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Development and characterization of active films based on STARCH-PVA, containing silver nanoparticles

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Abstract

In order to obtain antimicrobial packaging films, starch-PVA-based films with silver nanoparticles (AgNPs) have been developed and characterized as to their physical and antimicrobial properties and silver release kinetics to polar (A, B, C and D1) and non-polar (D2) food simulants. Antimicrobial activity against two bacteria, *Listeria innocua* and *Escherichia coli,* and two fungi, *Aspergillus niger* and *Penicillium expansum,* was studied. Silver-loaded starch-PVA films exhibited antimicrobial activity against the tested microorganisms, which depended heavily on the concentration of AgNPs. Their addition only led to notable physical changes in the colour and transparency of the films, which underwent significant changes and turned brownish-yellow and opaque, this being more notable when the silver concentration rose. Silver was released into aqueous simulants in its entirety within the first 60 minutes of contact. In the non-polar simulant (oleic acid), the release capacity of the films drastically decreased, being the only case where the established limit (60 mg/Kg simulant) was met. As a consequence, the use of the developed films as food packaging materials should be restricted to fat-rich foodstuffs.

Key words: antimicrobial activity; release kinetics; mechanical properties; optical properties.

1. Introduction

About one third of the food produced for human consumption is lost or wasted worldwide, this being approximately 1,300 million tonnes per year (FAO, 2011). These losses take place along the food supply chain due to physical, chemical and biological factors. For example, as a result of microbial growth, off-odors and changes in the aroma, color, and texture can be accelerated. Additionally, some microorganisms and their toxins may cause food recalls and serious foodborne outbreaks (Corrales, Fernández & Han, 2014).

The considerable pressure placed on achieving a reduction in these losses has increased the interest in developing new packaging materials which lead to the retardation of deterioration, the extension of the shelf-life, and the quality maintenance of the foodstuff. The incorporation of natural active substances in film matrices is a current alternative means of preventing food spoilage (Lanciotti, Gianotti, Patrignani, Belletti, Guerzoni & Gardini, 2004).

Heavy metals from mineral sources have been used in the form of salts, oxides, and colloids for thousands of years because of their antimicrobial properties. These metals can be incorporated into food-contact polymers to enhance the mechanical and barrier properties and to extend food shelf life (Pal, Tak & Song, 2007). Of the metals, silver exhibits a higher degree of toxicity to microorganisms while being less toxic to mammalian cells in minute concentrations (Rai, Yadav & Gade, 2009). Silver has strong inhibitory or bactericidal effects for a broad spectrum of bacteria, fungi, and viruses (Ghosh et al., 2010; Mohanty, Mishra, Jena, Jacob, Sarkar & Sonawane, 2012; Li, Xie, Shi, Zeng, OU-Yang & Chen, 2010). Moreover, its high thermal stability, low volatility and cost of production are remarkable (Duran, Marcarto, de Souza, Alves & Esposito, 2007, Martínez-Abad, Lagarón & Ocio, 2014b).

Silver in its metallic state is an inert material, but it can react with the environmental moisture to provide silver ions. The catalytic oxidation of metallic silver and the reaction

with dissolved monovalent silver ion probably contribute to the bactericidal effect (Martínez-Abad, 2014a; Pal et al., 2007; Rai et al., 2009). In spite of that, the exact mechanism of the action of silver species is not well known. Some studies describe it as based on the morphological and structural changes found in the bacterial cells (Rai et al., 2009). The mechanism of action of metallic silver, silver ions and silver nanoparticles (AgNPs) is linked with its interaction with the thiol group (-SH) compounds found in the respiratory enzymes of bacterial cells. For example, the interactions of silver with L-Cysteine residues cause the denaturation and loss of enzymatic functions (Feng, Wu, Chen, Cui, Kim & Kim, 2000; Martínez-Abad, et al., 2014b; Liau, Read, Pugh, Furr, & Russell, 1997). The mode of antibacterial action of AgNPs is probably similar to that of silver ions (Mohanty et al., 2010) and different authors report that the antimicrobial effect of silver nanoparticles depends on their size and shape (Ghosh et al., 2010; Rai et al., 2009; Raimondi, Scherer, Kotz & Wokaun, 2005).

Wet chemical reduction is the most frequently applied method for the synthesis of AgNPs. For the chemical synthesis, the use of different radiation sources and/or a combination of different strong reducing agents have been applied in the presence of stabilizers in order to prevent the unwanted agglomeration of the colloidal forms (Mohanty et al., 2012; Neto, Ribeiro & Zucolotto, 2008). Most of these methods, which make use of strong reducers, lead to environmental toxicity risks. As an alternative, the green synthesis of AgNPs has been developed, which involves the selection of a solvent medium and environmentally-friendly reducing agents and stabilizers (Raveendran, Fu & Wallen, 2003; Sharma, Yngard, & Lin, 2009). Some authors followed these steps by using sunlight or UV radiation (Pourjavadi & Soleyman, 2011; Vimala et al., 2011), reducing biopolymers such as poly(vinyl alcohol) (Bryaskova, Pencheva, Kale, Lad & Kantardjiev, 2010), poly(vynil pyrrolidone) (Morales, Morán, Quintana & Estrada, 2009), gelatin (Darroudi, Ahmad, Zamiri, Zak, Abdullah & Ibrahim,

2011; Pourjavadi & Soleyman, 2011), starch (Torres-Castro, González-González, Garza-Navarro & Guana-González, 2011), poly(ethylene glycol) (Vimala et al., 2011), or even plant extracts (Mohapatra, Kuriakose & Mohapatra, 2015; Roy, Sarkar & Ghosh, 2015). The preparation of nanoparticles within biopolymers provides several advantages due to the fact that macromolecular chains possess a large number of hydroxyl groups that can complex the metal ion, thus enabling a good control of the size, shape and dispersion of nanoparticles, increasing biocompatibility and biodegradability, and giving rise to species that are less toxic to mammalian cells (Mohanty et al., 2010).

The Food and Drug Administration/Centre for Food Safety and Applied Nutrition (FDA/ CFSAN – USA) accepted the use of silver nitrate as a food additive in bottled water and silver zeolites for use in all types of food-contact polymers (FDA, 2010), while in the European Regulation silver is accepted under 94/36/EC Directive as a colouring agent (E-174) with no restrictions. Silver is one of the most widely used antimicrobial additives in polymer films for food packaging applications (Martínez-Abad, Sánchez, Lagarón & Ocio, 2012). This approach has been tested on a wide variety of biopolymer matrices including hydroxyl propyl methyl cellulose (de Moura, Mattoso & Zucolotto, 2012), agar (Ghos et al., 2010; Rhim, Wang & Hong, 2013), (poly)vinyl alcohol (Bryaskova et al., 2010; Sedlarik, Galya, Sedlarikova, Valasek & Saha, 2009), gelatin (Kanmani & Rhim, 2014) or blends such as chitosan-cellulose (Lin, Chen, Huang, Cao, Luo, & Liu, 2015), starch-clay (Abreu, Olivera, Rodeigues, Cerqueira, Vicente & Machado, 2015) or chitosan-PVA-Glutaraldehide (Vimala et al., 2011).

Previous studies revealed that blend films based on starch-PVA presented several advantages over pure starch films. The incorporation of PVA into gelatinized starch matrices implied the formation of interpenetrated polymer networks with beneficial effects on the mechanical and water barrier properties of the films, these becoming much more extensible and stable during storage (Cano, Fortunati, Cháfer, Kenny,

Chiralt & González, 2015). These results suggest that starch-PVA-based films could be a proper alternative for the development of active films containing silver nanoparticles. These silver particles might be able to improve the physical properties of films and to control the food spoilage. To the best of our knowledge, no studies about the blend starch-PVA-silver nanoparticles have been published.

In the development of silver-loaded films, knowledge of the release kinetics of the active compound is needed in order to ensure that it complies with the current legislation for food packaging materials (Commission Regulation EU 10/2011), while assuring antimicrobial effectiveness.

The aim of the work was to develop active starch-PVA-based films which are able to deliver silver species. In this sense, the release kinetics of silver from starch-PVA films to different food simulants as well as their physical properties and antimicrobial activity against two bacteria *Listeria innocua* and *Escherichia coli* and two fungi, *Aspergillus niger* and *Penicillium expansum* were studied.

2 **Materials and Methods**

2.1 Materials

Pea starch (S) was purchased from Roquette Laisa España S.A. (Benifaió, Valencia, Spain), poly(vinyl alcohol) (PVA)(M_w: 89,000-98,000, degree of hydrolysis > 99 %, and viscosity: 11.6-15.4cP) and silver nitrate ($AgNO_3$) were obtained from Sigma Aldrich Química S.L. (Madrid, Spain) and glycerol, magnesium nitrate-6-hydrate ($Mg(NO_3)_2$), ethanol, 98% glacial acetic acid and oleic acid were provided by Panreac Química S.A. (Castellar de Vallès, Barcelona, Spain).

2.2. Preparation of film forming dispersions

Films were obtained by means of the solvent casting procedure after the preparation of film forming dispersions (FFDs) following the methodology described by Cano et al.

144 (2015). Starch (2% w/w) was dispersed in an aqueous solution at 95 ºC for 30 min,
145 while being stirred, to induce starch gelatinization. Thereafter, the dispersion was
146 homogenized using a rotor-stator homogenizer (Ultraturrax D125, Janke and Kunkel,
147 Germany) at 13,500 rpm for 1 min and 20,500 rpm for 3 min. Afterwards, PVA was
148 incorporated into the previously gelatinized starch dispersion in a S:PVA ratio of 2:1
149 and the dispersion was maintained at 90 ºC for 30 min under stirring. Finally, glycerol
150 was added at a starch:glycerol ratio of 1:0.25, on the basis of previous studies
151 (Jiménez, Fabra, Talens & Chiralt, 2012).
152 Starch-PVA film forming dispersions containing silver nanoparticles were obtained by
153 the reduction of silver nitrate salts using UV light (Monge, 2009) in the starch-PVA
154 dispersion itself, taking advantage of the stabilizing properties of the polymers (Torres-
155 Castro et al., 2011). The synthesis can be summarized as follows: different amounts of
156 40 mM $AgNO_3$ were added to the previously described starch-PVA dispersions so as to
157 obtain different S:$AgNO_3$ ratios: 1:0.006, 1:0.06, 1: 0.16 and 1:0.32. Each mixture was
158 maintained at 90 ºC for 30 min under stirring and UV radiation till the dispersion turned
159 brown due to the formation of AgNPs. Finally, glycerol was also added in the same
160 ratio as in the control film. The reduction of silver nitrate into an AgNPs formation was
161 monitored by using a DU 730 spectrophotometer (Thermo Scientific, England) at 420
162 nm.
163
164 2.3. Film formation
165 From the above described silver starch-PVA dispersions, dried films were obtained by
166 means of the casting method. Newly obtained dispersions were poured into a Teflon
167 plate at a surface density of solids of 85 g m^{-2}. For antimicrobial tests, films were
168 casted into Petri dishes, by using the same amount of the film-forming dispersion.
169 Films were dried at 22 ºC and 45 % HR for 48 h and afterwards, peeled off the casting
170 surface. They were conditioned at 25 ºC and 53 %RH in a chamber using a $Mg(NO_3)_2$

saturated solution until further analysis. The film thickness was measured at six random positions with a Palmer digital micrometer to the nearest 0.0025 mm, reaching values of between 0.058 and 0.067 mm. The control film (S-PVA) and four silver-loaded films were obtained with the increasing amounts of $AgNO_3$ (S-PVA1, S-PVA2, S-PVA3, S-PVA4). All the films were analyzed after one or five storage weeks, according to previous studies (Cano, Jiménez, Cháfer, González & Chirialt, 2014). For antimicrobial analysis and release studies, the films were only conditioned for one week.

2.4. Characterization of composite films

2.4.1. Moisture content

Moisture content (MC) was evaluated by drying. Firstly, the film samples were dried in a vacuum oven at 60 ºC for 24 h. Later on, the pre-dried samples were placed in desiccators containing P_2O_5 (RH 0 %) at room temperature until reaching a constant weight (around 2 weeks). Five replicates per film formulation were analysed.

2.4.2. Water vapour permeability (WVP)

Water vapour permeability (WVP) was evaluated in films equilibrated by following the gravimetric method, ASTM E96-95 (ASTM, 1995), using Payne permeability cups (Payne, elcometer SPRL, Hermelle/sd Argenteau, Belgium) of 3.5 cm diameter. Deionised water was used inside the testing cup to achieve 100 % RH on one side of the film, while an oversaturated magnesium nitrate solution was used to control the RH on the other side of the film. A fan placed on the top of the cup was used to reduce resistance to water vapour transport. Four replicates of each type of films were analysed at 25 ºC. The water vapour transmission (WVTR) was determined from the slope obtained from the regression analysis of weight loss data *vs* time, once the steady state had been reached, divided by the film areas.

198

199 2.4.3. Mechanical properties

200 Mechanical properties were measured using a Universal Test Machine (TA.XT plus,
201 Stable Micro Systems, Haslemere, England) following the ASTM standard method
202 D882 (ASTM, 2001). Equilibrated film specimens (2.5 cm wide and 10 cm long) were
203 mounted in the film-extension grips (A/TG model) which were set 50 mm apart. The
204 speed of the testing machine during stretching was 50 mm min^{-1} until breaking. Force-
205 distance curves were obtained and transformed into Stress-Hencky curves which
206 allowed tensile strength at break (TS, MPa), percentage of elongation at break (E, %)
207 and elastic modulus (EM, MPa) to be obtained. Eight replicates were carried out per
208 formulation.

209

210 2.4.4. Optical properties

211 The CIE-L*a*b* coordinates and internal transmittance (Ti) of the films was quantified
212 by means of the reflection spectrum on the white and black background from 400 to
213 700 nm with a MINOLTA spectrocolorimeter CM.36000d (Minolta Co. Tokyo, Japan)
214 with a 30 mm illuminated sample area, using D65 illuminant/ 10 º observer.
215 Measurements were taken on the side of film which was in contact with air during
216 drying and each formulation was analyzed in triplicate. Ti was calculated applying the
217 Kubelka–Munk theory for multiple scattering to the reflection spectra, following the
218 methodology described by Cano, et al., (2014).

219

220 2.4.5. Thermogravimetric analysis (TGA)

221 A thermogravimetric analyzer (Mettler Toledo, Switzerland) was used to obtain the
222 thermal weight loss (TG) curve, and its derivative (DTG), of the samples. To this end,
223 approximately 10 g of sample were poured into an alumina crucible and heated from 25
224 ºC to 600 ºC at 10 ºC/min, using nitrogen flow. The onset, peak and end temperatures

225 (T_0, T_p and T_e, respectively) were obtained for each degradation step in the films. The
226 measurements were taken in duplicate for each film.
227
228 2.4.6. Kinetics of silver release
229 The kinetic studies of silver release were carried out by following current legislation
230 (Commission Regulation EU 10/2011). Rectangular film strips of 12 cm^2 total area were
231 immersed in a glass tube with 20 mL of food simulants: simulant A (ethanol 10 % (v/v)),
232 simulant B (acetic acid (3 % (w/v)), simulant C (ethanol 20 % (v/v)), simulant D1
233 (ethanol 50 % (v/v)) and simulant D2 (oleic acid as a vegetal oil), following the
234 established relationship of 6 dm^2 kg^{-1}. Samples were kept at 20 ºC for 7 days. Simulant
235 samples were removed at different times and the released silver was quantified by
236 atomic absorption spectroscopy, Analyst 100 (Perkin Elmer, Madrid, Spain). Before the
237 injection, 5 ml of simulant were properly diluted in distilled water in the case of
238 simulants A, B, C and D1 and in ethanol in the case of simulant D2. The Ag
239 concentrations, expressed as mg kg^{-1} of simulant, were determined from the
240 absorbance values by using a standard curve for $AgNO_3$ solutions. Two replicates per
241 film formulation were performed. Finally, release kinetics were modelled using the
242 Peleg equation (Eq. 1) (Peleg, 1988), determining the k_1, k_2 and V_{eq} values for each
243 experimental series.
244 $$\frac{t}{(M_t - M_0)} = k_1 + k_2 t \quad (1)$$
245 where M_0 and M_t (mg Kg^{-1}) are the concentrations of Ag in the simulant at initial and t
246 (h) times, respectively, k_1 is the Peleg constant rate and k_2 is the Peleg constant
247 capacity. k_2 is also related to the release at $t \rightarrow \infty$ (Eq. 2):
248 $$V_{eq} = M_0 + \frac{1}{k_2} \quad (2)$$
249
250 2.4.7. Microbial analysis

The antimicrobial effectiveness of films was analysed by a method adapted from Kristo, Koutsoumanis, & Biliaderis, (2008) and Sánchez-González, González-Martínez, Chiralt & Cháfer, (2010). Stock cultures of *Escherichia Coli* (CECT 515), *Listeria. Innocua* (CECT 910) and *Asperguillus Niger* (CECT 20156), supplied by Colección Española de Cultivos Tipos (CECT, Burjassot, Spain), were kept frozen (-25ºC) in Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain), for bacteria, and Potato Dextrose Broth (Scharlab, Barcelona, Spain), for fungi, supplemented with 30% glycerol. The Department of Biotechnology (Universitat Politècnica de València, Valencia, Spain) provided *Penicillium expansum* from their culture collection.

Bacteria were regenerated by transferring a loopful of bacteria into 10 ml of TSB and incubating them at 37 ºC overnight. A 10 µl aliquot from the overnight culture was again transferred to 10 ml of TSB and grown at 37 ºC to the end of the exponential phase of growth. This culture, appropriately diluted, was then used for the inoculation of the agar plates in order to obtain a target inoculum of 10^2 UFC/cm^2. Tryptone soy agar with 3 % NaCl (Panreac química, S.A., Castellar del Vallés, Barcelona, Spain) was used as a model solid food system (TSA-NaCl). Aliquots of TSA-NaCl (20 g) were poured into Petri dishes. After the culture medium solidified, a properly diluted overnight culture was inoculated on the surface.

On the other hand, fungi were inoculated on potato dextrose agar (PDA) and incubated at 25 ºC until sporulation. The cells were counted in a hemocytometer and diluted to a concentration of 10^5 spores/ml. Aliquots of PDA (20 g) were poured into Petri dishes. After the culture medium solidified, a diluted spore solution was inoculated on the surface.

Films of the same diameter as the Petri dishes (containing or not an antimicrobial substance) were placed on the inoculated surfaces. Non-coated inoculated TSA-NaCl and PDA Petri dishes were used as controls. Plates were then covered with para-film to avoid dehydration and stored for 12 days at 25 ºC and 10 ºC, for fungi and bacteria

strains, respectively. The microbial counts on the TSA-NaCl and PDA plates were examined immediately after the inoculation and periodically throughout the storage period (0-3-5-7-10-12 days). To this end, the agar was removed aseptically from Petri dishes and placed in a sterile fitter stomacher bag (Seward, West Sussex, United Kingdom) with 100 ml of tryptone phosphate water (Sharlab S.A., Barcelona, Spain). The bag was homogenized for 2 min in a Stomacher blender (Bag Mixer 400, Seward, UK). Afterwards, serial dilutions were made and then poured onto plates for incubation, for 5 days at 25 ºC and for 24 - 48 h at 37 ºC, for fungus and bacteria respectively, before colonies were counted. PDA plates were used to obtain the fungus counts while a selective microbial medium was used for bacteria to obtain a high degree of selectivity and good colonies. *E. coli* was counted in Violet Red Bilis agar (Sharlab S.A., Barcelona, Spain) plates and *L. Innocua* in Palcam Agar Base (Sharlab S.A., Barcelona, Spain) supplemented with Palcam Selective Supplement (Sharlab S.A., Barcelona, Spain). All the tests were performed in triplicate.

2.5. Statistical analysis

Statgraphics Centurion XV.I (Manugistics Corp., Rockville, MD) was used to carry out the statistical analysis of the results through an analysis of variance (ANOVA). To differentiate the samples, Fisher's least significant difference (LSD) was used at the 95 % confidence level.

3. Results and discussion

3.1. Physical properties of films

Table1 shows the elasticity modulus (EM), tensile strength and elongation values at the break point of the films after the two different storage times (one and five weeks) under

303 controlled conditions. Control films (S-PVA) exhibited mechanical behaviour that was
304 halfway between what was observed in the pure pea starch and pure PVA films (Cano
305 et al., 2015).
306 As can be observed, EM and tensile strength values were enhanced at low
307 concentrations of silver (S-PVA1 and S-PVA2), afterwards decreasing as the silver
308 concentration rose while the films became significantly more stretchable at the highest
309 silver concentration. Several authors also obtained similar results, which were
310 attributed to the adsorption of silver to the polymer chains, in line with the van der
311 Waals interactions between the hydroxyl groups of PLA/starch and the partial positive
312 charge on the surface of the silver nanoparticles (Rhim et al., 2013; Shameli et al.,
313 2010). The contrasting behaviour of EM and TS induced by AgNPs from a determined
314 concentration level upwards could be attributed to an oversaturation effect of the
315 polymer network active points for silver adsorption, which leads to a plasticizing effect
316 of silver species in the matrix. In this sense, it is remarkable that the increase in the
317 ionic strength in the aqueous media when the concentration of silver nitrate increases,
318 implies a reduction of the free-volume of macromolecules before the film formation
319 which will affect the chain extension and aggregation level during the film formation
320 step. This effect will reduce the intermolecular forces among polymeric chains, giving
321 rise to weaker films.
322 Thermogravimetric analysis also revealed a decrease in polymer attraction forces when
323 the silver concentration increased. Table 2 shows the onset, peak and end
324 temperatures of the different degradation steps observed for the S-PVA films deduced
325 from the DGTA curves (Figure 1). Previous studies reported the polymer separation in
326 PVA-S blend films (Cano et al., 2015) and, coherently, each polymer degraded
327 independently. The first step was attributed to the starch degradation according to
328 previously reported data (Cano et al., 2015), and the second and third steps to the PVA
329 thermodegradation, as deduced from other authors: a main degradation stage followed

330 by a final decomposition of the previously formed compounds (Bonilla, Fortunati,
331 Atarés, Chiral, & Kenny, 2014). The addition of the silver compound significantly
332 reduced the thermal stability of the starch and PVA fractions in the film. Nevertheless,
333 whereas the degradation temperature of the starch phase decreased as the silver
334 concentration rose, the same temperature values were observed for PVA degradation
335 regardless of the silver content. The disappearance of the second PVA degradation
336 step was observed from the lowest concentration level of silver. This suggests that Ag
337 interacted to a different extent with the starch and PVA fractions in the film, but in both
338 cases, the chain extension and bonds in the film network were notably affected by the
339 presence of silver (and other ions: NO_3^-) in the system.
340 The moisture content of the films (Table 1) was not significantly affected either by the
341 incorporation of $AgNO_3$ or by the storage time, which indicates that the equilibrium
342 moisture content was reached after 1 storage week. Likewise, there were no
343 remarkable differences between the WVP values (Table 1) as a consequence of silver
344 addition. However, these values tended to decrease when low amounts of silver were
345 added, afterwards rising when the concentration increased. This could be related with
346 the structural differences in the polymer matrix, commented on above, referring to the
347 different chain arrangement as a function of the initial ionic strength in the aqueous
348 media and molecular interactions with the silver species. At a low silver concentration,
349 macromolecular chains would be more extended with linked silver species, which will
350 lead to an increase in the tortuosity factor for the diffusion of the water molecules
351 (Cussler, Highes, Ward & Aris, 1998; Rhim et al., 2013). On the contrary, higher
352 amounts of silver will inhibit the extension of macromolecules in the aqueous media,
353 giving rise to a less compact film structure, where water molecules can be transferred
354 more quickly.
355 The optical properties of the films were analysed in terms of internal transmittance at
356 450 nm (Ti), as a measure of the transparency of the films, and by means of clarity

357 (L*), hue (h*) and chrome (C*) , which are shown in Table 3. While the control starch-
358 PVA films were colourless and transparent, the films loaded with silver particles turned
359 from pale brown to dark brown, depending on the $AgNO_3$ concentration. In Table 3, a
360 significant ($p<0.05$) reduction in the values of transparency (Ti), luminosity (L*) and hue
361 (h*) of silver-loaded films can be observed, while the colour saturation (C*) increased.
362 This is due to the silver reduction forming the silver nanoparticles (AgNPs), which
363 generates a yellow to brownish colour, attributed to the characteristic surface plasmon
364 resonance of AgNPs (Puišo, Prosyšenvas, Guobienė & Tamulevišius, 2008; Zheng,
365 Rong, Zhang, Lianm & Zeng, 2001). A colour analysis of the films can consequently be
366 an efficient and easy tool with which to monitor the reduction process.
367 The extent of the changes in the optical parameters during film storage was dependent
368 on the concentration of AgNPs. Thus, films loaded with higher amounts of $AgNO_3$
369 exhibited greater changes (decrease in L*, h* and C*), which indicates that the silver
370 reducing process progressed throughout storage, and confirms that free Ag+ remains
371 in the films after 1 week of storage. Silver ions are known to readily reduce to
372 elemental particles in slightly reducing environments (Martínez Abad, 2014a).
373
374 3.2. Silver release
375 Silver release was studied as a function of time in five different food simulating liquids
376 (aqueous solutions with 10, 20 and 50 % of ethanol, 3% of acetic acid and a non-polar
377 medium, oleic acid). Figure 2 shows the total accumulated silver released into the
378 different simulants from the films throughout time. Simulants A, C and D1 exhibited a
379 very similar release profile, so the release profile for the D1 simulant (with 50 %
380 ethanol) is not shown. Simulants B (with low pH) and D2 (non-polar medium) behaved
381 differently, as can be observed in Figure 2.
382 In aqueous systems, the water uptake in the film enhanced the release of silver into the
383 simulants, as can be deduced from Figure 2. Once the film was immersed in the

aqueous simulants, most of the release took place within the 60 first minutes, depending on the type of aqueous simulant used. Samples released 100 % of their silver content after 1 h of immersion in the acidic medium (simulant B) and after 4 to 10 h in those media containing 10 to 50 % of ethanol, respectively, which represent 16, 157, 393 and 787 mg of silver / kg of simulant, for S-PVA1, S-PVA2, S-PVA3 and S-PVA4 films, respectively. This agrees with the hydrophilic nature of the films, which became completely hydrated, swollen and plasticized after very short times of immersion. During the hydration process, the polymer network becomes more open, favouring the migration of silver to the aqueous media.

Martínez-Abad, Sánchez, Lagarón, & Ocio, (2013) showed that the release kinetics was greatly affected by the silver speciation in the matrix (silver ions or solid nanoparticles). Thus, a 100 % burn release of silver within the first 30 minutes was obtained from hydrophilic ethylene-vinyl alcohol copolymer (EVOH) films loaded with free silver ions; nevertheless, the release kinetics dramatically slowed down when using silver nanoparticles, as they were retained in the polymer network. The behaviour shown by the silver-loaded S-PVA composite films suggests the presence of both kinds of silver specimens, silver nanoparticles and silver ions. The release of silver into a liquid system depends on different factors, such as the liquid migration to the polymer matrix and its swelling, the polymer solubility in the liquid phase and the diffusion of the active compound through the polymer matrix to the liquid.

The different release behaviour in non-polar simulant D2 can be explained by the limited diffusion of oleic acid in the highly polar polymer matrix, thus maintaining a closed network structure, which hinders the diffusion and release of silver into the simulant. As can be deduced from Figure 2-d, in contact with low-polar systems, i.e. fat-rich foods, the S-PVA polymeric matrix will exhibit a limited silver release, up to 50-78 %, depending on the initial silver concentration.

410 An empirical model (Peleg model, Eq. 1) was applied to fit the release kinetics of silver
411 to food simulants from the films. Parameter k_1 is inversely related to the mass transfer
412 rate at the very beginning of the process and k_2 is inversely related to the maximum
413 attainable value of the function (equilibrium value $-V_{eq}$, Eq. 2) (Abu-Ghannam &
414 McKenna, 1997; Turhan, Sayar & Gunasekaran, 2002, Atarés, Chiralt, & González-
415 Martínez, 2008). Figure 2 shows the experimental points and predicted curve, where
416 the close fit of the model can be observed in every case.
417 Table 4 shows the values of kinetic constant k_1 for the different films and simulants (A,
418 B, C and D1). The equilibrium value deduced from k_2 (not shown) did not significantly
419 differ from the values commented on above for each film, deduced from the total silver
420 concentrations in the respective formulation. For simulant D2, the values of k_1 and k_2
421 for each film are shown in Table 5. The estimated equilibrium value and the
422 corresponding percentage of release, with respect to the total amount of silver in the
423 film, are also shown.
424 The release rate (inverse of k_1) of silver decreased as the amount of ethanol in the
425 aqueous solution rose (Table 4). The increase in the ethanol concentration in the
426 simulant reduced the polarity of the medium and limited the hydration process of the
427 polymer network and the weakening effect on the matrix, leading to a slower diffusion
428 of silver through the film. Likewise, the release rates were affected by the initial silver
429 concentration in the films according to the different values of the driving force for the
430 mass transfer process; thus, the films loaded with the greatest silver concentration
431 released silver into the different simulants faster. This is especially true in the case of
432 samples S-PVA3 and S-PVA4, where a greater amount of silver ions, with higher
433 mobility, could remain.
434 The degree and rate of film hydration, which greatly facilitates molecular mobility and
435 silver diffusion, will be dependent on the water content and pH of the simulant. In the
436 case of simulant B (3% acetic acid), the release of silver was favoured (lower k1)

because of the greater solubility of the film at low pH. This is due to the partial hydrolysis of the polymer caused by acetic acid, which led to smaller and thus, more soluble fragments. This effect has also been reported by several authors working with starch and starch-PVA films under acidic environments (Yoon, Park & Byun, 2006; Olivato, Grossmann, Yamashita, Eiras & Pessan, 2011; Carvalho, Zambon, da Silva Curvelo & Gandini, 2005; Ortega-Toro, Collazo-Bigliardi, Talens & Chiralt, 2015).

Taking into account the total release into the different simulants, and the established overall migration limit (OML) for food contact packaging materials: 60 mg of substances/kg of food simulant or foodstuff (EN1186-1, 2002, Commission Regulation Nº 10/2011), the only films that fulfilled this requirement are S-PVA1 (for all types of foodstuffs) and S-PVA2 for fat-rich foodstuffs. Nevertheless, more research has to be carried out on real foodstuffs in order to check the viability of its application.

3.3. Antimicrobial activity.

The antimicrobial activity of silver-loaded films was analysed against two fungi, *A. niger* and *P. expansum*, and two bacteria, *L. innocua* and *E. coli*. The antimicrobial efficacy was evaluated through the analysis of the growth (or survival) of a determined infection level of the microorganism (10^5 spores/ml and 10^2 UFC/cm^2, for fungi and bacteria, respectively) following the above-described methodology.

Silver-loaded films exhibited antimicrobial activity, which depended on the concentration used. As expected, the highest antibacterial activity was observed for the formulation with the greatest silver concentration (S-PVA4), showing a bactericidal effect (defined as a decrease of 3 magnitudes in the bacterial load, in comparison with the control) for both bacteria. Several authors (Rhim et al., 2013; Bryaskova et al., 2010; Martínez-Abad et al., 2014a) also found that the antibacterial activity of films with embedded AgNPs increased as the concentration of silver rose.

S-PVA3 and S-PVA2 also exhibited a bactericidal effect but only throughout the first 5 or 7 days for *L. innocua* and *E. coli*, respectively. After this period, the inhibition level of these silver-loaded films decreased throughout the storage period. On the other hand, the antibacterial activity was greater in *E. coli* than in *L. innocua* (Figure 3) due to the difference cell wall structures of both types of bacteria. The presence of the negatively charge lipopolysaccharide in Gram negative bacteria attracts the positively charged silver ions or silver nanoparticles, thus dramatically increasing the permeability of the membrane. Moreover, the cell wall of gram positive cells (*Listeria*) presents a thicker cell wall of peptidoglycans, which makes the penetration of silver nanoparticles difficult (Bryaskova et al., 2010). In fact, formulations S-PVA2 and S-PVA3 exhibited reductions in the *E. coli* population of around 5 logs during the first 7 days of storage, while for *L. innocua*, the maximum reduction reached was 3 logs up to the 5^{th} day.

As regards the fungus, control films without silver were able to grow to a maximum of about 10^8 CFU/cm^2 for both fungi under the stated conditions (Figure 4). At low silver concentrations in the films (S-PVA1 and S-PVA2), almost no antifungal effect was observed: S-PVA1 reduced the initial microbial load by about 1 log and S-PVA2 reduced the plate counts of *P. expansum* by about 4 logs during the first 3 days of incubation. When using moderate concentrations of silver (S-PVA3), the growth of both fungi was inhibited up to the first five or seven days of storage, for *Aspergillus* and *Penicillium*, respectively. The level of reduction in the fungal population during this time period was remarkable; around 4-5 logs with respect to the control film. After this period, the inhibition level also decreased throughout storage, as commented on above, reaching a maximum value of 3 logs after 12 days of incubation.

Only when using the highest silver concentration (S-PVA4), was a completely fungistatic activity detected during storage, as no sign of cultivable counts were observed during the whole period. To the best of our knowledge, very little information related with the antifungal activity of AgNPs-loaded films has been found. Only Abreu

et al. (2015) reported antifungal tests against Candida albicans for agar films loaded with silver nanoparticles, but they observed no antifungal effect.

At this point, some considerations have to be taken into account. From the reported data, silver concentrations of around 10-500 ppm are needed to exert bactericidal activity in a rich medium, such as TSB supplemented with NaCl (Halminton-Miller & Shan, 1996; Nomiya, Yoshizawa, Tsukagoshi, Kasuga, Hirakawa & Watanabe, 2004; Ruparelia, Chatterjee, Duttagupta & Mukhereji, 2008; Sondi & Salopek-Sondi, 2004; Martínez-Abad et al., 2012). If water or salt buffers are used, the bactericidal concentrations are proven to be much lower, in the range of 0.01-1ppm (Bjarnsholt et al., 2007; Hwang, Katayama, & Ohgaki, 2007; Kim et al., 1998). Taking into account the release studies, 100 % of silver is delivered into the aqueous simulants by the films, giving rise to a minimum concentration of silver of 16 ppm (S-PVA1) in the liquid simulants. This suggests that the release of silver into the TSB/PDA medium in the Petri dishes was not completed (as occurred in liquid aqueous simulants), being retained in the polymer matrix and, so, not able to act as an antimicrobial. As commented on above, the release kinetics was closely related with the hydration of the polymer network and, therefore, the hydration level of the film in contact with the agar medium could be constrained as the water molecules entrapped in the agar gel are not able to diffuse so effectively, which will affect the silver release and antimicrobial activity of the films.

4. Conclusions

Starch-PVA based films embedding silver nanoparticles exhibited remarkable antibacterial activity against *Listeria innocua* and *Escherichia coli* and antifungal activity against *Aspergillus niger* and *Penicillium expansum,* which depended heavily on the concentration of AgNPs in the film. This incorporation did not imply relevant changes in the physical properties of the films, except for their colour and transparency; these both

underwent significant changes, becoming brownish-yellow and opaque, especially when the silver concentration in the films increased.

The antimicrobial effectiveness of silver-loaded films was limited by the release behaviour of silver from the films in contact with the agar plate, seemingly reduced as compared to food simulants. The silver was delivered to aqueous simulants (including the acidic one) in its entirety within the first 60 minutes of contact. Nevertheless, when using a non-polar simulant, the release capacity of the films drastically decreased. The silver released into the food simulants widely exceeds the maximum amount permitted (60 mg/Kg simulant) in all cases, except when using the non-polar simulant (oleic acid). So, the use of the developed films as food packaging materials should be restricted to fat-rich foodstuffs. For the purposes of optimizing the release capacity of the films with moderate silver concentrations, additional studies should be carried out in order both to reduce the burst release in contact with highly aqueous environments and also to comply with the current legislation.

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743 Table 1: Elastic modulus (EM), tensile strength at break (TS), percentage of elongation at break (E, %), moisture content (MC) and water vapour

744 permeability (WVP) of S-PVA and silver composite films after 1 (1W) and 5 (5W) storage weeks. Mean values and standard deviation.

745

	EM (MPa)		TS (MPa)		E (%)		MC (% d.b.)		WVP(g-mmkPa^{-1}h^{-1}m^{2})	
Film	1W	5W	1W	5W	1W	5W	1W	5W	1W	5W
S-PVA	506±63^{a1}	690±44^{a2}	26.8±1.4^{ab1}	32.3±1.6^{a2}	40±4^{ab1}	41±3^{a1}	8.2±0.3^{a1}	7.1±1.7^{ab1}	5.09±1.17^{a1}	5.1±0.4^{bc1}
S-PVA-1	638±38^{b1}	552±58^{b2}	29±3^{bc1}	32±3^{a1}	37±8^{ab1}	47±9a^{b2}	6.8±1.5^{a1}	6.4±1.9^{ab1}	4.6±0.4^{ab1}	4.6±0.5^{ab1}
S-PVA-2	771±42^{b1}	652±34^{a2}	30.7±1.6^{c1}	30.2±1.6^{a1}	33±4^{a1}	42±7^{a2}	6.5±1.1^{a1}	5.1±0.5^{a1}	3.8±0.2^{b1}	4.04±0.27^{a1}
S-PVA-3	518±65^{a1}	542±56^{b1}	25.3±1.3^{a1}	30±3^{a2}	41±6^{b1}	50±7^{b2}	7.2±1.2^{a1}	8±0.9^{b1}	5.2±0.5^{ac1}	5.4±0.7^{c1}
S-PVA-4	229±34^{c1}	262±45^{c1}	18.3±1.4^{d1}	22±3^{b2}	53±5^{c1}	54±5^{b1}	7.6±1.3^{a1}	7.1±1.3^{ab1}	6.1±0.4^{c1}	6.4±0.4^{d1}

746 ^{a,b,c,d} different letters in the same column indicate significant differences among formulations ($p<0.05$).

747 ^{1,2} different numbers in the same row indicate significant differences between storage times ($p<0.05$).

748

749 Table 2. Onset, peak, end temperatures (T_o, T_p, T_e respectively) and residual mass (RM) obtained from TGA analysis. Mean values and
750 standard deviation.
751

	First degradation (S)			Second degradation (PVA)			Third degradation (PVA)			RM 600 ºC
Films	T_0	T_p	T_e	T_0	T_p	T_e	T_0	T_p	T_e	(%)
S-PVA	163±12^{a}	210±7^{ab}	244±3^{a}	262.3±1.6^{a}	305.3±0.7^{a}	357.4±1,7^{a}	387,8±1,7^{a}	417±2^{a}	447±3^{a}	13.7±0.3^{a}
S-PVA1	153.36±1.14^{ab}	193.5±0.7^{b}	225.7±0.9^{ab}	243.8±0.4^{b}	286.8±0.8^{b}	338.7±1.3^{b}	369±5^{b}	396±5^{b}	421±5^{b}	17.0±1.7^{ab}
S-PVA2	135±3^{bcd}	181.9±0.8^{c}	227,8±1.5^{ab}	246.9±0.9^{b}	287±2^{b}	333±5^{b}				21.21±0.09^{ab}
S-PVA3	133.4±0.9^{cd}	169.6±0.4^{d}	216.83±1.15^{ab}	246.1±0.2^{b}	287.3±0.4^{b}	332±3^{b}				21.25±0.14^{ab}
S-PVA4	119.5±1.9^{d}	154±5^{e}	196±8^{b}	245.0±0.2^{b}	286.5±0.9^{b}	330.1±1.4^{b}				25±1^{b}

752 $^{a, b, c,}$ different letters in the same column indicate significant differences among formulations at the same time of the analysis (0 or 73 says) ($p<0.05$).
753

754 Table 3: Colour parameters (Clarity: L*, chrome: C_{ab}* and hue: h_{ab}*) and internal transmittance (Ti) of S-PVA and silver composite films after 1

755 (1W) and 5 (5W) storage weeks. Mean values and standard deviation.

756

	L*		C_{ab}*		h_{ab}*		Ti (450nm)	
Films	1W	5W	1W	5W	1W	5W	1W	5W
S-PVA	84.32 ± 1.08^{a1}	82.1 ± 1.6^{a1}	3.6 ± 0.4^{a1}	4.4 ± 1.4^{a1}	110 ± 9^{a1}	112 ± 7^{a1}	86.2 ± 0.5^{a1}	85.9 ± 0.2^{a1}
S-PVA-1	49.0 ± 1.4^{b1}	39.4 ± 1.3^{b2}	12.9 ± 0.6^{b1}	17.5 ± 0.4^{b2}	56 ± 1^{b1}	55.7 ± 0.6^{c1}	58.4 ± 1.9^{b1}	29 ± 3^{b2}
S-PVA-2	39.4 ± 0.7^{c1}	34.2 ± 0.4^{c2}	19.0 ± 0.3^{c1}	14.5 ± 0.2^{c2}	64.3 ± 0.6^{c1}	56 ± 3^{c2}	24 ± 2^{c1}	12 ± 2^{c2}
S-PVA-3	38.2 ± 1.0^{cd1}	33.0 ± 0.3^{cd2}	17.6 ± 0.4^{c1}	12.3 ± 0.6^{d2}	60.3 ± 1.1^{bc1}	49 ± 2^{c2}	20 ± 3^{c1}	10.5 ± 0.5^{cd2}
S-PVA-4	36.8 ± 1.3^{d1}	31.8 ± 0.4^{d2}	13.9 ± 1.7^{b1}	6.3 ± 0.9^{e2}	57 ± 3^{b1}	37 ± 8^{b2}	20 ± 3^{c1}	5 ± 3^{d2}

757 ^{a,b,c,d,e} different letters in the same column indicate significant differences among formulations ($p<0.05$).

758 ^{1,2} different numbers in the same row indicate significant differences between storage times ($p<0.05$).

759 Table 4: Values of the kinetic constant k_1 (h mg_{Ag} $kg_{simulant}^{-1}$) for the silver release of the

760 different films into the simulants (A, B, C and D1).

761

	Simulant			
Film	A	C	D1	B
SPVA-1	0.037	0.173	0.292	0.0493
SPVA-2	0.015	0.029	0.038	0.0102
SPVA-3	0.002	0.003	0.003	0.0011
SPVA-4	0.001	0.003	0.003	0.0001

762

763 Table 5: Values of Peleg's parameters of the different films in the D2 simulant: k_1 (h mg_{Ag}
764 $kg_{simulant}^{-1}$), k_2 (mg_{Ag} $kg_{simulant}^{-1}$), V_{eq} ($kg_{simulant}$ mg_{Ag}^{-1}) and the percentage released with
765 respect to the total amount of silver in the films.

766

Film	k_1	k_2	V_{eq}	% Released
SPVA-1	1.033	0.08	13	78
SPVA-2	0.189	0.015	66	42
SPVA-3	0.154	0.005	204	52
SPVA-4	0.087	0.003	400	51

767