact, this process would be fulfilled and release of the FSI would be enhanced at higher relative humidity. As a result, the silver fractions released to the peels are similar in the first 20 min and increase after 24 h depending on the humidity.

Appearance of the Films after Food Contact. It is desirable that the films are transparent and colorless in application for consumer acceptance. Color measurements contribute to objectively differentiate and evaluate changes in the color of the films. Except for samples with 10 wt % silver and in contact with chicken breasts, the film specimens were highly transparent (Figure 6). Table 5 reveals color changes as a
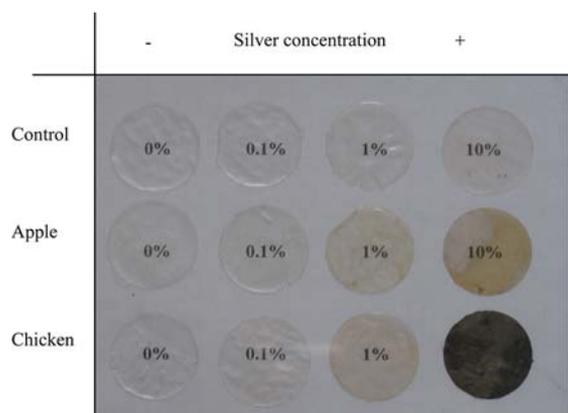
Figure 6. Images of the EVOH samples with different silver contents after 24 h of contact with low (apple) and high (chicken) protein content food samples.

function of silver content in the polymer and contact with chicken breasts or apple peels, as examples of high-protein and low-protein food samples, respectively. As can be observed from the results, the films presented good transparency as indicated by high lightness values (L) (97–99), except for samples with 10% silver in contact with apple peels (92.13) and chicken breasts (42.38). Samples in contact with apple peels show a decrease in transparency, an increase in yellowness, and a slight increase in redness, significant only for 10% silver content. In contact with chicken breasts, samples show an increasing yellowness and a slight increase in greenness with increasing silver content. This is significant only in samples with 1 wt % silver. Samples with 10 wt % silver radically change to opaque shiny silvery films. L values in these samples are not due to transparency of the films but are attributed to the reflectance of the light by the shiny metallic surface of the samples (Figure

Table 5. Color Measurements of the As-Prepared Films (Control) and after Contact with Low and High Protein Content Food Samples

sample	$L*$	$a*$	$b*$	$\Delta E*$
control				
0%	98.97 A	0.07 BC	0.00 A	0.00 A
0.1%	99.06 A	0.02 BC	0.52 A	0.53 A
1%	99.11 A	0.13 BC	0.89 AB	0.88 A
10%	97.68 A	0.18 BC	2.37 AB	2.74 A
apple peels				
0%	98.81 A	−0.08 BC	0.24 A	0.82 A
0.1%	98.61 A	−0.06 BC	0.82 AB	0.99 A
1%	98.40 A	0.05 BC	1.48 AB	1.63 A
10%	92.13 B	1.12 A	8.41 C	10.94 C
chicken breast				
0%	99.01 A	0.04 BC	0.46 A	0.50 A
0.1%	98.83 A	−0.17 C	1.80 AB	1.82 A
1%	97.31 A	−1.23 D	7.05 C	7.38 B
10%	46.38 C	0.53 AB	3.70 B	52.82 D

[a]Mean values with different letters in the same column represent significant differences ($p < 0.05$) among the samples according to a one-way analysis of variance (ANOVA) and Tukey's multiple-comparison tests.

6). Silver ions are readily reduced to elemental silver in the presence of weak reducing environments. Therefore, the increase in yellowness can probably be attributed to plasmon resonance of fine elemental silver particles formed after reduction by food components sucked in during the water sorption process. The remarkable appearance of films with 10 wt % silver after contact with high protein content could be due to complexation with sulfide in proteins (deep black), as well as reduction of these agglomerates into metallic silver.

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Notes
The authors declare no competing financial interest.

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