

Nano-biocomposite films with modified cellulose nanocrystals and synthesized silver nanoparticles



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ABSTRACT

Ternary nano-biocomposite films based on poly(lactic acid) (PLA) with modified cellulose nanocrystals (s-CNC) and synthesized silver nanoparticles (Ag) have been prepared and characterized. The functionalization of the CNC surface with an acid phosphate ester of ethoxylated nonylphenol favoured its dispersion in the PLA matrix. The positive effects of the addition of cellulose and silver on the PLA barrier properties were confirmed by reductions in the water permeability (WVP) and oxygen transmission rate (OTR) of the films tested. The migration level of all nano-biocomposites in contact with food simulants were below the permitted limits in both non-polar and polar simulants. PLA nano-biocomposites showed a significant antibacterial activity influenced by the Ag content, while composting tests showed that the materials were visibly disintegrated after 15 days with the ternary systems showing the highest rate of disintegration under composting conditions.

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1. Introduction

Polymer nanocomposites have attracted considerable attention in recent years as a result of their good performance, improved properties, design flexibility, lower life-cycle costs and a unique large applicability range in various industrial fields. Antimicrobial materials and surfaces are increasing their importance in areas such as healthcare, food active packaging, automotive and textiles to control harmful microorganisms (Hojatollah Bodaghi et al., 2013; Kuorwel et al., 2013; Manso, Cacho-Nerin, Becerril, & Nerin, 2013). However, all these systems need to combine a strong antibacterial efficiency with other characteristics, such as environmental safety, low toxicity, cost effectiveness and easy fabrication.

Silver is a powerful antimicrobial agent that has been used since ancient times. Recent technical innovations facilitate the

incorporation of silver-based materials to commercial formulations with antimicrobial properties (Fortunati, Latterini, Rinaldi, Kenny, & Armentano, 2011; Nocchetti et al., 2013; Rinaldi et al., 2013). Nanotechnology is one of the preferred innovations, allowing metals to be processed in the nanoscale to improve their chemical, physical and electronic properties (Busolo, Fernández, Ocio, & Lagaron, 2010; Lloret, Picouet, & Fernández, 2012). The synthesis of metallic nanoparticles with controlled shape, size and dispersion stability is a challenging research area due to their potential in electronics, optical, sensors and catalysis sectors (Cai, Kimura, Wada, & Kuga, 2009; Latterini & Tarpani, 2011; Storti, Elisei, Abbruzzetti, Viappiani, & Latterini, 2009). Preparation of metal colloids by reduction is a nominally simple reaction, but it requires a careful control of the synthesis conditions, since this process is sensitive to a balance between nucleation and crystal growth (Cai et al., 2009). This is a fundamental point since difficulties in dispersion of nanoparticles in a solvent are well known by their tendency to aggregate due to their high surface energy (Ifuku, Tsuji, Morimoto, Saimoto, & Yano, 2009).

Silver ions are active against a very broad spectrum of bacteria, yeast, fungi and viruses even at very low concentration

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(Lara, Ayala-Nuñez, Ixtepan-Turrent, & Rodríguez-Padilla, 2010; Nocchetti et al., 2013) with negligible toxicity towards human cells at the same concentration range (Williams, Doherty, Vince, Grashoff, & Williams, 1989). Moreover, colloidal nanosilver shows antimicrobial properties against Gram-positive and Gram-negative bacteria (Dallas, Sharma, & Zboril, 2011). These properties are particularly interesting for food active packaging (Lopez-Rubio, Lagaron, & Ocio, 2008). In the United States, the Center for Food and Drug Administration (FDA/CFSAN) accepts the use of silver nitrate as a food additive in bottled water. In addition to this, the European Food Safety Authority (EFSA) has concluded that there is no safety concern for the consumer if migration of silver ion does not exceed the group specific migration limit of $0.05 \text{ mg Ag kg}^{-1}$ food (<http://www.efsa.europa.eu/en/efsajournal/pub/1999.htm>, updated 02 March 2011). Recent studies have reported that migration levels of silver nanoparticles in poly(vinyl chloride) (PVC) nanocomposites are clearly below the conventional migration limits indicated in the current European legislation (Reglament N10/2011) but the effects of nanoscale particles remain unclear and further studies are necessary to assess the real possibilities of nano-Ag in food packaging applications (Cushen, Kerry, Morris, Cruz-Romero, & Cummins, 2013).

Nowadays, there is a trend to substitute petroleum based plastics by renewable plastic packaging with lower environment impact. In food packaging, PLA is a very promising material due to its excellent mechanical properties, transparency and commercial availability. Nonetheless, some of its properties, such as gas and water vapour permeability and thermal stability, are somewhat poor for some specific applications (Averous, Bordes, & Pollet, 2009). In this context, the addition of nanoparticles as polymer additives contributes to enhance the barrier to gases due to the synergistic tortuosity, crystal nucleation and chain immobilization effects (Martino, Ruseckaite, Jiménez, & Averous, 2010).

Cellulose nanostructures have been recognized as important bio-based fillers to enhance the biopolymer performance, in terms of mechanical, thermal and barrier properties (Fortunati, Peltzer, et al., 2012; Fortunati, Puglia, et al., 2013). Cellulose-based materials are widely used because of their biocompatibility, edibility, abundance in nature, excellent barrier properties and low cost (Brinchi, Cotana, Fortunati, & Kenny, 2013). The use of nanocelluloses has been widely proposed to enhance PLA properties (Bitinis et al., 2013; Frone, Berlioz, Chailan, & Panaitescu, 2012; Raquez et al., 2012).

The aim of this research was the design, develop and characterize an innovative nano-biocomposite based on PLA, surfactant-modified cellulose nanocrystals (s-CNC) and synthesized silver nanoparticles (Ag) to be applied for fresh food antimicrobial active packaging. The effect of cellulose crystal nano-dimensions and the combination of cellulose nanostructures with synthesized silver nanoparticles were evaluated in terms of microstructure, crystallinity, water and oxygen barrier, migration and antibacterial properties. Moreover, the post-use properties of the developed multifunctional formulations were evaluated by a laboratory-scale composting condition test.

2. Experimental

2.1. Materials

Poly(lactic acid), PLA, 3051D, with specific gravity 1.25 g cm^{-3} , molar mass $1.42 \times 10^4 \text{ g mol}^{-1}$ and melt flow index (MFI) $7.75 \text{ g 10 min}^{-1}$ (210°C , 2.16 kg) was supplied by Nature Works® (Minnetonka, MN, USA). PLA resin 3051D had 96% L-lactide to 4% D-lactide units. PLA pellets were dried in a vacuum oven at 98°C for 3 h before processing. Microcrystalline cellulose (MCC, dimensions

10–15 μm) was supplied by Sigma–Aldrich (Milan, Italy). AgNO_3 (Sigma–Aldrich, ACS reagent, $\geq 99.0\%$), polyvinylpyrrolidone (PVP, $M_w = 40 \text{ kDa}$, Sigma–Aldrich), chloroform (CHCl_3 , 99.4% GC-grade) were used as received.

2.2. Cellulose nanocrystals synthesis and modification

MCC was hydrolyzed in sulphuric acid (64%, wt/wt) at 45°C for 30 min (Fortunati, Peltzer, et al., 2012). Modified cellulose nanocrystals (s-CNC) were obtained after the addition of an acid phosphate ester of ethoxylated nonylphenol (Beycostat A B09 (CECCA S.A.)) in 1/4 (wt/wt) ratio as previously reported (Fortunati, Armentano, Zhou, Iannoni, et al., 2012; Fortunati, Armentano, Zhou, Puglia, et al., 2012).

2.3. Silver nanoparticles synthesis and characterization

Silver nanoparticles (Ag) were synthesized by selective reduction in the presence of PVP to control the particle growth. AgNO_3 (0.0856 g) was added to 50 mL of a CHCl_3 boiling solution containing 0.72 g of PVP. The solution was then heated up to the boiling point and maintained at this temperature for 5 h under vigorous stirring.

A Philips mod. 208 transmission electron microscope (TEM), operating at 80 kV of beam acceleration was used to analyze the average size and distribution of the silver nanoparticles. They were deposited in a 400 mesh copper coated with formvar support grid and were left overnight in a desiccator to evaporate the solvent. Histograms for size distribution were obtained by analyzing 150–200 nanoparticles in each sample. UV–vis absorption spectra of the Ag suspensions were recorded with a double beam Perkin-Elmer Lambda 800 spectrophotometer.

2.4. PLA nano-biocomposites processing

PLA nano-biocomposite films were prepared by solvent casting. PLA was dissolved in CHCl_3 (10%, wt/v) with vigorous stirring at room temperature (RT). The solutions were poured onto glass Petri dishes, and then allowed to dry for about 24 h at RT. PLA neat films (200 ± 50) μm thick were obtained (film thickness was measured using a micrometre to $\pm 0.001 \text{ mm}$, and five different measurements were performed).

For the preparation of the nanocomposite films, a predetermined amount of s-CNC suspension in chloroform was mixed with the previously prepared PLA solution. The obtained solutions were stirred for 4 h before they were cast onto glass Petri dishes and dried. The produced films were (200 ± 50) μm thick (film thickness was measured using a micrometre to $\pm 0.001 \text{ mm}$). Ternary films with 0.5 wt% or 1 wt% of Ag combined with 1 wt% of s-CNC (designed as PLA/1s-CNC/0.5Ag and PLA/1s-CNC/1Ag, respectively) were produced and characterized. For comparison, binary PLA/Ag systems with 0.5 wt% or 1 wt% of Ag were also prepared and designed as PLA/0.5Ag and PLA/1Ag, respectively. Films with thicknesses of (200 ± 50) μm were obtained (film thickness was measured using a micrometre to $\pm 0.001 \text{ mm}$).

All films were placed in a vacuum oven at 40°C for 2 weeks before characterization in order to remove remaining chloroform.

2.5. PLA nano-biocomposites characterization

2.5.1. Microstructure, transparency and Wide Angle X-ray Scattering (WAXS)

The microstructure of PLA nano-biocomposites and the filler dispersion in the matrix were investigated by using transmission electron microscope (TEM, JEOL JEM-2010). Samples for TEM analysis were previously ultra-microtomed (RMC, model MTXL) in order

to obtain slices 100 nm thick. A confocal laser scanning microscope (CLSM, Nikon PCM2000, Italy), equipped with a diode laser ($\lambda = 400$ nm) or an Ar-laser ($\lambda = 488$ nm) as light sources, was used to investigate the nanocomposite morphology and the nanoparticle dispersion.

The absorption spectra of the film samples were recorded with a Varian (Cary 4000, USA) spectrophotometer equipped with a 150 mm integration sphere for reflectance spectra recording. A bar of barium sulphate was used as reference to calibrate the spectrophotometer.

Wide Angle X-ray Scattering (WAXS) patterns of PLA nanobiocomposites were obtained with a Seifert diffractometer (JSO-DEBYEFLEX 2002) with CuK radiation ($\lambda = 0.1546$ nm), 40 kV voltage and 40 mA current. The diffraction intensities were recorded between 2° and 80° (2θ angle range) by steps of 1° min^{-1} .

2.5.2. Thermal characterization

Differential scanning calorimetry (DSC, Mettler Toledo 822e) measurements were performed from -25°C to 210°C , at $10^\circ\text{C min}^{-1}$ (the samples were rapidly cooled to the start temperature), performing two heating and one cooling scans (210°C to -25°C at $10^\circ\text{C min}^{-1}$). Melting and cold crystallization temperatures and enthalpies (T_m ($^\circ\text{C}$), T_{cc} ($^\circ\text{C}$), ΔH_m (J g^{-1}), ΔH_{cc} (J g^{-1})) were determined from the second heating scan as well as glass transition temperatures (T_g ($^\circ\text{C}$)) for all nano-biocomposites.

2.5.3. Oxygen transmission rate (OTR) measurements

OTR values were determined by using an oxygen permeation analyzer (Systech Instruments, model 8500, Metrotec S.A., Spain). Film thicknesses were measured with a Digimatic Micrometer Series 293 MDC-Lite (Mitutoyo) to ± 0.001 mm. Samples were clamped in the diffusion chamber at $(25 \pm 1)^\circ\text{C}$. Pure oxygen (99.9%) was introduced into the upper half of the sample chamber while nitrogen was injected into the lower half of the chamber where an oxygen sensor was placed. Oxygen transmission rate per thickness (OTR^*e) was determined for each formulation.

2.5.4. Water vapour permeability (WVP) tests

Water vapour permeability was calculated by following the UNE-ISO 53097:2002 standard and according to the following equation:

$$\text{WVP} \text{ (kg m/m}^2 \text{ s Pa)} = \frac{\text{WVT}}{S \times (\text{RH}_1 - \text{RH}_2)} \times e \quad (1)$$

where e is the film thickness (m), S is the saturation pressure (Pa) at the testing temperature, RH_1 is the relative humidity of the climatic chamber where tests were performed and RH_2 is the relative humidity inside the capsule. At least three repetitions per experiment were performed as previously reported (Martucci & Ruseckaite, 2010). Samples with a diameter of 95 mm were fixed with paraffin to the top of aluminium capsules containing CaCl_2 as drying agent. These capsules were placed in a climatic chamber at $20.0 \pm 0.1^\circ\text{C}$ and $50 \pm 2\%$ relative humidity. Samples were weighed periodically until the steady state was reached. The mass change (± 0.0001 g) versus time was recorded at specific intervals (t) and then plotted. Linear regression was used to calculate the slope of the fitted straight line, which represents WVT as follows:

$$\text{WVT} = \frac{\Delta m}{t \times A} \quad (2)$$

where Δm is the mass change of the capsule test (kg) at time t (s) and A is the test area (m^2). WVT ($\text{kg s}^{-1} \text{ m}^{-2}$) is the vapour transmission rate through a mean film thickness e (m).

2.6. Overall and specific migration tests: kinetics of silver release

In order to determine the overall migration of the samples under study, rectangular strips of 10 cm^2 total area of each film were immersed in glass tubes with 10 mL of ethanol 10% (v/v) (simulant A) and isooctane (alternative simulant to D2). Food simulants are used to demonstrate the compliance of plastic materials not yet in contact with food (Commission Regulation EU 10/2011). Samples in ethanol 10% (v/v) were kept in a controlled chamber at 40°C during 10 days according to the Regulation EU No. 10/2011 (Commission Regulation EU 10/2011), while samples in isooctane were kept at 20°C during 2 days, according to the EN 1186-1:2002 (European Standard EN 1186-1:2002). After the incubation period, films were removed and simulants were evaporated to complete dryness. Furthermore, residues were weighed with an analytical balance (Sartorius ATILON) to ± 0.1 mg in order to determine the overall migration value in mg kg^{-1} of simulant. Three independent determinations were performed for each sample and the final overall migration value was the average of the three determinations.

The same food simulants, ethanol 10% (v/v) and isooctane, were used to carry out the specific migration tests. In this case, rectangular strips of 12 cm^2 total area of each film were immersed in glass tubes with 20 mL of simulants to keep the relation $6 \text{ dm}^2 \text{ L}^{-1}$ as indicated by the EN 13130 standard. Samples in ethanol 10% (v/v) were kept in a controlled atmosphere at 40°C for 10 days, while samples in isooctane were kept at 20°C for 2 days. After the incubation period, films were removed and simulants were treated to determine the Ag^+ amount. Prior to analysis isooctane extracts were evaporated to dryness and re-suspended in 5 mL of HNO_3 1% (wt/v). Ethanol extracts were analyzed with no previous treatment. The determination of Ag in the migration extracts was performed by using Inductive Coupled Plasma spectroscopy with Mass Spectrometry detection (ICP-MS) model 7700x Agilent (Santa Clara, CA, USA) in argon (0.35 L min^{-1}) at 1600 W radiofrequency and S/C temperature 2°C . The calibration curve was obtained with Ag^+ standards in HNO_3 1% (wt/v), in a concentration range between $5 \mu\text{g kg}^{-1}$ and $1000 \mu\text{g kg}^{-1}$. Each standard was analyzed by triplicate.

A release kinetics study was also performed in ethanol 10% (v/v) for 20 days to study the gradual release of Ag^+ to the simulant at 40°C (Fortunati, Peltzer, Armentano, Jiménez, & Kenny, 2013). Diffusion coefficients were calculated from the beginning of the release process by using a mathematical model based on the Fick's second law. In this study, we propose the use of a simplified release model described by Eqs. (3) and (4), which are based on the Crank equation (Crank & Gupta, 1975) and described by Chung, Papadakis, and Yam (2002) where $M_{p,0}$ is the initial amount of analyte in the polymer matrix and $M_{F,t}$ is the amount of migrant transferred from the film to the food simulant from time zero to time t (s).

$$\frac{M_{F,t}}{M_{p,0}} = \frac{2}{L_p} \left[\frac{Dt}{\pi} \right]^{0.5} - \frac{Dt}{\alpha L_p^2} \quad (3)$$

$$\alpha = \frac{K_{FP} V_F}{V_p} \quad (4)$$

D is the diffusion coefficient (cm s^{-1}) and L_p is the film thickness.

2.7. Antibacterial activity

The microorganisms used in this study were *Escherichia coli* RB (*E. coli* RB) and *Staphylococcus aureus* 8325-4 (*S. aureus* 8325-4). *E. coli* RB was provided by the "Zooprofittico Institute of Pavia", Italy whereas *S. aureus* 8325-4 was kindly supplied by Timothy J. Foster (Department of Microbiology, Dublin, Ireland).

E. coli RB was routinely grown in Luria Bertani Broth (LB) (Difco, Detroit, MI, USA) and *S. aureus* 8325-4 in Brian Heart Infusion (BHI) (Difco) overnight under aerobic conditions at 37 °C using a shaker incubator (New Brunswick Scientific Co., Edison, NJ, USA). These cultures, used as source for the experiments, were reduced at a final density of 1×10^{10} cells mL⁻¹ as determined by comparing the OD₆₀₀ of the sample with a standard curve relating OD₆₀₀ to cell number.

To evaluate the antimicrobial activity of PLA and PLA nano-biocomposite films, 100 µL (1×10^4) of overnight diluted cell suspensions of *E. coli* RB or *S. aureus* 8325-4 were added to each sample seeded at the bottom of 96-well tissue culture plates and incubated at different temperatures (37 °C, 24 °C and 4 °C) for 3 h and 24 h, respectively. These temperatures were chosen to evaluate their influence on the antibacterial activity exerted by PLA and PLA nano-biocomposite films as food packaging systems. Some food is kept refrigerated at 4 °C but it may be possible that under transportation food could be kept at higher storage temperatures (24 °C and/or 37 °C). Furthermore, 96-well flat-bottom sterile polystyrene culture plates (TCP), used as control, were incubated at the same temperatures and times. At the end of each incubation period, the bacterial suspension was then serially diluted, and plated on the LB (*E. coli*) or BHI (*S. aureus*) agar plates, respectively. Those plates were then incubated for 24 h/48 h at 37 °C. Cell survival was expressed as percentage of the CFU of bacteria survived on PLA nano-biocomposite films to CFU of bacteria grown on PLA and PLA/1s-CNC without Ag.

Further analysis of the antibacterial activity of these samples was carried out by determining the release of Ag⁺ with a Perkin-Elmer series ICP-MS, analysing those solutions obtained by the interaction of solvent with samples (area 1 cm²) at 37 °C at 3 different incubation times (3 h, 24 h and 72 h). Solutions were regularly analyzed by ICP-MS to determine the concentration of Ag⁺, once the instrumental setup had been calibrated with a standard solution. Experiments were conducted in duplicate.

2.8. Disintegrability in composting conditions

Disintegrability of PLA and PLA nano-biocomposites was followed through a disintegration test in composting conditions according to the ISO-20200 standard. A specific quantity of compost, supplied by Gesenu S.p.a. (Italy), was mixed together with the synthetic biowaste, prepared with certain amount of sawdust, rabbit food, starch, sugar, oil and urea. The water content of the substrate was around 50 wt% and the aerobic conditions were guaranteed by mixing it softly. The samples were buried at 4–6 cm depth in perforated boxes, containing the prepared mix, and incubated at 58 °C. The (20 mm × 20 mm × 0.02 mm) films were recovered at different disintegration steps, washed with distilled water, dried in oven at 37 °C for 24 h, and weighed. The disintegrability value was obtained normalizing the sample weight, at different stages of incubation, to the initial ones. Photographs of the samples were taken for visual comparison.

2.9. Statistical analysis

S. aureus and *E. coli* cells grown on PLA were used as positive controls. Continuous data were expressed as the average and standard deviations (SD). Two-group comparisons were performed by application of the Student's *t* test. All analyses were performed by using GraphPad Prism 4.0 (Graph Pad Software Inc., San Diego, CA, USA). Two-tailed *p* values <0.05 were considered statistically significant. The same statistical procedure was followed to evaluate the addition of different concentration of Ag and s-CNC on the overall migration and the release of Ag⁺.

3. Results and discussion

3.1. Surfactant modified cellulose nanocrystals

Fig. 1 (panel A) summarizes the different steps and strategies used to prepare cellulose based nano-biocomposites in this study. Cellulose nanocrystals are recovered in water suspension after acid hydrolysis and the TEM micrograph (left side of the scheme) shows that CNC appears well individualized with typical dimensions ranging from 100 to 200 nm in length and 5–10 nm in width as previously reported (Fortunati, Armentano, Zhou, Iannoni, et al., 2012; Fortunati, Armentano, Zhou, Puglia, et al., 2012). Because of the high stability of aqueous cellulose nanoparticle suspensions, water is the preferred medium to prepare nanocomposite films. However, this fact restricts the choice of the matrix to hydrosoluble polymers, which are inherently highly sensitive to humidity. In recent works we demonstrated that the freeze-drying step is critical for the production of nano-biocomposites with uniform cellulose nanocrystal dispersion since the CNC tend to agglomerate into flakes during the freeze-drying (see Fig. 1, panel A) (Fortunati, Armentano, Zhou, Iannoni, et al., 2012; Fortunati, Armentano, Zhou, Puglia, et al., 2012). The use of surfactants to obtain a stable suspension of cellulose nanocrystals in organic media is a procedure used by different authors (Ljungberg et al., 2005; Petersson, Kvien, & Oksman, 2007). The surfactant used in this research is able to hinder hydrogen bonding between the cellulose nanocrystals during the freeze drying and to further increase the distribution of cellulose in organic solvents, such as chloroform (Averous et al., 2009). The FESEM image shown in Fig. 1 (panel A), right highlights the crystal shape and size of surfactant modified cellulose (s-CNC): cellulose nanocrystals maintained the original acicular structure and no morphological modifications occurred during the dispersion in the organic solvent.

3.2. Silver nanoparticle characterization

The chemical synthesis of silver nanoparticles (Ag) was carried out in chloroform, in order to obtain a particle suspension within the same solvent used for PLA. The preparation of Ag particles in CHCl₃ was carried out following a previously reported procedure (Rinaldi et al., 2013). The formation of Ag was monitored spectrophotometrically in the reaction solutions. The presence of an intense absorption band centred at 430 nm in the extinction spectrum (Fig. 1, panel B-a), which was assigned to the surface plasmon resonance band of Ag, confirmed the formation of silver colloids in CHCl₃.

TEM was used to obtain information on the size and morphology of metal nanostructures. Fig. 1, panel B-b shows that the obtained suspensions were characterized by spherical silver nanoparticles, with no aggregates. The statistical analysis of nanoparticle diameter indicated a Gaussian distribution, centred at (7.8 ± 0.4) nm with (1.1 ± 0.1) nm as full width at half maximum (FWHM), indicating a good dispersion.

3.3. PLA nano-biocomposites: microstructure, transparency and WAXS analyses

Nano-biocomposite morphology was evaluated by TEM and the main results are reported in Fig. 2 for the ternary system reinforced with s-CNC nanocrystals and loaded with the highest content of silver nanoparticles. Fig. 2a shows the good dispersion of s-CNC in the nanoscale, since well-defined single crystals were detected in the cross-section of the ternary nanocomposite. As previously reported for similar multifunctional systems (Averous et al., 2009) the surfactant was able to cover the single crystals during the solvent, casting process allowing a better dispersion of cellulose in the

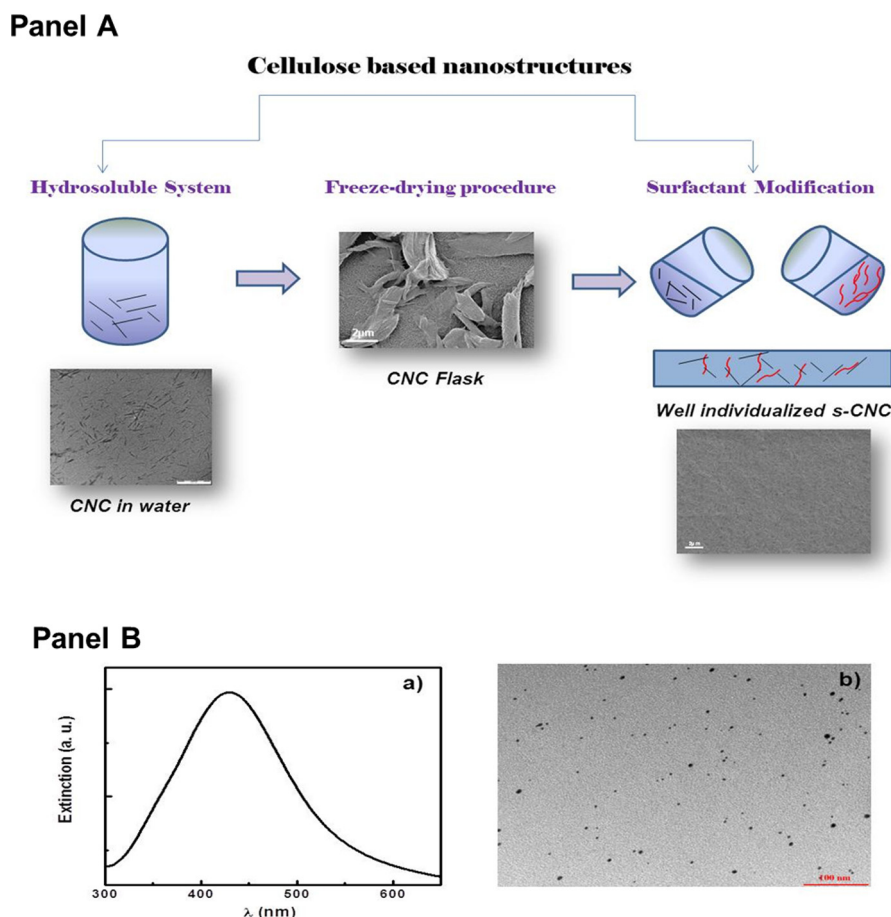


Fig. 1. (Panel A) General scheme of strategy used for the preparation and modification of cellulose nanocrystals and for cellulose based nano-biocomposites by solvent casting. (Panel B) Extinction spectrum of Ag suspension in CHCl_3 (a) and TEM image of synthesized Ag nanoparticles (b).

polymer matrix. Due to the different contrast properties and their small dimension, it was not possible to observe the distribution of silver nanoparticles in Fig. 2a. For this reason the TEM acquisition parameters were optimized to permit the silver nanoparticles observation and to study their distribution in the PLA fracture surface (Fig. 2b). TEM analysis of PLA/1s-CNC/1Ag ternary system shows the high homogeneity of the Ag nanoparticles distribution in the polymer fracture surface and the presence of well individualized nanoparticles (Fig. 2b insert) with spherical shape.

The optical images of the nano-biocomposites were recorded with a confocal laser scanning microscope taking advantage of the light scattered by Ag (Fig. 2c and d) and enabled to obtain information on the distribution of the metal particles in the films. The direct visualization of the Ag particles in the nano-biocomposite structure evidenced that these films were characterized by a quite homogeneous particle dispersion, which did not appear to be affected by the presence of cellulose crystals. The good dispersion obtained is likely due to the high affinity between PLA and the PVP stabilizer shell. Results from the UV–vis spectrophotometry tests on the nanocomposite films are shown in Fig. 3. The nanocomposite formulations show optical properties similar to the neat PLA film, without a significant reduction in the amount of reflected light in a wide range of vis region (region above 500 nm), documenting the good transparency of composite films. However, some yellowing was observed in the case of binary and ternary nano-biocomposites, being more evident with the increasing Ag content (Fig. 3). In particular, in the 800–500 nm range and below 250 nm all the films show similar spectra, overlapping, within the experimental error, with the spectrum of neat PLA. An intense absorption

centred at 290 nm, attributed to cellulose crystals, was observed for the ternary composites. Lower light reflection intensities were detected for all the composite samples in the 300–480 nm region, compared to the neat PLA film. This behaviour is likely due to the light extinction related to the presence of silver nanoparticles (Baia, Muresan, Baia, Popp, & Simon, 2007); the lower percentage of reflected light (higher absorption) in the UV and blue portion of the spectrum by the composite materials, compared to the neat PLA (Fig. 3), is also responsible of the mentioned yellowish effect. Furthermore, higher intensity in the absorption band was detected in the binary and ternary nano-biocomposites with the highest Ag content (1 wt%). The percentage of the reflected radiation at 430 nm was lower for the ternary composites, suggesting that cellulose nanocrystals might enhance the SPR absorption of Ag, probably due to their higher dielectric constant. It should be noted that in all nanocomposites the SPR band did not enlarge, compared to the suspension spectrum, confirming that the metal particles were not aggregated in the polymer structure.

The structures of pure PLA and PLA nano-biocomposites were characterized by WAXS to study the effect of the silver nanoparticles content and their combination with surfactant-modified cellulose nanocrystals on the crystallinity of the PLA matrix. Fig. 4 shows WAXS curves for PLA binary and ternary nano-biocomposites. As expected by the low content of D-LA in its composition (4%), PLA films only showed a wide diffraction band at $2\theta = 16.5^\circ$, suggesting the PLA amorphous nature. No particular effect of silver nanoparticles on the PLA crystallinity was detected (Fig. 4a). However, the presence of a shoulder at around $2\theta = 19^\circ$ for the PLA/1s-CNC/1Ag nano-biocomposite indicated an increased

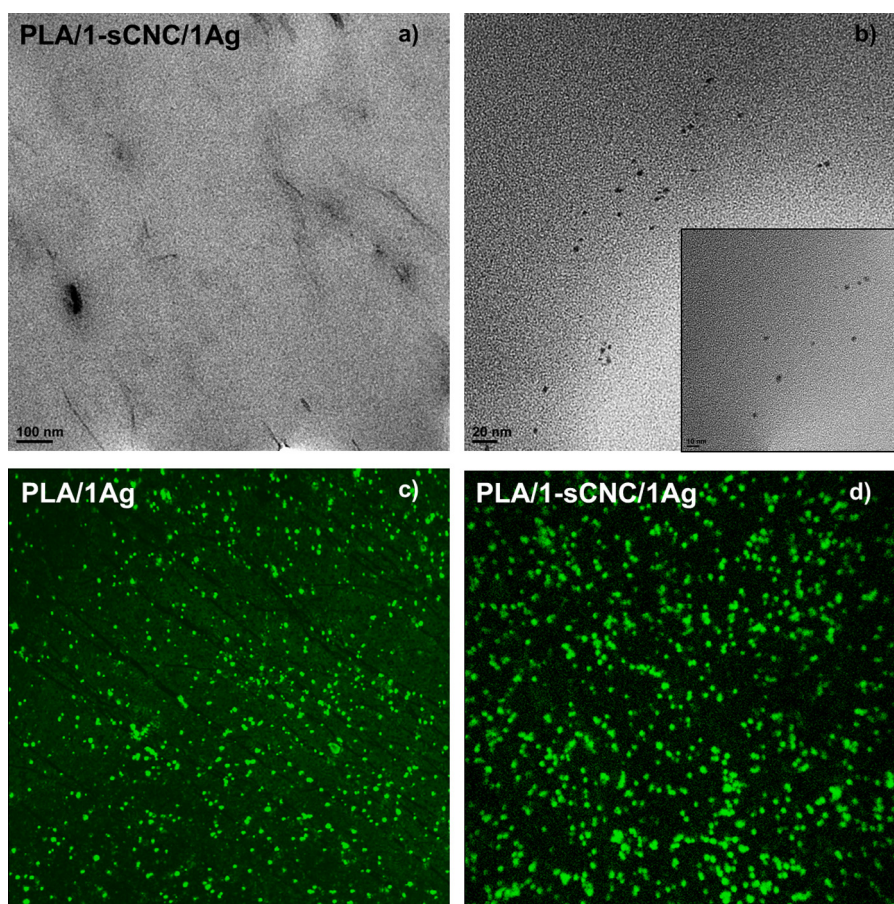


Fig. 2. TEM images of modified cellulose (a) and silver distribution (b and insert) for PLA/1s-CNC/1Ag ternary bio-nanocomposite. Confocal images recorded on PLA/1Ag (c, scan area $100 \times 100 \mu\text{m}^2$) and PLA/1s-CNC/1Ag (d, scan area $130 \times 130 \mu\text{m}^2$).

ability to crystallize of PLA when reinforced with s-CNC combined with the higher content of silver (Fig. 4b).

3.4. Thermal properties

Differential scanning calorimetry was used to investigate the glass transition, crystallization and melting of PLA and PLA nano-biocomposites. Table 1 summarizes the thermal parameters. The heating thermogram for the PLA neat film produced by solvent

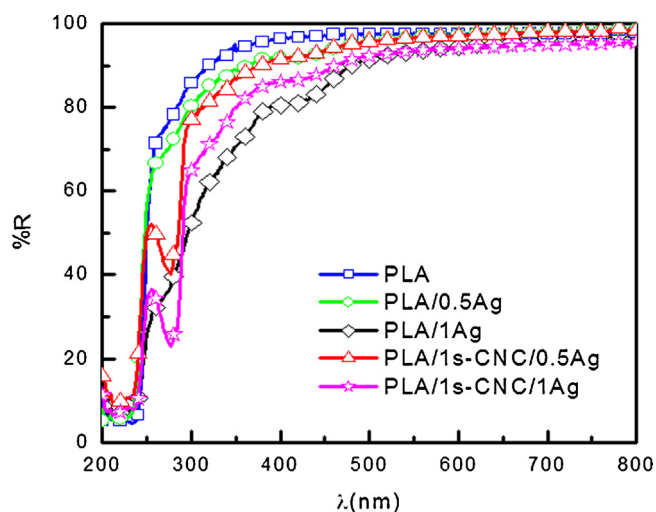


Fig. 3. Reflection spectra of PLA (squares), PLA/0.5Ag (circle), PLA/1Ag (diamonds), PLA/1s-CNC/0.5Ag (triangles) and PLA/1s-CNC/1Ag (stars).

casting displayed successively the glass transition temperature (T_g) at around 45°C , and the melting endotherm at T_m (151.2 ± 0.5) $^\circ\text{C}$, very similar results to those previously reported (Fortunati, Peltzer, et al., 2012), while no exothermic crystallization was observed during the cooling scan. Therefore, the main change in the baseline for the heat flow was attributed to the glass transition (Pei, Zhou, & Berglund, 2010). The heating thermograms for PLA reinforced nano-biocomposites showed the glass transition temperature, the exothermic peak related to the cold crystallization temperature T_{cc} and the melting endotherm at T_m . The low T_g values detected for neat PLA and PLA based nano-biocomposites at the first heating scan were due to the presence of residual solvent in the films after the casting process. A deeper analysis of all thermograms revealed a sharp endothermic peak associated to the glass transition, typically attributed to stress relaxation on heating (Fortunati, Armentano, Iannoni, & Kenny, 2010). Other studies reported similar relaxation phenomena in plasticized PLA, giving an additional confirmation of the presence of residual solvent with a plasticizing effect in these films (Burgos, Martino, & Jiménez, 2013).

The glass transition temperatures of the nano-biocomposites increased around $5\text{--}7^\circ\text{C}$ with the introduction of silver nanoparticles and this result was also observed in the case of ternary systems loaded with both silver and surfactant modified s-CNC. It was reported that although semi-crystalline, PLA-3051D shows slow nucleation and crystallization rates and consequently these samples should behave as amorphous after normal quenching. (Fortunati, Armentano, Zhou, Puglia, et al., 2012). In our case, it was observed that the ability to re-crystallize increased for PLA nano-biocomposites, with a reduction in the cold crystallization temperature with the increase in silver contents. This effect was

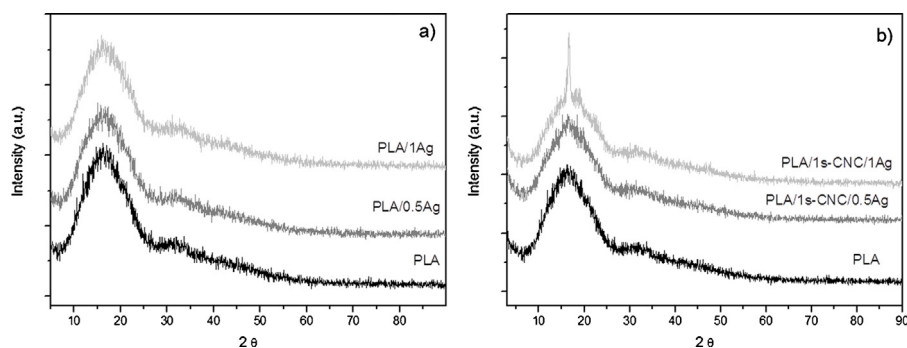


Fig. 4. Wide Angle X-ray Scattering patterns of binary (a) and ternary (b) PLA based nano-biocomposites.

enhanced by the combination of Ag with the s-CNC nanocrystals. The presence of silver nanoparticles in the PLA matrix promotes the cooling crystallization phenomena, while the combination of Ag with s-CNC favours the nucleation effect and the crystal growth (Fortunati, Armentano, Zhou, Iannoni, et al., 2012). Moreover, multiple melting peaks, not detected in the case of the PLA neat film, were observed in the case of PLA nano-biocomposites. The presence of multiple melting peaks could be related to the formation of different crystal structures or to lamellar populations with different perfection degrees (Fortunati, Armentano, Zhou, Iannoni, et al., 2012). Thermal analysis clearly evidenced that the nucleation effect was enhanced when homogeneous cellulose nanocrystals are dispersed in PLA and their good interaction with silver nanoparticles is achieved.

3.5. Barrier properties

Table 1 summarizes the oxygen transmission rate (OTR^e) values and the water vapour permeability (WVP) coefficients for PLA and PLA binary and ternary nano-biocomposites. A reduction in OTR^e values around 20% and 35% with respect to the PLA film was detected for PLA/0.5Ag and PLA/1Ag binary systems, respectively. Larger reductions in OTR^e were detected in the case of ternary systems. Thus, PLA/1s-CNC/0.5Ag showed a reduction of ca. 41% while a higher reduction (56%) was obtained in the case of the PLA/1s-CNC/1Ag ternary system with the highest content of silver nanoparticles. These results underline the positive effect and synergies of cellulose and silver nanoparticles on the PLA barrier properties, highlighting the increased ability to crystallize of silver in PLA matrices. It is known that the permeability properties are, in fact, linked to factors that will influence the tortuous path of gas molecules through the nanocomposite structure, such as particles shape and size, degree of exfoliation, reinforcement loading, orientation, crystallinity and porosity (Pettersson & Oksman, 2006). The combination of s-CNC and Ag nanoparticles produced an efficient barrier effect due to their good dispersion shown in TEM micrographs (Fig. 2) and the increased ability to crystallize demonstrated by WAXS and DSC analyses.

WVP results are also reported in Table 1. A reduction of about 40–45% was detected in the case of binary systems with silver nanoparticles and a similar behaviour was observed in the case of PLA/1s-CNC/0.5Ag ternary systems. Larger reductions, reaching 63%, were detected for the ternary system loaded with 1 wt% of silver nanoparticles, highlighting the positive effect of the increase in silver content and synergies with cellulose nanostructures.

3.6. Overall and specific migration: kinetics of silver release

Results of overall migration for PLA based nano-biocomposites in both food simulants (i.e. isooctane and ethanol 10%, v/v) are

shown in Fig. 5, panel A). It should be noted that results for all samples are well below the legal overall migration limit (60 mg kg^{−1} of simulant) (Commission Regulation EU 10/2011). It is interesting to highlight that the overall migration of pure PLA in ethanol 10% v/v was higher than in isooctane, and this behaviour was expected due to the hydrolysis of PLA and the formation of low molecular weight oligomers more prone to migrate (Schmidt et al., 2011). However, nano-biocomposites showed high overall migration in isooctane (0.05 < *p*). This is probably due to the increase in the polymer swelling caused by the isooctane which diminishes the resistance to the release of additives to the medium by increasing the free volume in the polymer matrix (Alin & Hakkarainen, 2011).

The overall migration in PLA/1Ag and PLA/1s-CNC/1Ag was twofold higher than in the case of PLA/0.5Ag and PLA/s-CNC/0.5Ag, due to the higher initial amount of Ag in the sample (0.05 < *p*), confirming the direct influence of Ag stabilized by PVP on the release of additives to the surrounding medium.

On the other hand, it is also important to know if the concentration of Ag in the simulant exceeded the specific limit of migration established by the European Food Safety Authority (EFSA), who evaluated the use of several silver-based substances intended to come into contact with food and defined a general specific migration limit of 0.05 mg of silver per kg of food (EFSA, 2005). Results of the specific migration of silver in both food simulants at the contact conditions indicated in the current legislation are shown in Table 1. The amount of silver transferred to isooctane from all the tested formulations was well below that specific migration limit, while after 10 days of incubation the release of Ag⁺ to ethanol 10% (v/v) in ternary nano-biocomposites with 1 wt% of Ag exceeded that limit. This behaviour could be due to the increase in the polarity in the food simulant causing the decrease in the additives release from PLA as it was described elsewhere (Fernández, Soriano, Hernández-Muñoz, & Gavara, 2010). Those composites with cellulose showed higher migration of Ag⁺ in the polar food simulant (0.05 < *p*) due to the enhancement of the hydrolytic degradation produced by the presence of cellulose nanocrystals (Xiang, Joo, & Frey, 2009). In addition, interactions between the carbonyl group of PVP and the primary hydroxyl functionalities of cellulose could favour the release of Ag⁺ to this simulant as it was well described by Masson et al. (1991).

Controlled release of additives to their surrounding environment is an important feature in many applications, particularly for food packaging and pharmaceuticals. The control of the antimicrobial agent release from packaging films is critical to preserve food products against microbial growth. In this study, the Ag⁺ release from the PLA-based nano-biocomposites, with and without cellulose nanocrystals, was quantified for 20 days in ethanol 10% (v/v). The diffusion coefficient (*D*, cm² s^{−1}) was calculated by fitting the experimental data to Eq. (3), and the ratio of the Ag⁺ concentration

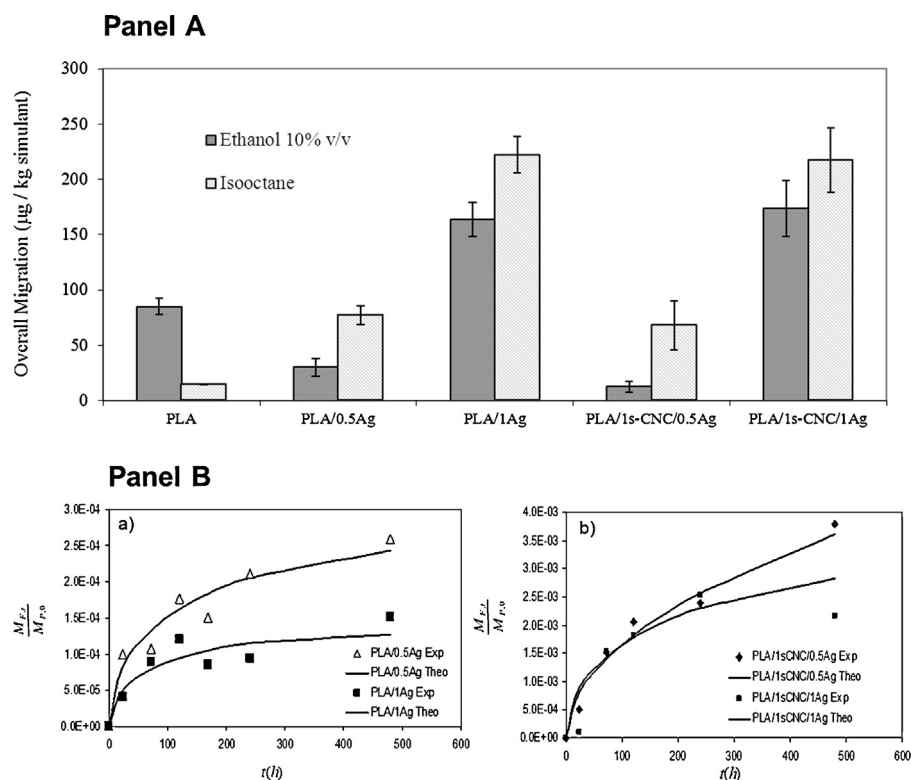


Fig. 5. (Panel A) Overall migration in ethanol 10% (v/v) and isooctane for PLA and PLA nano-biocomposites. (Panel B) Migration kinetics of silver with increasing exposure time for binary (a) and ternary (b) PLA nano-biocomposites at 40 °C.

in solution at time t ($M_{F,t}$) to the Ag^+ concentration in the polymer matrix at $t=0$ ($M_{F,0}$) was plotted against the square root of time. The value of D , calculated for each sample, was determined by minimizing the root mean squares errors (RMSE) of the measured and estimated values. Xiang, Taylor, Hinestroza, and Frey (2013) indicated that an active compound can be released from a biodegradable delivery system by diffusion through the polymer matrix, by pores present in the matrix and/or after the degradation of the polymer backbone. Fig. 5, panel B shows the gradual release of Ag^+ in ethanol 10% (v/v) during 20 days of incubation at 40 °C. It was observed that experimental data fit

well to Eq. (3) and the D value was further calculated. The presence of cellulose nanocrystals speeded up the release of Ag^+ in the food simulant as indicated by the higher diffusion value for PLA/1s-CNC/1Ag and PLA/1s-CNC/0.5Ag in comparison with those without cellulose in their formulations. These results were unexpected, since a lower release of Ag^+ in ternary nano-biocomposites should be theoretically observed due to the increase in the polymer crystallinity and the path tortuosity, as it was noticed in the WVP and OTR^e results. This effect could be attributed to the higher hydrolysis of the PLA in the simulant for these nano-biocomposites.

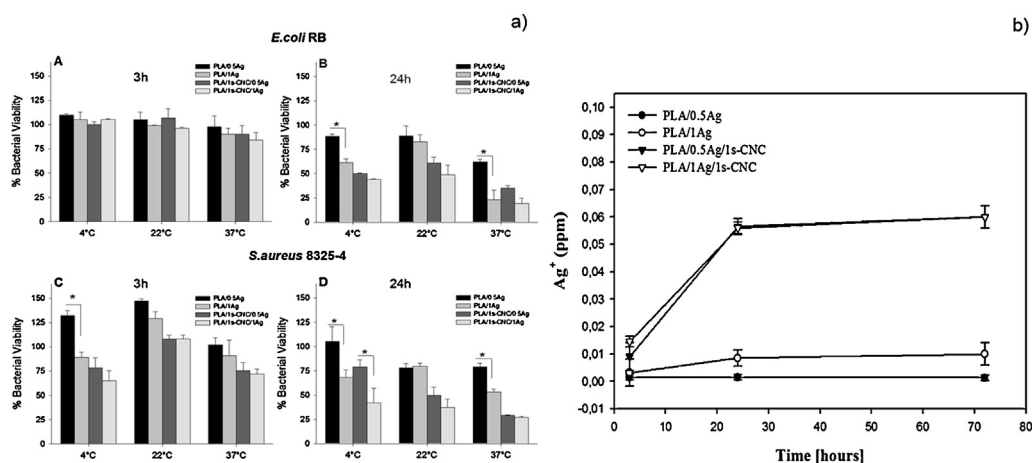


Fig. 6. (a) Antibacterial properties of PLA nano-biocomposites films at different temperatures. *E. coli* RB (A and B) and *S. aureus* 8325-4 (C and D) cells were incubated on PLA and PLA bio-nanocomposite films for 3 h (A and C) and 24 h (B and D) at 4 °C, 22 °C, and 37 °C, respectively, as reported in Section 2. Results are expressed on a biomaterial-basis and are presented as an average $\pm$ standard deviation ($0.05 > p$). (b). Ag^+ release upon degradation of PLA nano-bio-nanocomposite films. The Ag^+ ions release was detected on the same samples incubated in physiological conditions at 37 °C for 3 h, 24 h and 72 h, respectively. Results are expressed on a biomaterial-basis and are presented as an average $\pm$ standard deviation.

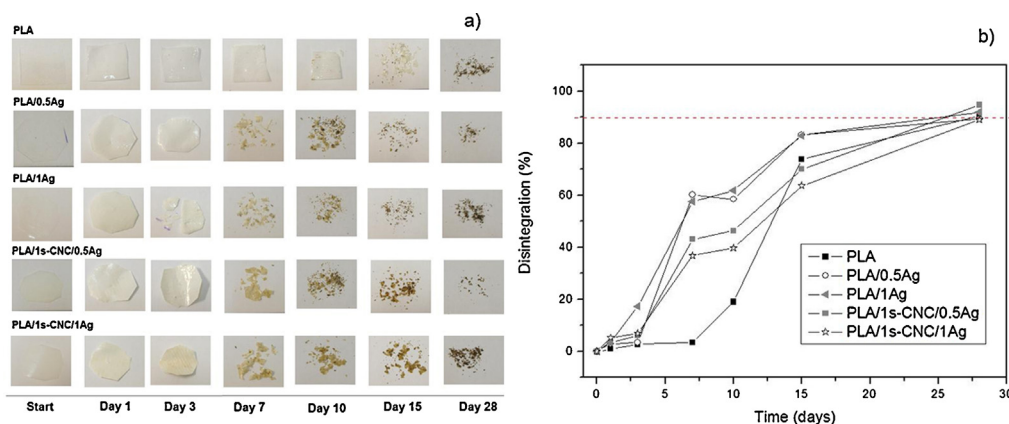


Fig. 7. (a) PLA and PLA nano-biocomposites before (0 days) and after different stages of disintegration in composting at 58 °C. (b) Disintegrability percentage values of PLA and PLA nano-biocomposites at different stages of incubation in composting.

3.7. Antibacterial properties

Fig. 6a shows the viability of *E. coli* (Fig. 6a-A and a-B) and *S. aureus* (Fig. 6a-C and a-D) cells onto PLA nano-biocomposite films after 3 h and 24 h incubation at 4 °C, 22 °C and 37 °C, respectively. As expected, the cell viability for both bacterial strains on PLA and PLA/1s-CNC films without Ag nanoparticles was quite similar at each incubation time and temperature (data not shown) ($p > 0.05$). On the contrary, PLA and PLA/1s-CNC films enriched with different concentrations of Ag nanoparticles (0.5 wt% or 1 wt%) showed an Ag dose-dependent antibacterial activity against *E. coli* and *S. aureus* strains but with some differences. The antibacterial activity was greater on *E. coli* than on *S. aureus* cells regardless of the incubation times and temperatures. These results are in agreement with previous studies showing that Ag nanoparticles are more toxic to *E. coli* than to *S. aureus* (Li et al., 2010; Wen-Ru et al., 2011). The reduced antibacterial activity on PLA nano-biocomposites for *S. aureus* may be due to the structural difference in the cell wall of Gram-positive if compared to Gram-negative cells. In fact, since the thick peptidoglycan layer of the staphylococcal cell wall prevents the penetration of the nanoparticles inside the cytoplasm, the antimicrobial effect is limited and seems related to the interaction with the bacterial surfaces or to the capture of the released silver ions. Only once inside the bacterial cell, the Ag^+ ions are known to interact with thiol groups of the proteins and to inactivate the enzyme activity (Liau, Read, Pugh, Furr, & Russel, 1997); silver ions turning DNA into a condensed form leading to the damage or even the death of the microorganisms (Kim et al., 2007). Recent studies showed that Ag nanoparticles in the range of 5–20 nm could more easily reach the nuclear content of bacteria by disrupting their membranes and increasing bacterial killing (Bin Ahmad et al., 2012; Lu, Rong, Li, Yang, & Chen, 2013). As regards the incubation time, the percentage of bacterial surviving fraction was higher after 3 h (Fig. 6a-A and a-C) if compared to a longer incubation time (24 h, Fig. 6a-B and 6a-D) for both bacterial strains ($0.05 > p$). The percentage of cell viability for both bacterial strains was slightly dependent on the incubation temperature and time: the cell surviving fraction was more reduced at 37 °C if compared at 22 °C and 4 °C after 24 h incubation time (Fig. 6B and D) ($0.05 < p$). This effect was less evident after 3 h (Fig. 6a-A and a-C). The antibacterial activity was also dependent on the Ag content, showing the highest value for samples containing 1 wt% Ag. These results are confirmed by previous studies performed on different PLA nano-biocomposites with higher Ag contents (Shameli et al., 2010). In addition, the antibacterial activity was significantly higher in the presence of 1 wt% s-CNC ($0.05 > p$) for both strains incubated at 37 °C for 24 h. Fig. 6b shows the release of Ag^+ ions from PLA nano-biocomposite films in

physiological conditions at 37 °C: even if the ions release was quite low, it was more evident for PLA nano-biocomposites containing 1s-CNC and reached their maximum after 24 h. These results were in agreement with the migration kinetics previously discussed. The higher release of Ag^+ ions from PLA/1s-CNC samples may explain their greater antibacterial activity on both strains at 37 °C after 24 h if compared to PLA/Ag samples at the same incubation time and temperature. In summary, the antibacterial activity for both strains was stronger on PLA/1s-CNC containing 1 wt% Ag nanoparticles if compared to PLA/1Ag when performed at longer incubation times. However, the effect was more significant for *E. coli* cells if compared to *S. aureus* cells.

3.8. Disintegrability in composting conditions

The biodegradation of PLA in compost is one of its most attractive properties for this polymer in massive applications, such as food packaging to limit the serious problem of waste disposal (Kale, Auras, Singh, & Narayan, 2007). The effect of nanoparticles on PLA biodegradation in compost has been reported over the past few years (Fukushima, Tabuani, Abbate, Arena, & Ferreri, 2010; Fortunati, Puglia, Santulli, Sarasini, & Kenny, 2012) and some delay in biodegradability of the biopolymer was observed. This effect is due to the improvement in water barrier properties of the nano-biocomposites, which could hinder the water diffusion through the bulk and consequently decrease the hydrolysis rate (Lee et al., 2002).

The study of the disintegration in composting conditions of PLA nano-biocomposites was carried out to evaluate the effect of the addition of silver nanoparticles and s-CNC nanocrystals on the disintegrability properties of the PLA matrix. Fig. 7a shows photographs of all samples at different composting times. Whitening and deformation of the films surface were detected just after the first day of incubation for all materials and these effects were enhanced after 3 days in composting conditions. These results are indicative of the beginning of the hydrolytic degradation, which was faster than expected by the low thickness of films (30–40 μm). The loss of transparency observed for all samples could be attributed to changes in the refractive index due to the water absorption and the formation of low molecular weight compounds after the hydrolytic degradation (Fukushima et al., 2010). It has been indicated that the PLA hydrolysis begins in the amorphous region of the polymer structure and all these effects would also result in changes in the polymer crystallinity and consequently in loss of transparency (Zaidi, Kaci, Bruzaud, Bourmaud, & Grohens, 2010). Moreover, it should be taken into account that the degradation experiments took place at 58 °C, which is higher than the T_g

Table 1
Thermal, barrier and migration behaviour of PLA and PLA nano-biocomposites.

Samples	Thermal properties				Barrier properties				
	First heating scan				OTR* <i>e</i> (cm ³ mm m ⁻² day ⁻¹)	Reduction in OTR (%)	WVP *10 ¹⁴ (kg ms ⁻¹ m ² Pa)	Reduction in WVP (%)	
	T _g (°C)	ΔH _m (J/g)	T _m (°C)	T _m (°C)					
PLA	45.0 ± 2.0	25.8 ± 0.7	151.2 ± 0.5	-	30.5 ± 1.0	-	1.04 ± 0.18	-	-
PLA/0.5Ag	52.6 ± 1.1	24.1 ± 0.4	147.2 ± 0.2	-	24.3 ± 0.1	20	0.64 ± 0.07	39	
PLA/1Ag	49.5 ± 0.1	25.4 ± 0.4	145.4 ± 0.1	-	19.7 ± 0.6	35	0.58 ± 0.09	44	
PLA/1s-CNC/0.5Ag	49.3 ± 1.2	27.7 ± 0.7	143.1 ± 0.1	149.8 ± 0.5	18.1 ± 0.7	41	0.59 ± 0.08	43	
PLA/1s-CNC/1Ag	48.6 ± 0.4	27.8 ± 0.1	143.4 ± 0.3	150.4 ± 0.2	13.4 ± 0.4	56	0.39 ± 0.01	63	
Migration studies									
				Ethanol 10% v/v (10 days, 40 °C) (μg kg ⁻¹ simulant)	Isooctane (2 days, 20 °C) (μg kg ⁻¹ simulant)		D (cm ² s ⁻¹)		RMSE (%)
PLA/0.5Ag				4.0 ± 1.0	1.0 ± 0.7		1.50 × 10 ⁻¹⁶		0.005
PLA/1Ag				4.2 ± 1.1	0.95 ± 0.24		8.50 × 10 ⁻¹⁷		0.005
PLA/1s-CNC/0.5Ag				40 ± 7	1.1 ± 0.8		2.50 × 10 ⁻¹⁴		0.18
PLA/1s-CNC/1Ag				94 ± 11	2.4 ± 1.0		2.3 × 10 ⁻¹⁴		0.22

of the matrix. This could increase the chain mobility, inducing the crystallization of the PLA matrix. At the 7th day, fragmentation and weight loss of the nano-biocomposites were already observed for all samples except for PLA neat film, which started fragmentation after the 10th day.

Fig. 7b shows the evolution of disintegrability values at different incubation times. It was observed that after 28 days of composting test, all materials reached 90% of disintegration. The addition of silver nanoparticles increased the degradation rate of PLA matrix reaching 60% at 10 days for PLA/0.5Ag and PLA/1Ag in comparison with 20% for the neat PLA film. These results are in agreement with those reported by other authors in PLA nanocomposites (Fukushima et al., 2010; Paul et al., 2005). The addition of cellulose nanocrystals also increased the disintegrability rate, but lower values were detected at 7 and 10 days for the PLA/1s-CNC/0.5Ag and PLA/1s-CNC/1Ag ternary systems than those obtained for binary nano-biocomposites. This behaviour could be attributed to the double effect caused by the addition of a hydrophilic filler, such as cellulose nanocrystals, which are expected to accelerate the degradation rate of the PLA nano-biocomposites. But, on the other hand, s-CNC could also limit the water diffusion by the increase in barrier properties and consequently delaying hydrolysis and further degradation. These results underlined that all the studied nano-biocomposites started the disintegration process before and with higher rate than neat PLA films, suggesting their perspective advantages in industrial applications when short biodegradation times are required.

4. Conclusions

The high potential benefits offered by nanotechnology in the development of tailor-made nano-biocomposites with specific applications is here shown by the combination of two different synthesized nanostructures: silver nanoparticles and cellulose nanocrystals in a biodegradable polymer matrix. Ternary nano-biocomposites based on PLA containing modified s-CNC and Ag nanoparticles were prepared by the solvent casting method and further characterized. Results showed the homogeneous particle dispersion in the polymer matrix, not affecting the original PLA transparency. The s-CNC induced PLA crystallization, which combined with the higher content of silver, resulted in clear enhancement of barrier properties to oxygen and water vapour (41% and 56% for PLA/1s-CNC/0.5Ag and PLA/1s-CNC/1Ag ternary nano-biocomposites, respectively). Reduction of about 40–45% in barrier properties was detected for binary systems, which was maximized for ternary systems loaded with 1 wt% of Ag, suggesting the positive effect of silver and its combination with cellulose nanostructures. Overall and specific migration values were well below the limits indicated by EFSA, while after the release of Ag⁺ to ethanol 10% (v/v) in ternary nano-biocomposites with 1 wt% of Ag exceeded the permitted limits. Higher migration was observed in those samples with cellulose, may be due to the interaction between the carbonyl groups PVP and the primary hydroxyl functionalities of cellulose that lead to the release of Ag⁺.

The addition of s-CNC resulted important for the improvement of the antibacterial activity of PLA/Ag. Finally the nano-biocomposites studied here started their disintegration process before and with a higher rate than pure PLA suggesting their perspective advantages in industrial applications when short biodegradation times are required.

In summary, the combination of the antibacterial properties of silver nanoparticles with cellulose nanocrystals in the PLA shows high potential to improve functional active properties with important implications in the development of new biodegradable materials for fresh food packaging applications.

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