

Morphology, Physical Properties, Silver Release, and Antimicrobial Capacity of Ionic Silver-Loaded Poly(L-lactide) Films of Interest in Food-Coating Applications

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ABSTRACT: In the present study, silver ions were incorporated into a poly-(L-lactide) (PLA) matrix by a solvent casting technique using different solvents and glycerol as plasticizer. The effect of the different formulations on the morphology, thermal, mechanical and color properties were first evaluated. Additionally, a thorough study of the silver ions release to an aqueous environment was also monitored over time by anodic stripping voltammetry and correlated with the antimicrobial performance against *S. enterica*. The incorporation of silver contents of up to 1 wt % did not affect morphology, thermal or mechanical properties of the films. A sustainable, antibacterial effectiveness was found for the films in liquid medium and a breakpoint of 10–20 $\mu\text{g L}^{-1}$ silver was established under the stated conditions, evincing silver ion releasing technologies may be applied to liquid environments while complying with current legislation. This study provides insight into the structure properties relationship of these antibacterial polylactide materials of significant potential in coating applications. © 2014 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* **2014**, *131*, 41001.

KEYWORDS: applications; biopolymers and renewable polymers; packaging; polyesters

Received 3 February 2014; accepted 8 May 2014

DOI: 10.1002/app.41001

INTRODUCTION

Although the antimicrobial efficacy of silver has been recognized since ancient times,¹ it is during the last decade that its use has become more and more popular. Because of its unspecific mechanism of action, silver ions are active against a very broad spectrum of bacteria, yeasts, fungi and viruses and are not toxic to human cells.^{2,3} Therefore, a wide variety of materials used in daily life are recently incorporated with silver or silver salts as key components to control microbial proliferation, ranging from textile clothing,⁴ stainless steel coatings in home appliances⁵ and food-contact materials^{6,7} (see Refs. 8–10 for review). In the US, the Center for Food Safety and Applied Nutrition in the Food and Drug Administration¹¹ accepts the use of silver nitrate as a food additive in bottled waters and in the EU, silver is accepted under directive 94/36/EC as a coloring agent (E-174) with no restrictions.¹² 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 $\leq 0.05 \text{ mg kg}^{-1}$ food for the whole group.¹³ This does not include the presence of nanoparticles in food contact products for the time being. Regardless of the

stringent regulations, silver ions still remain the most widely used antimicrobial polymer additive in food applications.^{14,15}

The globally increasing demand for minimally processed, easily prepared and ready-to-eat “fresh” food products has encouraged manufacturers to develop new technologies as an alternative to thermal processing. These new alternative technologies such as lower thermal, pulsed electric fields or high pressure treatments may in some instances allow pathogenic bacterial growth. However, even if food-borne pathogens are totally eliminated by efficient thermal treatments, microbial recontamination of the food surface could take place during the post-processing steps, when the risk of cross-contamination is elevated. As a result, a reduction in food shelf-life is observed and the risk of food-borne illnesses is greatly increased. Therefore, new preservation techniques, such as incorporation of antibacterial substances into the food products or the packaging or the surfaces where food contact takes place, are currently being investigated and applied. As bacterial contamination occurs primarily on 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.^{16,17} Additionally, targeted, sustained or controlled of the antimicrobial would allow the use of less quantities of the

antimicrobial while assuring the desired effect over the shelf-life of the product.

The approach of the use of silver in the food industry has been mostly bounded to silver zeolites^{18–20} 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 the incorporation of silver filler contents of up to 5 wt %. This high silver content could limit the application of these systems in antimicrobial packaging, due to possible permeability and dispersion problems, surpassing the migration limits during the shelf-life of the product or a negative environmental impact. Additionally, these very low migration rates assure the safety of the treated surface, but, even then, silver ions may not sufficiently migrate as to kill bacteria within the product. For the correct development and final application of silver in the food packaging 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. Additionally, the extreme instability of silver ions in the presence of complex organic matter, like foods, advises the need for a sustained and targeted release of silver ions.^{21,22} A feasible approach to this challenge inside the range of food packaging polymers might be the use of poly-lactide (PLA) based polymers. PLA is a biodegradable and compostable polymer that can be derived from renewable resources such as starch. Consumers' demands, and the requirements by regulatory authorities, to pursue more environmentally friendly and less polluting packages, have prompted interest into PLA materials, owing to its high mechanical strength and transparency, ease in processing and relatively low prices in the market compared to other biopolymers.²³ Additionally, PLA and its copolymers are the most widely used plastics for controlled drug delivery systems because of their biodegradability, biocompatibility, and ease of processing.²⁴

A previous study demonstrated that PLA-silver compounds could be capable of efficiently killing two of the most common food-borne pathogens in developed countries, *Salmonella enterica* and a human norovirus surrogate, both by surface contact with a bacterial suspension and on artificially inoculated vegetable samples.²⁵ In the present article, a wider set of PLA-silver materials were investigated on the morphology, thermal properties, tensile tests and color alteration. The influence on the use of different solvent mixtures or the addition of glycerol as a plasticizer was further investigated. Sustained release of silver ions from the films was monitored after successive daily and weekly washings by ASV under laboratory conditions to accurately correlate the silver ions concentration with their antibacterial performance against *S. enterica*, and establish breakpoints for silver under these conditions.

MATERIALS AND METHODS

Film Preparation

PLA supplied by Natureworks Llc was used for preparation of the cast films. The polylactide with a D-isomer content of ~2%

had a number-average molecular weight (M_n) of about 130,000 g mol⁻¹, and an average molecular weight (M_w) of about 150,000 g mol⁻¹ as reported by the manufacturer. Polymer pellets were dissolved in tetrahydrofuran (THF) or in a mixture of THF and dimethylformamide (DMF; 3 : 1 w/w) in the ratio 5 : 95 (w/w) at 50°C under stirring to generate PLA-THF and PLA-DMF films, respectively. Glycerol (Panreac, Barcelona, Spain) was added with the pellets to a 10 wt % dry weight to produce PLA-THF-G and PLA-DMF-G films. After dissolution, the suitable amount of silver nitrate (Sigma-Aldrich) was added to the solution as to achieve films with 0, 0.01, 0.1, 1, and 5 wt % silver nitrate weight in dry conditions. The solution was cast onto glass Petri dishes to obtain a 30- to 50- μ m-thick films after solvent evaporation for 3 h at 50 and 60°C for PLA-THF and PLA-DMF films, respectively. The remaining solvent in the films was further allowed to diffuse out in a vacuum oven at 50°C for 18 h. Films were stored in a 0% relative humidity (RH) desiccators protected from light with aluminium wrapping before undergoing testing and to a maximum of 14 days.

Morphology

To investigate the morphology of the films, SEM microphotographs (XL30 ESEM, Phillips) were taken with an accelerating voltage of 20 keV on the sample surface and on the thickness of the films after cryofracturing the samples immersed in liquid nitrogen. Further optical images were taken with an optical microscope Eclipse 90i (Nikon).

FT-IR Analysis

Transmission FTIR and ATR-FTIR using the GoldenGate of Specac Ltd. (Orpington, UK) experiments were recorded within a N₂ purged environment using a 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. Acquisition of the IR spectrum of a PLA pellet as purchased from the manufacturer was performed by coupling an ATR accessory GoldenGate of Specac Ltd. (Orpington, UK) to the FTIR equipment.

Differential Scanning Calorimetry (DSC)

Thermal properties were studied by differential scanning calorimetry (DSC) using a Perkin-Elmer DSC-7 calorimeter (Perkin-Elmer Cetus Instruments, Norwalk, CT). Samples with a typical weight of 3–4 mg were held at 60°C for 5 min in the nitrogen purged chamber to eliminate humidity, then cooled down to 0°C and finally heated to 180°C. The rate of both heating and cooling runs was 10°C min⁻¹. The values of glass transition temperature (T_g), melting points (T_m), and melting enthalpy (ΔH_m) were taken from this heating run. Calibration was performed using indium and dodecane as reference samples. All tests were carried out in triplicate.

Mechanical Properties

Tensile tests were performed according to ASTM Standard D 638 in stamped dogbone-shaped specimens of the samples. An Instron Testing Machine (Model 4469; Instron, Canton, MA) was used, with a crosshead speed of 10 mm min⁻¹, at ambient conditions of typically 23°C and 60%RH. At least, four specimens of each film were tensile tested as to obtain statistically meaningful results.

Color Analysis of Treated Samples

The change in color of the films after 24 h contact with the food matrix was determined using a handheld Minolta Chroma-meter CR300 (Minolta Camera, 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^* value ranges from 0 (black) to 100 (white); a^* value ranges from -80 (green) to 100 (red); and b^* value ranges from -80 (blue) to 70 (yellow). Samples were evaluated per triplicate and four measurements were taken at random locations on each of the studied films. ΔE^* was calculated as a global parameter of color alteration according to the following equation [eq. (1)]:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (1)$$

Release Study

A voltammetric method was used to determine the release of free silver ions (FSI) over time from the films to a slightly acidic aqueous environment. For this purpose, 1 g of the cast films with different silver contents was immersed in 100 mL slightly acidified (1 mM HNO₃ to stabilize silver in its ionic form) distilled water at 25°C and stored without stirring before testing. The FSI content for each measurement was determined on an aliquot extracted from the polymer film containing solution by differential pulse anodic stripping voltammetry (ASV) with an Autolab III (EcoChemie) potentiostat setup under conditions stated in Metrohm application bulletin n° 207/2e “Analysis of silver by stripping voltammetry.” The ASV working range was 0.001–0.1 ppm of silver. After each ASV measurement, the film containing solution was sterilized by filtration and set apart before its use to determine antimicrobial capacity. Subsequently, the films were reimmersed in new fresh slightly acidified water and reincubated again under the same conditions for a subsequent release measurement. This procedure was correlatively repeated in two sets of batches, each day for 14 days to measure release and antimicrobial performance and each week for 10 weeks to evaluate antimicrobial performance on the long term. All experiments were carried out in duplicate.

Antimicrobial Testing

Salmonella enterica CECT 554 strain was obtained from the Spanish Type Culture Collection (CECT; Valencia, Spain) and stored in Phosphate Buffer Saline (PBS) with 10% Tryptone Soy Broth (TSB, Conda Laboratories, Madrid, Spain) and 10% glycerol at -80°C until needed. For experimental use, the stock culture was maintained by regular subculture to Tryptone Soy Agar (TSA) slants at 4°C and transferred monthly.

Previous to each study, a loopful of bacteria was transferred to 10 mL of TSB and incubated at 37°C overnight. A 100- μ L aliquot from the overnight culture was again transferred to TSB and grown at 37°C to the mid-exponential phase of growth. This culture served as the inoculum for antimicrobial assays.

To assess the effectiveness of PLA-THF and PLA-DMF films, susceptibility tests were carried out employing the macrodilution method M26-A described by the Clinical and Laboratory Standards Institute (CLSI) with modification. Briefly, 8 mL of the aqueous solution containing the silver released over each

consecutive day or week were added into 2 mL 5× M9 medium. A bacterial suspension in mid-log phase was then inoculated in each test tube to achieve an initial inoculum size of $\sim 5 \times 10^5$ CFU mL⁻¹ and incubated at 37°C for 24 h. Then, 0.1 mL of each sample was sub-cultivated on TSA plates for viable count after incubation at 37°C for 24 h. Samples treated analogously but without silver were used as controls. Each of these experiments was performed in duplicate.

RESULTS AND DISCUSSION

Morphology

SEM images of the cryofractured films as well as optical microscopy images of the samples were taken to evaluate the effect of the solvent, the incorporation of silver or the presence of glycerol in the morphology of the films. No differences in morphology were found for silver contents below 0.1 wt %, indicating that small silver concentrations do not measurably affect the morphology of the cast films (data not shown). PLA-THF films with 1 wt % silver showed the presence of star-like crystals [Figure 1(C)] which were not present in films without silver [Figure 1(A)]. These crystals were confirmed to contain silver as measured by EDX (data not shown) and are thought to be made of crystallized silver nitrate aggregates. Silver nitrate is highly soluble in water but it is not soluble in organic solvents, while biopolyesters, on the contrary, are mostly soluble in solvents with low polarity, like chloroform or THF. Silver nitrate was found to be soluble in THF only at very low concentrations. Therefore, a combination of DMF and THF (1:3) was further selected, as it was the combination which yielded the highest solubility of the active compound without compromising the film-forming capacity of PLA. PLA-DMF films, on the other hand, presented a rough surface with slits and hollows, indicating that the casting conditions did not allow a continuous microstructure to be well formed at the surface [Figure 1(E)]. No such silver aggregates were found in PLA-DMF films with silver, indicating that the higher solubility of the antimicrobial in a solvent mixture of DMF and THF (1 : 3 w/w) resulted in a better distribution of the biocide. When glycerol was added into both PLA-DMF and PLA-THF films, this was not found to be miscible with the polyester as expected.²⁶ Instead, it was found to be confined in segregated phases homogeneously distributed along the surface and thickness of the films [only cross-sections are shown in Figure 1(G,H)]. Crystals of the silver salt were not found in the films when glycerol was added and it was not possible to locate silver compounds in PLA-THF-G or PLA-DMF-G by EDX mapping. Therefore, as an alternative approach to ascertain if silver had more affinity for any of the two phases, PLA-THF-G and PLA-DMF-G were irradiated with UV light at 254 nm for 5 h. Silver was, thus, reduced to elemental silver, yielding a characteristic brown color. As it can be seen from the observation of PLA-THF-G in Figure 2, silver was found to be mainly confined within the glycerol phases, probably due to its higher solubility in the more polar component. This phenomenon was also observed in all samples with glycerol and silver (data not shown).

FTIR Analysis

Infrared spectra of PLA-THF and PLA-DMF samples with different silver contents were analyzed to evaluate possible changes

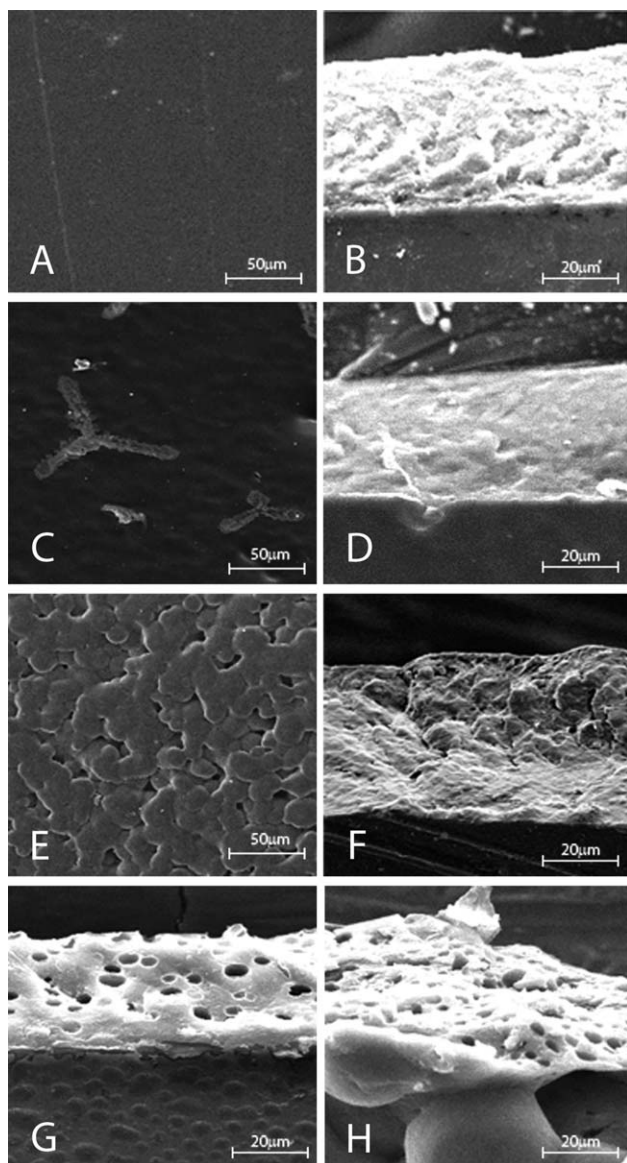


Figure 1. SEM images of the PLA films. (A) Surface and (B) Cross-section of reference PLA films without silver. (C) Surface and (D) Cross-section of PLA-THF films with 1 wt % silver. (E) Surface and (F) Cross-section of PLA-DMF films with 1 wt % silver. (G) Cross-section of PLA-THF-G films with 1 wt % silver (H) Cross-section of PLA-DMF-G films with 1 wt % silver.

in molecular organization due to the incorporation of silver. In particular, differences in the crystalline content were investigated due to its distinct IR absorption patterns of amorphous and crystalline components in characteristic bands. All of the PLA samples showed a band around 921 cm^{-1} , which is well assigned to the coupling of the C—C backbone stretching with the CH_3 rocking mode and sensitive to the 10_3 helix chain conformation of PLA α crystals.²⁵ The presence of β -crystals can be ruled out by the absence of the characteristic band at 908 cm^{-1} in all spectra.²⁷ On the other hand, the amorphous fraction in PLA can be ascribed to the band at 955 cm^{-1} .²⁶ As intensity of the band at 921 cm^{-1} increases while that of the band at 955 cm^{-1} decreases during the crystallization process, and their

changes are synchronized, it was possible to determine the relative crystallinity of neat PLA pellets, PLA-THF and PLA-DMF film samples by using the ratio of the two bands.^{26,30} No significant differences were found in the ratio of these bands among samples with silver contents up to 1 wt %, either in PLA-THF or in PLA-DMF samples (Table I). This indicates that the amount of crystalline fraction in the polymer may not be altered even if relatively high concentrations of silver are incorporated. The cast films, however, displayed a significantly higher crystallinity fraction as compared to the PLA pellets (absorbance ratio of 1:12). This could be explained by a faster cooling of neat PLA during extrusion and pelletization, which is expected to difficult polymer crystallization.

Thermal Properties

To evaluate further the influence of the casting conditions, the presence of glycerol or the incorporation of an increasing load of silver on the different polymer formulations, the material thermal properties were analyzed by DSC. Figure 3 shows typical thermograms of the polymer pellets before film preparation, PLA-THF films and PLA-DMF films both with 1 wt % silver content. The values of the glass transition temperature (T_g), melting point (T_m), and melting enthalpy (ΔH_m) for all samples are shown in Table II. Strong differences are observed between the polymer pellets and the cast films. In the low temperature frame, cast films show significantly lower T_g values and a substantially lower increase in specific heat associated with the glass transition (ΔC_p) (Figure 3). The lower ΔC_p points to a higher degree of crystallinity, while the lower rigidity of the amorphous phase might be associated with a plasticization of the polymer by possible remnants of casting solvents within the films. At higher temperatures, a cold crystallization exotherm could not be observed in the cast films, and two melting peaks appear instead of one. This phenomenon can be explained by the melt-recrystallization model ascribed to the PLA melts as well as to other semicrystalline polymers.³¹ The model suggests that small and imperfect crystals change successively into more stable crystals through a melt-recrystallization mechanism. That

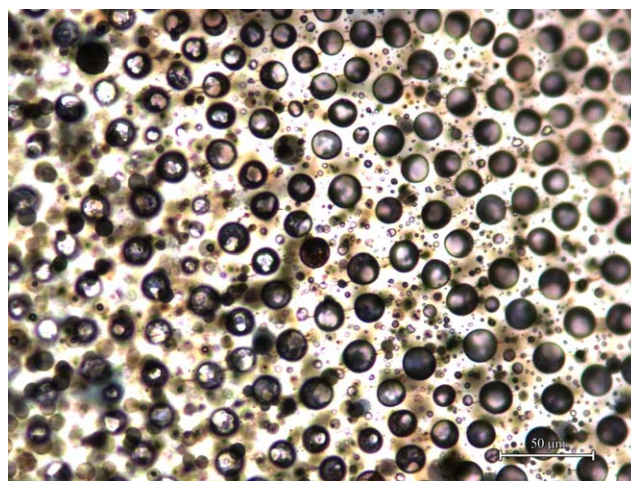


Figure 2. Optical microscopy image of PLA-THF-G films with 1 wt % silver after 5 h UV irradiation at 254 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table I. Absorbance Ratio of Transmission FT-IR Bands at 955 and 922 cm^{-1} as a Function of Silver Content in the Different PLA Cast Films

Silver content (wt %)	Absorbance ratio (955 cm^{-1} /922 cm^{-1})	
	PLA-THF	PLA-DMF
0	0.985 aA ^a	0.997 aA
0.01	0.930 aA	0.984 aA
0.1	0.949 aA	0.983 aA
1	0.992 aA	0.997 aA
5	0.997 aA	1.159 bB

^aMean 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.

is, the melting and recrystallization are competitive in the heating process. Accordingly, an endothermic peak appears when the rate of melting overwhelms that of recrystallization, and the exothermic peak appears when the rate of recrystallization overwhelms that of melting. Because the recrystallization proceeds slowly, it is gradually suppressed with increasing cooling or heating rates. In their fabrication, polymer pellets are rapidly cooled down after extrusion. This hinders crystallization, and allows the polymer to crystallize from early stages throughout the heating run until crystals are finally melt in the form of one single endotherm (T_{m2}). In cast films, solvent is evaporated slowly, which might allow a higher degree of crystallization to be achieved. As melting of crystals overwhelms crystallization in these cases, no visible exotherms are observed during the heating run, while two melting endotherms appear instead, the second (T_{m2}) being thought to be the final melting of crystallites formed throughout the heating process. For all polymer formulations, the addition of

increasing amounts of silver did not significantly alter the thermal properties of the cast films, except for PLA-THF and PLA-DMF films with 5 wt % content, which display significantly lower T_g . This may be due to weakening of the amorphous phase by the presence of the silver aggregates as postulated above. The results indicate that a relatively high load of silver (up to 1 wt %) can be incorporated in the films without altering their thermal properties. Differences can be observed, however, when the different casting methods and the addition of glycerol are evaluated. PLA-THF films display a significantly lower T_{m1} as compared to PLA-DMF films. This suggests crystallites in PLA-DMF are bigger or more perfect than in PLA-THF films. Both effects could be due to a slower evaporation of the THF : DMF solvent mixture. Because T_{m2} is the result of the final melting of recrystallized crystals, its value is almost constant with both solvents.^{31,32} The addition of glycerol, however, produced an overall decrease in both T_{m1} and T_{m2} .³²

Mechanical Properties

PLA is known to be inherently brittle, which has prompted much research on improving this by for example adding different additives.³³ Mechanical properties of PLA-THF, PLA-DMF, PLA-THF-G, and PLA-DMF-G with increasing silver content were evaluated. The materials elastic modulus, elongation at break and maximum tensile strength are presented in Table III. No significant differences in the elastic modulus of PLA-THF, PLA-DMF, and PLA-THF-G with or without silver were observed. This suggests that incorporation of silver in these films does not alter their mechanical properties and that differences among the samples may be ascribed to the actual variations between different castings. On the other hand, PLA-DMF-G films displayed a significantly lower elastic modulus and higher deviation among samples. This may be attributed to the absence of a continuous film structure at the surface of films, as observed in Figure 1(E). Elongation at break is significantly increased with the presence of glycerol, while the maximum tensile strength significantly

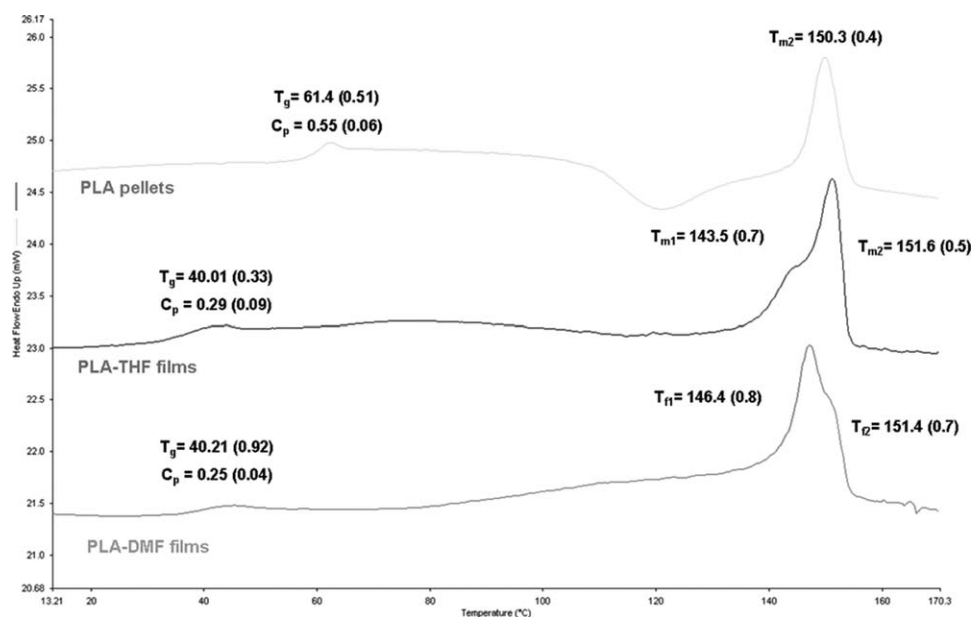


Figure 3. Typical thermograms of PLA pellets, PLA-THF and PLA-DMF films. Values for the different parameters are embedded in the image.

Table II. Thermal Properties of PLA Films Cast with THF (PLA-THF) or a 3 : 1 (w/w) Mixture of THF : DMF (PLA-DMF) Incorporating Different Silver Contents as Measured by DSC

Sample	T_g (°C)	T_{m1} (°C)	T_{m2} (°C)	ΔH_m (J g ⁻¹)
Polymer pellets	63.79 A ^a	–	150.29 A	6.59 A
PLA-THF 0%	41.24 B	144.87 A	152.03 A	31.93 B
PLA-THF 0.01%	40.05 B	143.15 A	150.67 A	29.45 B
PLA-THF 0.1%	40.34 B	144.10 A	151.10 A	28.09 B
PLA-THF 1%	40.88 B	143.14 A	151.25 A	28.35 B
PLA-THF 5%	37.86 BC	143.87 A	153.03 A	25.17 B
PLA-THF-G 0%	41.54 B	141.58 A	148.70 B	27.46 B
PLA THF-G 0.1%	42.40 B	141.11 A	148.87 B	31.54 B
PLA-THF-G 1%	39.74 B	142.25 A	146.12 B	26.76 B
PLA-THF-G 5%	41.86 B	141.08 A	146.87 B	28.99 B
PLA-DMF 0%	40.91 B	145.92 A	151.59 A	29.29 B
PLA-DMF 0.01%	43.22 B	145.89 A	152.22 A	29.88 B
PLA-DMF 0.1%	42.84 B	145.50 A	151.64 A	27.31 B
PLA-DMF 1%	41.37 B	145.48 A	151.70 A	30.87 B
PLA-DMF 5%	37.36 C	143.53 A	149.28 A	29.17 B
PLA-DMF-G 0%	44.72 B	142.11 A	149.20 B	31.50 B
PLA-DMF-G 0.1%	45.35 B	141.95 A	149.05 B	35.10 B
PLA-DMF-G 1%	49.24 B	140.89 A	148.20 B	28.47B
PLA-DMF-G 5%	47.66 B	144.20 A	148.65 B	34.88 B
PLA- THF	39.97 b	143.97 b	151.62 a	28.79 a
PLA-THF-G	41.38 b	141.51 c	147.64 b	28.47 a
PLA-DMF	41.14 b	146.37 a	151.29 a	29.51 a
PLA-DMF-G	46.77 a	140.14 c	148.78 b	32,49 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.

decreases in both PLA-THF-G and PLA-DMF-G films, as would be expected.³¹ PLA-DMF films seem to be somewhat more ductile than PLA-THF films although differences were not found significant. Again, the incorporation of silver did not significantly affect either parameter on both PLA-THF and PLA-DMF films.

Color Analysis

Transparency is highly desirable in many applications and PLA is a highly transparent polymer. Color measurements contribute to objectively differentiate and evaluate changes in the color of the films. All films tested showed a high transparency, except PLA-DMF films containing silver and PLA-DMF-G films with ≥ 1 wt % silver content (Table IV). In these cases, the decrease in transparency (ΔL^*) and a significant increase in yellowness (Δb^*) was associated with the increase in silver content. In PLA-DMF samples with 1 and 5 wt % silver content, where these changes are higher, a significant increase in redness is also observed. In PLA-THF and PLA-THF-G films, however, the slight changes in color are not significant. Silver is known to readily reduce to elemental particles in slightly reducing environments. The silver particles formed, if small enough, generate a yellow to brownish color depending on their size and shape due to plasmon resonance.^{34–36} DMF is a weak reducing agent and the reduction of silver when in contact with this solvent has been reported previously.^{36–40} How-

ever, the increase in yellowness in the films is mostly noticeable for high silver concentrations. Additionally, slightly acidified conditions during casting were found to decrease this color alteration (data not shown). Interestingly, color alteration by the addition of silver is attenuated if glycerol is present. This could be attributed to a better stabilization of silver which was found to be preferentially confined within the glycerol domains (Figure 2).

Release Study

For several applications of antimicrobial films in foods or other related fields, it is desirable that the release of the antimicrobial be activated upon contact with moisture and then sustainably maintained over the shelf-life of the product. To evaluate how the different silver contents, the solvent mixture used or the plasticizer might affect the controlled release, silver ion content from PLA-THF, PLA-DMF, and PLA-DMF-G films with different silver content was measured by means of ASV over successive daily washings. ASV has been proven a useful tool to monitor the release of traces of silver from polymer matrices.^{41–44} The release of PLA-THF-G films was not monitored in detail, as preliminary results showed that release was not sustained (data not shown). The cumulative amounts of silver recovered throughout the experiment from PLA-THF films and PLA-DMF films with and without glycerol are presented in Figure 4(A,B), respectively.

Table III. Mechanical Properties of the Various Films Tested

Sample	Modulus (Mpa)	Elongation at break (%)	Max. tensile strength (MPa)
PLA-THF 0%	1664 ± 165 A ^a	4.4 ± 0.4 C	42.3 ± 2.7 AB
PLA-THF 0.1%	1745 ± 141 A	4.0 ± 0.1 C	47.8 ± 2.7 AB
PLA-THF 1%	1583 ± 189 A	4.5 ± 0.5 C	40.9 ± 2.9 ABC
PLA-THF 5%	1725 ± 234 A	5.0 ± 0.3 C	44.7 ± 4.5 AB
PLA-THF-G 0%	1588 ± 38 A	28.4 ± 3.3 AB	34.9 ± 2.0 ABC
PLA THF-G 0.1%	1621 ± 95 A	28.4 ± 9.6 AB	36.4 ± 1.8 ABC
PLA-THF-G 1%	1514 ± 175 A	27.5 ± 4.0 AB	33.6 ± 0.3 ABC
PLA-THF-G 5%	1596 ± 44 A	33.2 ± 10.1 A	35.9 ± 0.2 ABC
PLA-DMF 0%	1601 ± 104 A	8.0 ± 1.1 C	45.4 ± 3.3 AB
PLA-DMF 0.1%	1682 ± 57 A	7.2 ± 1.3 C	47.1 ± 4.7 A
PLA-DMF 1%	1576 ± 81 A	5.6 ± 0.4 C	42.1 ± 2.7 AB
PLA-DMF 5%	1498 ± 123 A	9.1 ± 1.7 BC	42.0 ± 1.8 ABC
PLA-DMF-G 0%	1185 ± 49 A	12.7 ± 1.8 BC	31.4 ± 2.1 ABC
PLA-DMF-G 0.1%	671 ± 216 B	16.5 ± 5.9 ABC	31.6 ± 3.9 BC
PLA-DMF-G 1%	705 ± 178 B	14.2 ± 6.3 ABC	29.8 ± 4.5 BC
PLA-DMF-G 5%	674 ± 205 B	18.5 ± 7.4 ABC	25.6 ± 1.2 C
PLA- THF	1679 ± 206 a	4.6 ± 0.5 b	44.5 ± 4.2 a
PLA-THF-G	1580 ± 155 a	25.6 ± 10.5 a	33.9 ± 3.1 b
PLA-DMF	1595 ± 115 a	7.3 ± 1.9 b	43.6 ± 3.7 a
PLA-DMF-G	808 ± 198 b	15.6 ± 5.3 b	30.0 ± 3.7 b

^aMean 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.

Table IV. Colour Analysis of PLA Films Cast with THF (PLA-THF) or a 3 : 1 (w/w) Mixture of THF : DMF (PLA-DMF) Incorporating Different Silver Contents

Sample	L*	a*	b*	ΔE
Standard plate	94.28 A ^a	0.56 A	2.83 A	-
PLA-THF 0%	93.85 A	0.67 A	3.11 A	0.78 A
PLA-THF 0.1%	94.56 A	0.66 A	3.25 A	0.52 A
PLA-THF 1%	92.27 A	0.84 A	5.40 AB	3.28 AB
PLA-THF 5%	93.44 A	0.69 A	3.35 A	1.01 AB
PLA-THF-G 0%	94.43 A	0.47 A	3.22 A	0.44 A
PLA THF-G 0.1%	94.12 A	0.51 A	3.33 A	0.68 A
PLA-THF-G 1%	94.32 A	0.52 A	3.37 A	0.56 A
PLA-THF-G 5%	94.30 A	0.17 A	4.13 A	1.37 AB
PLA-DMF 0%	93.71 A	0.61 A	3.11 A	0.64 A
PLA-DMF 0.1%	91.06 B	1.14 A	9.17 BC	7.18 AB
PLA-DMF 1%	72.59 D	4.75 B	14.25 DE	24.95 AB
PLA-DMF 5%	61.99 E	3.39 B	16.86 E	35.33 B
PLA-DMF-G 0%	93.71 A	0.45 A	3.52 A	0.93 A
PLA-DMF-G 0.1%	93.82 A	0.66 A	3.29 A	0.68 A
PLA-DMF-G 1%	84.01 BC	-0.22 A	16.95 E	17.53 AB
PLA-DMF-G 5%	81.31 C	1.12 A	12.38 CD	16.26 AB

^aMean 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.

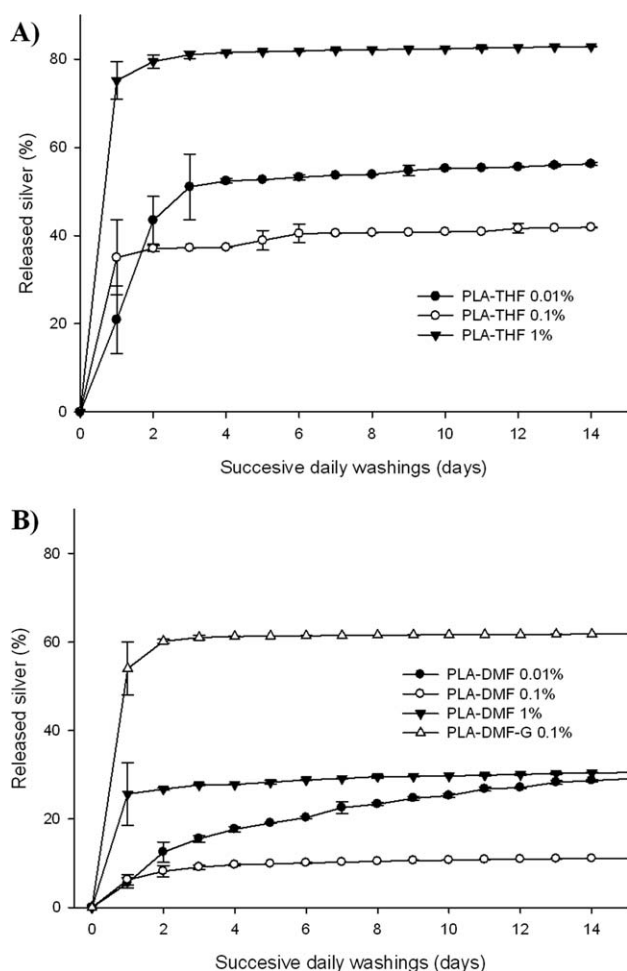


Figure 4. Total accumulated release of silver from (A) PLA-THF and (B) PLA-DMF and PLA-DMF-G films with increasing silver contents after successive daily washings.

In all tested films, there is an initial burst release before 24–48 h, after which ions are discharged in much slower and sustained kinetics. This correlates with previous works.²⁵ However, despite following analogous patterns, there are remarkable differences between the samples depending on the solvent used in the casting, the silver contents incorporated in the film or the addition of glycerol. With increasing silver load, a higher initial burst release and a smaller slope of sustained release is observed in the films. This effect is far more noticeable in PLA-THF films [Figure 4(A)]. As an example, in PLA-THF films with 1 wt % silver, the amount of ions released within 24 h is far greater than the cumulative release throughout the rest of the experiment. In films PLA-THF with 0.01 wt % silver, however, this burst release regime is prolonged for 2–3 days and the amount of silver released daily afterward is higher [Figure 4(A)]. On the other hand, when the release patterns of films with equal amount of silver but different solvent are evaluated, PLA-THF films show stronger burst release and lower slope of sustained ion release, as compared to PLA-DMF films [Figure 4(A,B)]. This is especially evident at higher silver concentrations, while no such differences are observed between solvents when 0.01 wt % silver is incorporated. These differences appear to be in good agreement with the morphological analysis and could

so be explained in terms of solubility. As explained in the morphological analysis, agglomerates of silver nitrate salts were found to crystallize and precipitate during solvent evaporation depending on solubility. Upon immersion in an aqueous solution, these agglomerates would be more rapidly dissolved, producing a burst release of silver ions. Hence, a higher silver content would increase the amount of agglomerated silver available for burst release. The use of a more compatible solvent mixture, like DMF : THF, would help increase the solubility of the silver salt, which could explain the slower burst release but a faster sustained release of silver compared to PLA-THF films. The release of silver from all these films is, generally speaking, higher than previously reported results on PLA or other polyester films.^{45,46} In these cases, however, the release was found to be much more sustained, either because the silver was in form of silver nanoparticles or because ions were stabilized within a mineral carrier. Considering the more sustained release of PLA-DMF films with lower silver contents, the effect of glycerol in the release profiles of these films was further studied in these films [Figure 4(B)]. In this case, the addition of the plasticizer led to a drastic increase in burst release, whereas the sustained release was slightly decreased. As seen in previous analysis, glycerol was not found to be miscible with the PLA matrix (Figures 1 and 2) and did not promote substantial changes in the crystalline structure of the polymer (Tables I and II). Additionally, a significant amount of silver was observed to be confined within the glycerol phases allowing a faster release of the antimicrobial entrapped in this phase. These results evidence that the selection of different solvents or the addition of a plasticizer such as glycerol may serve as an additional tool to tailor the release capacities of silver based antimicrobial polyesters. This may be useful for implementation of these technologies in the food or in other areas. For example in food products there may be a need for an initial higher biocide capacity followed by a slower sustained release to prevent recontamination.

Long-Term Antimicrobial Performance After Weekly Washings

To evaluate the antimicrobial effectiveness of the produced films, susceptibility assays were performed against the food-borne pathogen *Salmonella enterica* in M9. The synthetic medium contains glucose as a sole carbon source and could stand for a hypothetical environment of surface contamination.²² First, the released silver over successive weekly washings was tested as to evaluate endurance of the antimicrobial activity of the PLA-THF [Figure 5(A)] and PLA-DMF [Figure 5(B)] films after a relatively long term in contact with moisture. Control films without silver did not produce any changes in viable counts as compared to samples without film, indicating antimicrobial effectiveness was only due to the release of silver. All tested films released enough silver during the first week as to decrease viable counts of *Salmonella* below the detectable threshold [10 CFU mL^{-1} ; Figure 5(A,B)]. Antibacterial effectiveness of PLA-THF and PLA-DMF films with 0.01 wt % was not sufficient from the second week on as samples were able to reach the same viable counts as the controls. In PLA-DMF-G samples with 0.1 wt % silver, a reduction of viables of at least 2 log is noted until week 4 [Figure 5(B)]. After that time, the antibacterial effect is gradually lost thorough weeks 5–7. When PLA-DMF films with the same silver content were evaluated, no viable

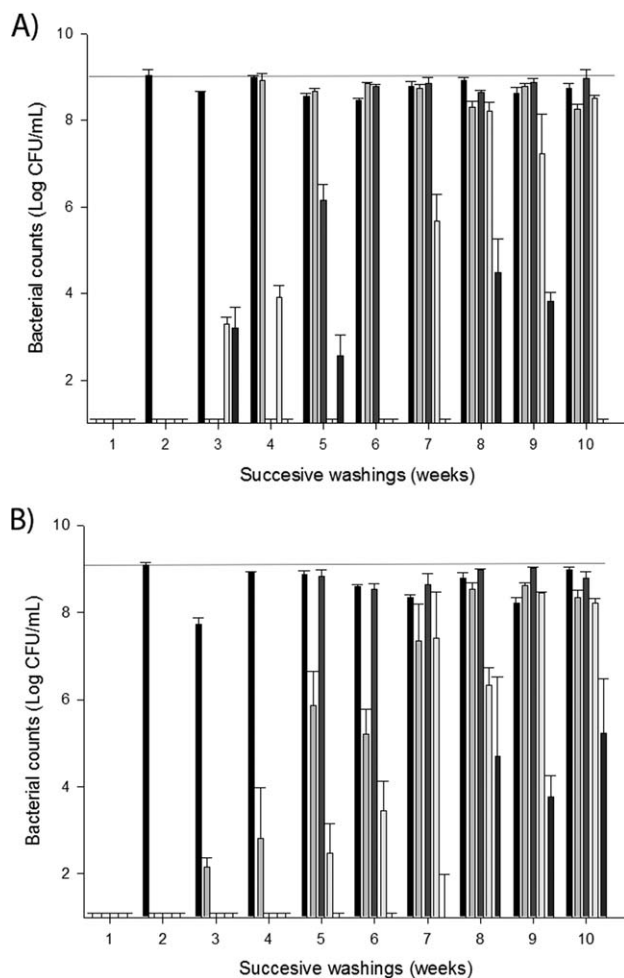


Figure 5. Viability of *Salmonella enterica* after incubation with silver released from (A) PLA-THF and (B) PLA-DMF films with 0.01 wt % silver, 0.1 wt % silver and glycerol, 0.1 wt % silver, 1 wt % silver, and glycerol, and 1 wt % silver. Line depicts average bacterial counts for reference samples without silver.

counts were detected until week 4, after which efficacy is totally lost. When 1 wt % silver is incorporated in PLA-DMF and PLA-DMF-G films, no detectable counts are observed until week 4 and 7, respectively. While PLA-DMF films with 1 wt % silver demonstrate antibacterial efficacy thorough the 10 weeks in contact with water, this is substantially reduced or lost after week 7 for PLA-DMF-G films with the same silver content. This indicates that the presence of glycerol does not improve the long-term efficacy of PLA-DMF films. The same effect is observed when THF is used as solvent. In PLA-THF-G and PLA-THF films with 0.1 wt % silver, a reduction of viables as compared to controls without silver is observed after week 3 and 5, respectively. PLA-THF-G and PLA-THF films with 1 wt % silver content exert an antibacterial effect for at least 7 and 10 weeks, respectively. In both cases, the addition of glycerol reduces the long-term efficacy of the films. Glycerol is usually added to drug releasing polymers as a plasticizer in order to increase the release capacities. However, release analysis revealed that only the burst release was increased when glycerol was incorporated in the films, whereas the sustained release was slightly decreased. Antimicrobial per-

formance of these films in this relatively long-term study is therefore in line with the release analysis and points out that the addition of glycerol may enhance the antimicrobial performance at the burst release stages but would be detrimental if a more sustained release is desirable. When the effect of the use of a different solvent mixture in the casting is compared among films with the same characteristics, it was found that the effectiveness of films formed with only THF were less sustained and more erratic. As an example, in PLA-THF samples with 1 wt % silver no viable bacteria were detected on weeks 1, 2, 4, 6, 7, and 10, while with analogue PLA-DMF films the same effect is achieved sustainably until week 8. This behavior can again be attributed to the different compatibility of silver with the solvent mixture used and is in line with the release results discussed above. The inconsistency of the sustained effectiveness of PLA-THF films throughout longer periods advises against their use for applications in which a long lasting biocide effect is desired. These films would be preferentially suitable for applications in which a greater burst effect is desired in the first stages in contact with moisture.

Antimicrobial Performance After Daily Washings

Considering that the films demonstrated high antimicrobial efficacy immediately after contact with the solution, it is important to ascertain if this release may be sufficiently sustained as to allow a continuous effect over time in shorter terms. Therefore, a second assay was conducted evaluating the effectiveness of the released silver from PLA-THF and PLA-DMF films with 0.01 and 0.1 wt % silver after daily washing. Additionally, silver in the solutions was quantified before inoculation, in order to accurately assess the required silver concentration in solution as to exert an antibacterial effect and establish breakpoints for silver under the stated conditions. As silver release was not constant in all samples, the released silver from only one replicate of the release study was incubated per triplicate with *S. enterica*. Released silver and the corresponding viable count number detected for each sample and through 14 days are presented in Table V. As shown in the release results, a considerable amount of the silver is released within the first 1 or 2 days, the release being more sustained in film samples with 0.01 wt % silver. Accordingly, no bacterial counts were detected for this period in any of the samples tested. In PLA-THF films, the antibacterial effect was found to be less sustained than in PLA-DMF films. With 0.01 wt % silver, no viables are detected until days 2 and 5, respectively, while the same effect is achieved with 0.1 wt % silver until days 4 and 9, respectively. Moreover, the release and subsequent antibacterial effect follows a more irregular pattern in PLA-THF than in PLA-DMF films. This behavior again correlates with the release results as well as with the morphology of both films as discussed above. The addition of glycerol to PLA-DMF films resulted in a reduction of the sustained antimicrobial effect from 9 to 5 days as compared with the same material without glycerol. Interestingly, the silver detected on the test tubes previous to inoculation can be quite well correlated with its corresponding antimicrobial effect in any of the samples. As an example, a silver concentration of ≤ 8 ppb did not produce any reduction of viable counts for any of the samples tested. On the other hand, no detectable viables were found when the test tubes contained ≥ 18 ppb silver. For samples where about 10–20 ppb silver were detected, the

Table V. Release and Antibacterial Performance of Silver Released from PLA-THF and PLA-DMF Films with 0.01 and 0.1 wt % Silver Over Consecutive Daily Washings

Time (day)	PLA-THF				PLA-DMF				PLA-DMF-G	
	Silver contents (wt %)									
	0.01 wt %		0.1 wt %		0.01 wt %		0.1 wt %		0.1 wt %	
	R ^a	B ^b	R	B	R	B	R	B	R	B
1	263	<1	2742	<1	45	<1	728	<1	3991	<1
2	264	<1	257	<1	59	<1	230	<1	502	<1
3	17	5.24 ± 0.13 ^c	16	<1	31	<1	122	<1	51	<1
4	12	4.89 ± 0.38	13	5.66 ± 0.24	18	<1	63	<1	15	<1
5	3	8.57 ± 0.03	≤1	8.97 ± 0.13	12	<1	20	<1	9	6.58 ± 0.16
6	10	5.54 ± 0.27	241	<1	10	6.23 ± 0.25	21	<1	6	8.92 ± 0.03
7	4	8.97 ± 0.10	13	<1	30	<1	15	<1	4	8.99 ± 0.06
8	≤1	8.78 ± 0.07	12	<1	3	8.78 ± 0.15	17	<1	5	9.03 ± 0.12
9	≤1	8.89 ± 0.02	6	7.01 ± 0.55	15	<1	16	<1	6	8.79 ± 0.09
10	3	9.04 ± 0.04	17	<1	3	8.87 ± 0.07	6	8.95 ± 0.19	3	8.98 ± 0.02
11	≤1	8.85 ± 0.11	2	8.89 ± 0.03	10	5.52 ± 0.13	13	<1	≤1	8.83 ± 0.08
12	≤1	8.65 ± 0.15	124	<1	2	9.01 ± 0.08	5	8.26 ± 0.09	4	8.73 ± 0.15
13	5	8.54 ± 0.21	7	8.69 ± 0.11	11	4.73 ± 0.31	9	5.42 ± 0.33	7	8.79 ± 0.12
14	≤1	7.98 ± 0.05	18	<1	≤1	8.54 ± 0.15	3	8.96 ± 0.10	2	9.06 ± 0.05

R^a: released silver ions (ppb) B^b: Bacterial counts (log CFU mL⁻¹) ^c: Standard deviation.

The initial bacterial counts as determined directly after inoculum were 5.75 ± 0.10 log CFU mL⁻¹. In reference samples without film or with PLA films without silver, bacterial counts after 24-h incubation were 8.97 ± 0.04 and 8.92 ± 0.12 log CFU mL⁻¹, respectively. All experiments were carried out in triplicate.

antibacterial effect varied substantially, which suggests that this might be the threshold range to be considered as a breakpoint for the tested strain and under the stated conditions. The very low concentrations necessary to exert an antibacterial effect evidence the outstanding potential of silver as antimicrobial and are in line with previous results in other synthetic media.^{22,47–49} Although literature has extensively reported the use of polymers incorporating silver nanoparticles for their possible use in food packaging applications, nanoparticles are only beginning to be considered within the frames of existing legislation in most countries, including the EU. Antimicrobial materials based on the release of ionic silver are, on the other hand, permitted and widely used in most countries.^{11–13} These materials are mostly based on silver exchange from resins or other inorganic fillers, like montmorillonites (MMT), zeolites^{41,46,50} being the most widely used of all, which are applied in all kinds of contact surfaces to keep them safe.¹⁴ In these cases, however, filler contents were in the range of 1–10 wt %. In the present study, the developed PLA films without further inorganic fillers led to a strong antibacterial effect with filler content of 0.01 and 0.1 wt %, respectively, which could allow the possible application of silver based polymers in food packaging or food–contact surfaces. The EFSA has stated a stringent migration limit of 50 µg silver/kg food (or ppb). The results in the present study evidence that a bactericidal effect can be achieved in solution even below this value, following a controlled and sustained release over storage time or even after successive washing (Table V).

CONCLUSIONS

The incorporation of silver into PLA films by casting revealed the solvent used greatly influenced the properties of the materials.

Silver was found to be less compatible with PLA-THF films, which was reflected in crystallization of silver at the surface at high silver concentrations, a higher burst release much depending on silver concentration and an inconsistent pattern of antimicrobial effectiveness when evaluated over a long period of contact with moisture. PLA-DMF, on the contrary, were more compatible, displayed more sustained release patterns and demonstrated a high antimicrobial performance over months contact. However, the film-forming conditions were detrimental for their mechanical properties and transparency at high silver concentrations. The addition of glycerol produced silver to be preferentially confined within this segregated phase producing a higher release of silver in the first stages. Antimicrobial performance was correlated with silver ion concentration in solution and the necessary concentration of silver to exert an antimicrobial effect was found to be in 10–20 ppb range. This evinces application of silver based antimicrobial technologies may be not only limited to surfaces, as in current applications. It may as well be possible to generate enough silver ions in a liquid environment to efficiently kill a common food-borne pathogen, such as *S. enterica*, without surpassing the stringent migration limitations stated by the EFSA. However, in the PLA-THF and PLA-DMF films tested, the sustained release proceeded in all cases by an initial burst release of 5–80% of the whole silver content upon immersion in water, which may impose some limitations for their use as such in direct food contact for instance. Currently, other strategies are being developed to tailor the release, such as multilayers, which will be the subject of further studies.

ACKNOWLEDGMENTS

The authors thank the Spanish ministry of economy and competitiveness (MAT2012-38947-C02-01) for financial support. A. Martínez-Abad was supported by the Spanish Council for Scientific Research (CSIC) under grant JAE_pre092.

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