



MMT-supported Ag nanoparticles for chitosan nanocomposites: Structural properties and antibacterial activity

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ABSTRACT

Multifunctional bionanocomposites have been prepared by loading chitosan matrix with silver-montmorillonite antimicrobial nanoparticles obtained by replacing Na⁺ ions of natural montmorillonite with silver ions. This filler has been chosen for its twofold advantage to serve as silver supporting material and to confer new and better performance to the obtained material. It has been proved that the achievement of the intercalation of chitosan into the silicate galleries of montmorillonite as well as the interaction between chitosan and Ag ions and silver particles lead to an enhancement of the thermal stability, to an improvement of mechanical strengths and to a reduction of the liquid water uptake of the obtained bionanocomposites. Results also show that silver ions are released in a steady and prolonged manner providing, after 24 h, a significant reduction in the microbial growth of *Pseudomonas* spp.

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1. Introduction

Chitosan, a crystalline polysaccharide obtained from crustaceans, is the deacetylation form of chitin, the second most abundant natural polymer. Because of the great advantage provided by the use of polymers obtained from renewable sources, chitosan has been one of the most attractive biopolymers due to its biocompatibility, biological activity and biodegradability. It has been used to design and fabricate new materials with functional properties applied in various fields, ranging from waste management and medicine to food processing and packaging (Wang, Chen, & Tong, 2006).

The enhancement of barrier and mechanical properties of chitosan-based films as well as the improvement of their dimensional stability has been achieved through the development of nanocomposites obtained by adding plasticizer and various types of layered silicates such as montmorillonite to the polymer matrix (Gunster, Pestreli, Unlu, Atici, & Gungor, 2007; Kasirga, Oral, & Caner, 2012; Lavorgna, Piscitelli, Mangiacapra, & Buonocore, 2010; Paluszkiwicz, Stodolak, Hasik, & Blazewics, 2011; Petrova et al., 2012; Tang et al., 2009; Wang et al., 2005; Xu, Ren, & Hanna,

2006). In order to further impart new functionalities to biopolymers for broadening their application fields, the use of suitable nanoparticles is highly demanded. In particular, silver nanoparticles, alone or supported on inorganic platelets (Patakfalvi, Oszka, & Dekany, 2003; Sotiriou, Meyer, Knijnenburg, Panke, & Pratsinis, 2012), can be used as filler into polymeric structures with the aim to endow antibacterial and antimicrobial properties to the obtained nanocomposites, thus making them suitable for a variety of target applications such as textile, biomedical and food packaging materials (Sotiriou et al., 2012). As for the latter field of application, several studies have demonstrated the effectiveness against microbial growth in foods of silver nanoparticles loaded in biopolymers such as sodium alginate, polyvinylpyrrolidone, cellulose based absorbent pads and hydroxypropyl ethylcellulose (de Azeredo, 2013).

Chitosan has already been investigated as matrix to incorporate silver compounds or silver nanoparticles (Zhang, Luo, & Wang, 2010). In particular, different silver compounds were incorporated into various forms of chitosan matrix, including solution (Bin Ahmad et al., 2012; Hsu et al., 2011), gel (An, Luo, Yuan, Wang, & Li, 2011; Krishna Rao, Ramasubba Reddy, Lee, & Kim, 2012) and film (Lopez-Carballo, Higuera, Gavara, & Hernandez-Munoz, 2013; Pinto et al., 2012; Regiel, Irusta, Kyzioł, Arruebo, & Santamaria, 2013). All these papers mainly deal with the structural characterization of the obtained nanocomposites and with the study of their antimicrobial and antibacterial activity. However, the knowledge

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about the silver ions release and the capability to control their release kinetic is essential in order to assess the applicability of silver nanocomposites in sectors such as medicine and food packaging. Wang, Liu, Ji, Ren, and Ji (2012) reported that the release of silver ions from chitosan-Ag/PVP nanocomposite films shows a burst release process in the first day whereas Martinez-Abad, Lagaron, and Ocio (2012) showed that the release of Ag^+ from a silver-based EVOH film in water takes place within 30 min, all samples reaching the equilibrium before the first hour. However, it is possible to design silver engineered nanoparticles, i.e. inorganic porous hosts with silver partially immobilized, able to release silver ions with a fine control and high effectiveness over time. In fact the use of bamboo charcoal supporting silver compounds delayed the sustained release of silver ions in aqueous environment over 70 h for diffusion effects (Yang, Wu, Liu, Lin, & Hu, 2009), whereas Ag and Ag_2O nanoparticles supported on nanostructured SiO_2 were able to release Ag^+ ions by controlled dissolution of the oxide layer and further oxidation of metal silver (Sotiriou et al., 2012).

Moreover, in the last years, an innovative class of inorganic engineered nanoparticles based on layered silicates such as montmorillonite supporting silver ions or silver metal have been proposed (Incoronato, Buonocore, Conte, Lavorgna, & Del Nobile 2010; Praus, Turicova, & Valaskova, 2008). In these nanoparticles the release of silver ions is controlled by both diffusion effects and oxidation reactions of silver to silver ions which depend on the size of silver nanoparticles and on the water wettability. Incoronato et al. (2010) embedded the obtained silver-montmorillonite particles into agar, zein and polycaprolactone matrices and showed that the water uptake of the polymeric matrix is the key parameter associated with the antimicrobial effectiveness of these active systems.

However, to the best of our knowledge, there is no paper reporting on the design of a chitosan-based nanocomposite films in which tailored functionalities were introduced using silver-montmorillonite nanoparticles. In this work we propose novel active nanocomposite films consisting of chitosan filled with silver-montmorillonite nanoparticles obtained by ion exchange reaction. In the novel multifunctional bionanocomposite, the inorganic carrier has been used as filler exhibiting a twofold advantage. It serves as silver supporting material for a slow and sustained release of silver ions in an aqueous medium and to confer better performance to the obtained films such as dimensional stability and mechanical properties. The resulting films were widely characterized in order to have a deeper understanding of the correlations between their structure and properties; their *in vitro* antimicrobial activity was also assessed in order to verify their effectiveness as active systems.

2. Experimental

2.1. Materials

Chitosan (CS) powder (molecular weight in the range 310–375 kDa and deacetylation degree >75%), silver nitrate (AgNO_3) and glacial acetic acid (HAc) were purchased from Sigma-Aldrich (Milan, Italy). The unmodified pristine clay (Na^+ -montmorillonite) was purchased from Southern Clay Products, Inc., TX. Glycerol by Fluka (Italy) was used as plasticizer.

2.2. Methods

2.2.1. Preparation of silver/montmorillonite nanoparticles

Silver-montmorillonite (Ag-MMT) nanoparticles were prepared by silver ions exchange reaction (Incoronato et al., 2010). Five grams of Na-MMT were dispersed in 100 ml of 0.2 mol/l NaCl solution for 4 h while stirring. The solid was then separated by

centrifugation at a speed of 10,000 rpm for about 15 min and then washed with deionized water for three times. The washed Na-MMT was brought in contact with AgNO_3 solutions at different concentration (500, 1000 and 5000 ppm) in order to enhance the ions exchange. In particular, Na-MMT was dispersed firstly in a 500 ppm AgNO_3 solution, at 70 °C for 3 h under stirring, covering the vessel in order to minimize the reduction extent by UV light. Afterwards the particles were separated and re-dispersed subsequently in the 1000 and 5000 ppm solutions for other three hours. At the end of the treatment the solid and liquid part of the slurry were separated by centrifugation at a speed of 10,000 rpm for 15 min, the particles were washed with deionized water for three times and dried in a vacuum oven at 80 °C overnight.

2.2.2. Preparation of chitosan based nanocomposite films

Chitosan solution was prepared by dissolving 2 g of CS powder in 100 ml of acetic acid solution (1%, v/v), homogenized using a magnetic stirring plate at 90 °C and 150 rpm for 20 min and successively cooled. The glycerol plasticizer (25% wt on CS solid base) was then added to the CS solution while stirring for 20 min at 60 °C. Nanocomposite samples were obtained by dispersing different amounts of Ag-MMT in 100 ml of 1% (v/v) acetic acid solution for 1 h at room temperature. The obtained dispersion was added to the CS solution, stirred for 1 h at room temperature and then sonicated for 30 min at 25 °C in a bath-type ultrasound sonicator. Known amounts of the dispersion were then poured into glass plates ($D = 14$ cm) and dried at $T = 22$ °C and $\text{RH} = 53\%$ for three days, until the solvent was completely evaporated and a self-standing film was obtained. This procedure allows the obtainment of films having an average thickness 120 ± 5 μm . The cast film was dried overnight in a vacuum oven at 25 °C. Chitosan/Ag-MMT nanocomposites containing 3 and 10% (w/w) of Ag-MMT nanoparticles on CS solid base were obtained and coded as CS/3AgMMT and CS/10AgMMT respectively. Neat chitosan, with and without glycerol, and chitosan/Na-MMT films containing 10% (w/w) of Na-MMT were prepared for comparison purposes and respectively coded as CS, CS-No Gly and CS/10NaMMT.

2.2.3. X-Ray analysis

The crystalline structures of both Na-MMT and Ag-MMT powders and chitosan and chitosan-based nanocomposites were evaluated using Wide Angle X-Ray Diffraction (WAXD) measurements. An Anton Paar SAXSess diffractometer (40 kV, 50 mA) equipped with a $\text{Cu-K}\alpha$ radiation ($\lambda = 0.1546$ nm) source and an image plate detector was used. The spectra were collected in the transmission mode by scanning the 2θ range between 1.5 and 40°. All spectra were corrected only for the dark current and the empty holder background.

2.2.4. X-Ray Photoelectron Spectroscopy (XPS)

X-Ray Photoelectron Spectroscopy (XPS) analysis was performed to detect the chemical state of the silver in the Ag-MMT powder. An ESCALAB MKII (VG Scientific Ltd, UK) instrument, with an $\text{AlK}\alpha$ (14 kV, 350 W) excitation source and a 5-Channeltron detection system, was used. Photoelectron spectra were collected at 20 eV constant pass energy of the analyzer. The electron take-off angle was 45° with respect to the sample. Pressure during analysis was around 10^{-9} mbar. Ag-MMT powder were suspended in ethanol and deposited on a gold plate. The spectra were processed by the CasaXPS software by using a peak fitting routine with symmetrical Gauss–Lorentian function.

2.2.5. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy analysis was carried out by using a FEI Tecnai G12 Spirit Twin microscope operated at 120 kV (LaB6 cathode, point resolution 0.35 nm). Images were recorded on

a CCD camera with the resolution of 4096×4096 pixels. Powder samples of Ag-MMT were dispersed in distilled water and the suspension was treated with ultrasound for 2 min. A drop of a very dilute suspension was placed on a holey-carbon coated copper grid and allowed to dry by evaporation at room temperature before observation.

2.2.6. Infrared spectroscopy (FTIR)

FTIR spectra of chitosan nanocomposites were collected in ATR mode by using a Nicolet FTIR spectrophotometer in the range of $4000\text{--}400\text{ cm}^{-1}$ at a resolution of 4 cm^{-1} .

2.2.7. Thermogravimetric analysis

The thermogravimetric analysis of the nanocomposite films was measured by using a TGA 2950 (TA Instruments), submitting the samples to a heating run from 30°C up to 800°C at a heating rate of $10^\circ\text{C}/\text{min}$, under air flow.

2.2.8. Dynamic mechanical analysis

Dynamic mechanical properties of the nanocomposites (storage modulus, loss modulus and $\tan \delta$) were obtained by using a DMA Q8000 (TA Instrument) in tension mode, 1 Hz frequency, from 20 to 250°C with a heating rate of $3^\circ\text{C}/\text{min}$. The specimens consist of strips of $7 \times 10\text{ mm}^2$. The glass transition was defined as the temperature where $\tan \delta$ is at the maximum value.

2.2.9. Water uptake

Films were cut in small pieces ($1.2\text{ cm} \times 1.2\text{ cm}$), weighted, placed in closed beakers containing 30 ml of water at various pH (i.e. 3.6, 6, 9) and stored at 25°C . The water uptake kinetics were evaluated by periodically measuring the weight of the film, after gently bottling its surface with a tissue, by means of a balance (Sartorius model SPB32, with an accuracy of 0.0001 g), until equilibrium was reached. The water uptake (W.U.) was calculated as follows:

$$\text{W.U.} = \frac{m_{\text{WetFilm}} - m_{\text{DryFilm}}}{m_{\text{DryFilm}}} * 100$$

where m_{DryFilm} is the weight of dry film and m_{WetFilm} is the weight of film during water sorption. Each data is the average value of three replicas.

2.2.10. Silver release rate

The concentration of silver released in an aqueous solution from the nanocomposite films was measured by Inductively Coupled Plasma Mass Spectrometry (PerkinElmer ICP-MS 2100 Inc. Shelton, CT, USA). Nanocomposite films were immersed in water in closed beakers at ambient room temperature. The ratio nanocomposite area/water volume was equal to $1\text{ cm}^2/10\text{ ml}$. At various time intervals, the beaker was emptied out and re-filled with fresh water. The withdrawn samples were analyzed by ICP/AES and the amounts detected over time were summed in order to obtain cumulative release data. A commercial silver ICP multistandard solution was used for calibration.

2.2.11. Antibacterial activity

The antibacterial activity of the nanocomposite systems was tested against a mix of *Pseudomonas fluorescens* (DSMZ 50090) and *Pseudomonas putida* isolated from spoiled mozzarella cheese. Plate Count Broth (PCB) (Oxoid, Milan, Italy) (tryptone 5 g/l , glucose 1 g/l and yeast extract 2.5 g/l) was used as culture medium (Incoronato et al., 2010). The *Pseudomonas* strains were maintained on slants of appropriate media and stored at 4°C as stock cultures. Prior to use, exponentially growing cultures grew overnight in the appropriate liquid medium at their optimal temperature. After 24 h, equal volumes (1%) of each suspension of microorganisms were mixed and serially diluted. An aliquot of the mix was inoculated in PCB

to obtain the final concentration of 10^3 number of colony forming units (CFU) mL^{-1} . To ensure a good degree of reproducibility in the inocula preparation procedure, the cell counts were standardized through the direct plate count technique. For the growth experiments PCB was spread in different tubes containing the various active and control films. All tubes were inoculated with prepared microbial cocktail (1%) and incubated at 25°C for 72 h under stirring conditions. Periodically (at 0, 24, 48 and 72 h), aliquots of 1 ml were taken from each tube for microbiological analyses. To avoid modifications in the microbial concentration due to sampling, each tube was used only for a single measure. Each sample was decimal diluted with sterile saline solution (9 g/l NaCl). The serial dilutions of sample were plated on *Pseudomonas* Agar Base (PAB), modified by adding *Pseudomonas* CFC selective supplement after autoclaving at 121°C for 15 min and incubated at 25°C for 48 h. The control samples were: tubes of inoculated PCB without any film (CNT), tubes with neat chitosan films (CS) and tubes with chitosan film containing 10% NaMMT (CS-NaMMT). All analyses were performed twice.

3. Results and discussion

A preliminary characterization of chemical and physical properties of Ag-MMT particles has been previously reported by some of the authors (Incoronato et al., 2010). It has been proved that the total content of silver in the Ag-MMT particles is equal to 0.037 g/g . The presence of the characteristic UV silver surface plasmon bands at 290, 350, 450 nm shows that residual Ag^+ ions or silver nanoparticles with average size of 2 nm, 10 nm and 40 nm are simultaneously present.

In order to have a deeper understanding of the chemical structure of silver atoms, the Ag-MMT powder has been submitted to XRD and XPS characterization. In Fig. 1(a) the XRD spectra of Na-MMT and Ag-MMT powders are compared.

Unmodified Na-MMT clay presents an intense diffraction peak at around 2θ equal to 7.2° , corresponding to the basal $d_{(001)}$ spacing between the silicate platelets of about 1.2 nm, alongside with other peaks associated to the main crystalline phases of the clay. In particular the diffraction peaks at 2θ equal to 7.2 , 19.6 , 29.3 , 34.9 and 36° are ascribed to magnesium montmorillonite $\text{MgO} \cdot \text{Al}_2\text{O}_3 \cdot 5\text{SiO}_2 \cdot x\text{H}_2\text{O}$; the peaks at 2θ equal to 7.2 (i.e. over imposed peak) and 35.5° are ascribed to the hexagonal calcium–magnesium montmorillonite $\text{Ca}_{0.2}(\text{Al,Mg})_2\text{Si}_4\text{O}_{10}(\text{OH})_2 \cdot 4\text{H}_2\text{O}$ and finally the diffraction peak at 2θ equal to 21 and 26.6° are assigned to hexagonal quartz SiO_2 (Paluszkievicz et al., 2011). After modification with silver, the diffraction spectrum of Ag-MMT clay shows that the main diffraction peaks assigned to the montmorillonite phases modify both in terms of shape and intensity. These evidences reflect the structural modifications which take place when Ag^+ ions replace Na^+ ions inside the layered structure of MMT. In particular it is likely that MMT clay loses its layered structure giving rise to nanoparticles with collapsed or exfoliated structure (Patakfalvi et al., 2003). The peak at 2θ equal to 37.9° in the XRD pattern of Ag-MMT corresponds to the (1 1 1) reflection of metallic Ag. It is also worth noting that the diffraction peak at 2θ around 32° may be assigned to the presence of $\text{AgO}/\text{Ag}_2\text{O}$ crystalline domains which generate from ions in basic conditions (Sotiriou et al., 2012).

The presence of both Ag metallic and $\text{AgO}/\text{Ag}_2\text{O}$ oxides is also confirmed by XPS analysis as reported in Fig. 1(b). The absorption bands at 368.4 eV and 375 eV are assigned to the electrons $3d_{5/2}$ and $3d_{3/2}$ of silver atoms. In particular, the $\text{Ag}3d_{3/2}$ peak at 375 eV can be assigned to the presence of Ag ions (Huang et al., 1996; An et al., 2011). The band at 368.4 eV is deconvoluted in two peaks at 369.2 and 368.1 eV attributed respectively to the 3d electrons of

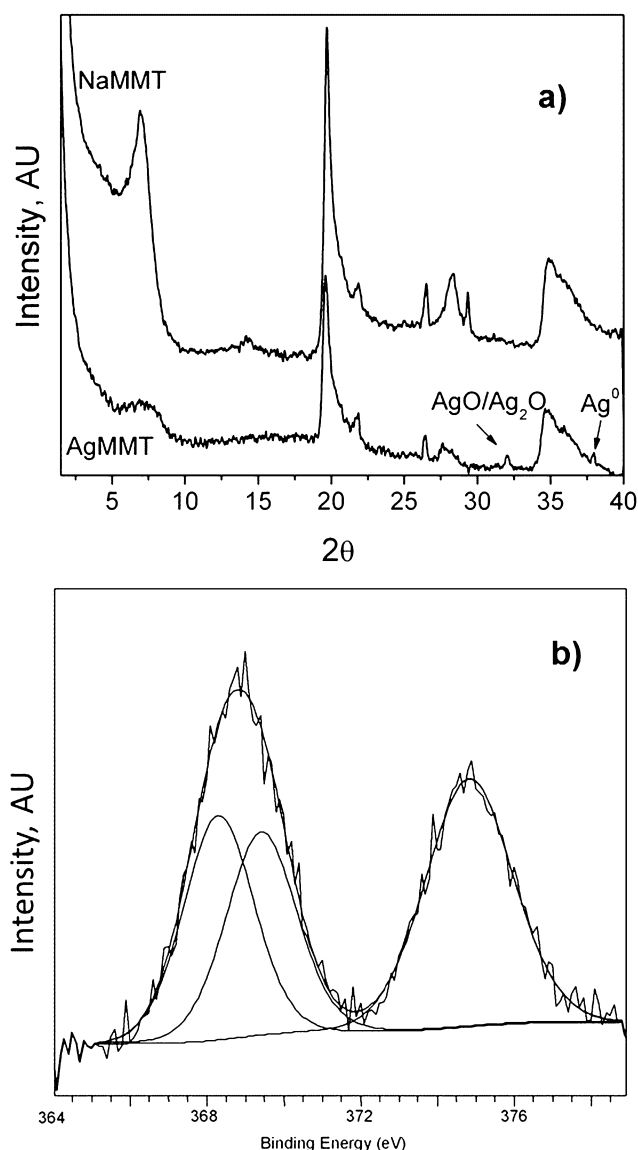


Fig. 1. (a) XRD spectra of Na-MMT and Ag-MMT clays and (b) XPS spectra of Ag-MMT clays.

oxide and metallic silver. Based on the results of XPS and WAXD analysis, and taking into account both the basal spacing $d_{(001)}$ of MMT (1.2 nm) and the size of nanoparticles which is larger than 2 nm, it is hypothesized that the silver nanoparticles are mainly located on the surface of single platelet as well as MMT tactoids. This is confirmed by TEM micrographs of Ag-MMT particles shown in Fig. 2.

The Ag and Ag₂O nanoparticles are deposited on the MMT lamellae with a preferential location on the edges. The particles are well-separated from each other and show a size in the range from 2 to 30 nm, thus confirming the previous result based on the UV plasmon bands (Incoronato et al., 2010). Most of silver nanoparticles have been detached from silicate platelets by sonication process and result homogeneously dispersed around the MMT lamellae. In the insert image of Fig. 2(a) the presence of nanoparticles with average size of about 2–3 nm alongside with the presence of MMT platelet fragments can be observed. They are likely originated by collapsing of MMT structure during the ion exchange process and their presence confirms the damage of MMT layered morphology by the ionic exchange process as already shown by XRD analysis.

In Fig. 3 are reported the XRD spectra of neat chitosan and of bionanocomposites containing 10 wt% of Ag-MMT and Na-MMT (Fig. 3a)) and a schematic representation of the CS/Ag-MMT (Fig. 3b)).

Neat chitosan film shows the characteristic crystalline peaks at 2θ equal to 8°, 11.2° and 18° as well as a broad peak at 23° ascribed to the amorphous structure corresponding to the chitosan with ammonium acetate groups. The crystalline structure of chitosan is strongly dependent on its origin and molecular constitution as well as its processing treatment (Rhim, Hong, Park, & Ng, 2006). It is known that the presence of nanoparticles such as Na-MMT inhibits the formation of crystalline domains (Abdollahi, Rezaei, & Farzi, 2012). However, in the present case the simultaneous presence of glycerol and Na-MMT clay has not any negative effects on the crystalline structure of chitosan. In fact the spectrum of Na-MMT nanocomposite presents the characteristic diffraction peaks of crystalline chitosan alongside with the characteristic peaks of MMT at 2θ equal to 19.6° and 7.2°. However, the diffraction spectrum of chitosan modified with Ag-MMT nanoparticles show a complete disappearance of the peaks ascribed to the chitosan crystalline structure. This modification may be ascribed to the presence of Ag ions which, interacting with NH and OH groups of chitosan, hinder the crystalline formation leading to the obtainment of an amorphous chitosan structure (Reicha, Sarhana, Abdel-Hamida, & El-Sherbinyb 2012). In Fig. 3(a) it can be also observed that the basal (001) diffraction peak of unmodified and silver modified clays, initially at 2θ equal approximately to 7.2° ($d_{(001)}$ spacing of 1.2 nm), shifts to around 4.5° ($d_{(001)}$ spacing of 2 nm) after formation of chitosan composites. This confirms that the highly hydrophilic chitosan macromolecules intercalate in the silicate galleries.

In a previous paper (Lavorgna et al., 2010) some of the authors showed by means of FTIR spectroscopy that in chitosan nanocomposites filled with MMT (1) glycerol molecules displace acetic acid from chito-ammonium acetate groups and form hydrogen bonding with amine groups and (2) Na-MMT clay interacts with chitosan macromolecules through hydrogen bonding between OH groups on MMT edge platelets and amine groups of chitosan. In Fig. 4 FTIR spectra of Ag-MMT and Na-MMT chitosan nanocomposites are compared with spectrum of chitosan film.

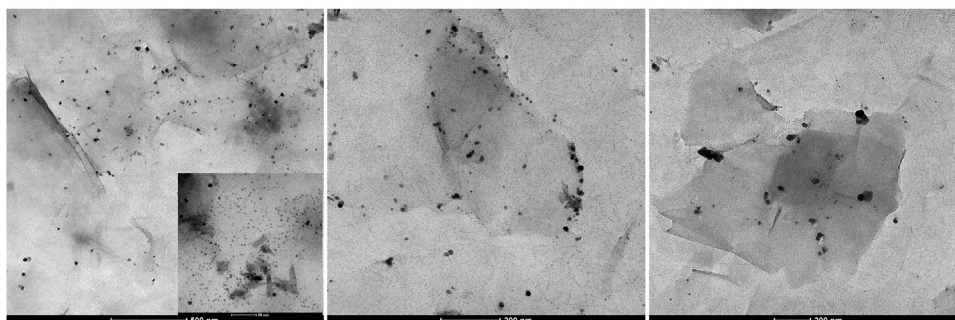


Fig. 2. TEM images of Ag-MM at different magnifications.

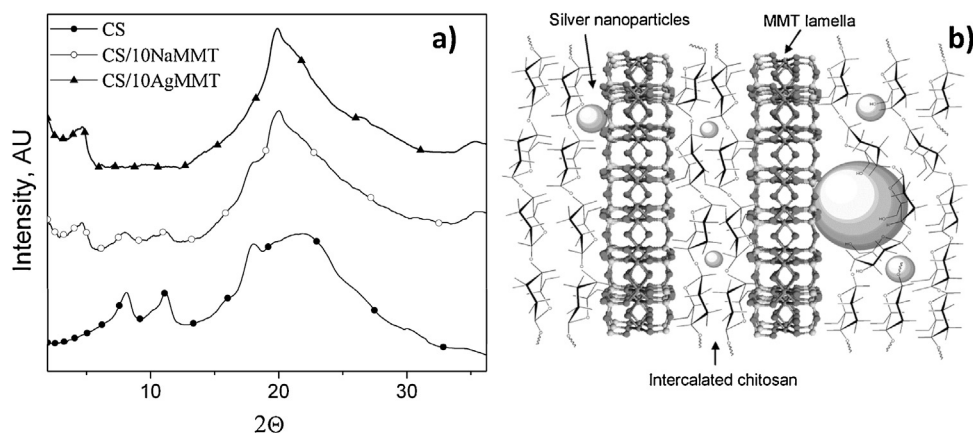


Fig. 3. (a) XRD spectra of neat chitosan and Na-MMT and Ag-MMT bionanocomposites and (b) schematic representation of CS/AgMMT.

The spectra related to the nanocomposites differ for the relative intensity as well as the wavenumber position of some characteristic absorbance peaks. In particular the presence of silver modified silicate nanoparticles brings about a reduction of the intensity of peak at 1630 cm^{-1} assigned to both carbonyl stretching and water bending modes and of peak at 1380 cm^{-1} assigned to CH bending modes. Furthermore the peaks at 1590 cm^{-1} , assigned to NH bending modes of amide groups (Wang et al., 2005), slightly shift toward lower wavenumbers (approximately 3 units as cm^{-1}). These spectral features may be tentatively ascribed to the interaction of amine chitosan with Ag^+/Ag^0 by electrostatic bond similar to that described by Vigneshwaran et al. (2007) as well as to the chelation of Ag with both amino groups and hydroxyl of chitosan (An et al., 2011; Shameli et al., 2010).

The effect of the simultaneous presence of AgMMT nanoparticles and glycerol on the thermal and mechanical properties of the obtained nanocomposites has been evaluated by thermogravimetric (TGA) and dynamic mechanical (DMA) analysis. Thermograms reported in Fig. 5 show that the thermo-oxidative degradation of the samples consists of three degradation steps: the first step ($30\text{--}200^\circ\text{C}$) is associated with the loss of absorbed water and glycerol; the second step ($200\text{--}450^\circ\text{C}$) corresponds to the degradation and deacetylation of chitosan, whereas the last one ($450\text{--}750^\circ\text{C}$) may be assigned to the oxidative degradation of the carbonaceous

residue formed during the second step (Wang et al., 2005, 2006b). In Table 1 the thermal parameters determined from TGA curves are reported: the temperature at which thermal degradation causes a loss of 20% and 50% weight (T_{20} and T_{50}), the peaks temperature for every step ($T_{\text{max I}}$, $T_{\text{max II}}$, $T_{\text{max III}}$) and the residue percentage at 750°C .

Neat chitosan shows a greater mass loss in the first step, due to the unbound glycerol removal. Moreover, it can be evidenced that, especially at high temperatures, the presence of nanoparticles enhances the thermal stability of the material. In fact, the onset temperatures of the third thermal degradation step increases with respect to neat chitosan by 28°C and 60°C , with the incorporation of 3 and 10 wt% of AgMMT respectively (Table 1). The observed thermal stabilization is likely due to the barrier provided by the platelets to the diffusion of oxygen (Wang et al., 2005) and to the intercalation of chitosan molecules in the silicate layers which brings about a restricted thermal motion. The residues at 750°C result lower than those expected on the basis of the nominal experimental composition. This may be attributed to the presence of absorbed water in the AgMMT powder as well as to the loss of inorganic filler during the thermal oxidative degradation.

As for the DMA analysis, the storage modulus and $\tan \delta$ of the chitosan and nanocomposites, as a function of temperature, are shown in Fig. 6a and b. As expected, the storage modulus of chitosan

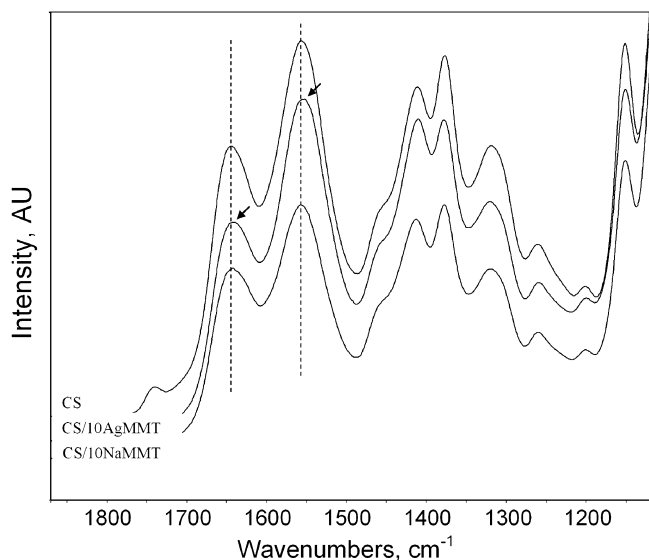


Fig. 4. FT-IR spectra of (a) CS, (b) CS/AgMMT and (c) CS/NaMMT.

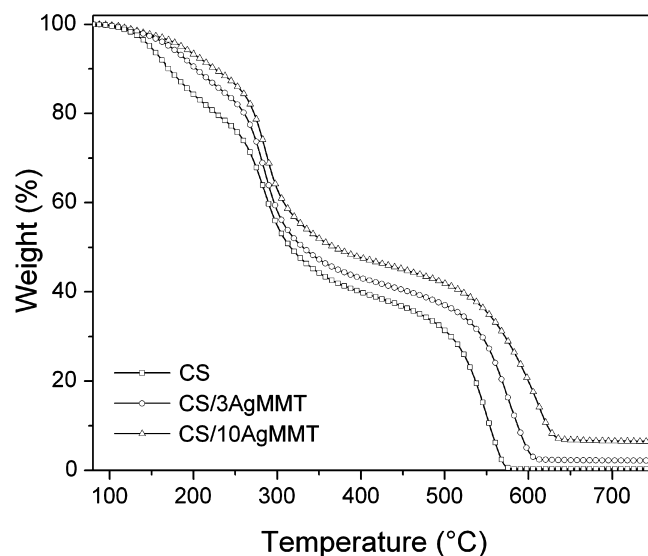


Fig. 5. Thermogravimetric analysis curves for (□) CS; (○) CS/3AgMMT and (Δ) CS/10AgMMT.

Table 1
Thermal parameters of samples.

Sample	T_{20} (°C)	T_{50} (°C)	$T_{\max}$ I	$T_{\max}$ II	$T_{\max}$ III	Residue at 750 °C
CS	227.21	315.83	170	285	550	
CS/3AgMMT	260.55	330.68	182	285	578	2.2%
CS/10AgMMT	272.39	368.04	204	287	610	6.5%

decreases by adding glycerol which acts as plasticizer. However, a recovery of storage modulus is obtained by adding 10% wt of AgMMT nanoparticles. This recovery, which is evident all over the investigated range of temperature, is more significant than that observed by some of the authors for chitosan nanocomposites incorporating 10%wt of NaMMT (Lavorgna et al., 2010). This suggests that interactions between chitosan and silver nanoparticles take place, allowing a better load transfer between matrix and fillers and thus providing high mechanical strengths.

The $\tan \delta$ plot of neat chitosan shows a prominent relaxation process at around 160 °C as reported in Fig. 6b. This peak is attributed to the glass transition temperature of chitosan arising from the relaxation of two glucopyranose rings via the glucosidic oxygen and assisted by a cooperative hydrogen bonds reordering (Quijada-Garrido, Iglesias-Gonzalez, Mazon-Arechederra, & Barrales-Rienda, 2007). The glass transition temperature decreases until 110 °C when glycerol is added, confirming its plasticizing effect on the chitosan polymeric network. Moreover, in the

nanocomposites it can be observed that the glass transition temperature increases as the nanoparticles amount increases. In particular, the introduction of 10% wt of Ag-MMT brings the value of the glass transition temperature to the value of neat chitosan, counteracting the negative effect of glycerol. Furthermore, it is observed a lower value of the relaxation strength as intensity of $\tan \delta$ which is ascribed to the action of Ag-MMT hindering the movement of the chains of chitosan in proximity of the transition.

Resistance of water swelling is one of the main characteristics that determines the suitability of chitosan films and chitosan based nanocomposites for many applications. Water uptake values at equilibrium (after 6 days of immersion) of neat chitosan and bionanocomposites containing 10% wt of Na-MMT and 10%wt of Ag-MMT nanoparticles are reported in Fig. 7.

Firstly, it can be noticed that the water uptake behavior is pH-dependent. CS, CS/10NaMMT and CS/10AgMMT nanocomposites swell greatly in the acidic medium compared to the neutral or basic one. This may be ascribed both to the protonation of amine groups to ammonium ions and to the displacement of glycerol in the formation of hydrogen bonding network. In fact, because of the amino groups reformed in the network, the equilibrium swelling ratio of chitosan and its bionanocomposites in the acidic solution is larger than that in the neutral one. The electrostatic repulsion of the protonated NH_3^+ groups along the chitosan chain can lead to an expansion of the network and hence to a higher swelling degree (Liu & Kim, 2012).

Moreover, it can be inferred from the data at pH 3.6 and 6 that the presence of Ag-MMT particles allows a reduction of the water uptake in comparison to the Na-MMT bionanocomposite. The latter shows an increment of the water liquid uptake compared to neat chitosan which is to be attributed to the high water affinity of the silicate particles or platelets as well as to a reduction of hydrogen bonding extent (Lavorgna et al., 2010). However, results reported in Fig. 7 show that the Ag ions or Ag metallic surface, as also confirmed by DMA and FTIR analysis, increase the network extent of chemical and physical cross-linking between the chitosan macromolecules conferring to the obtained mate-

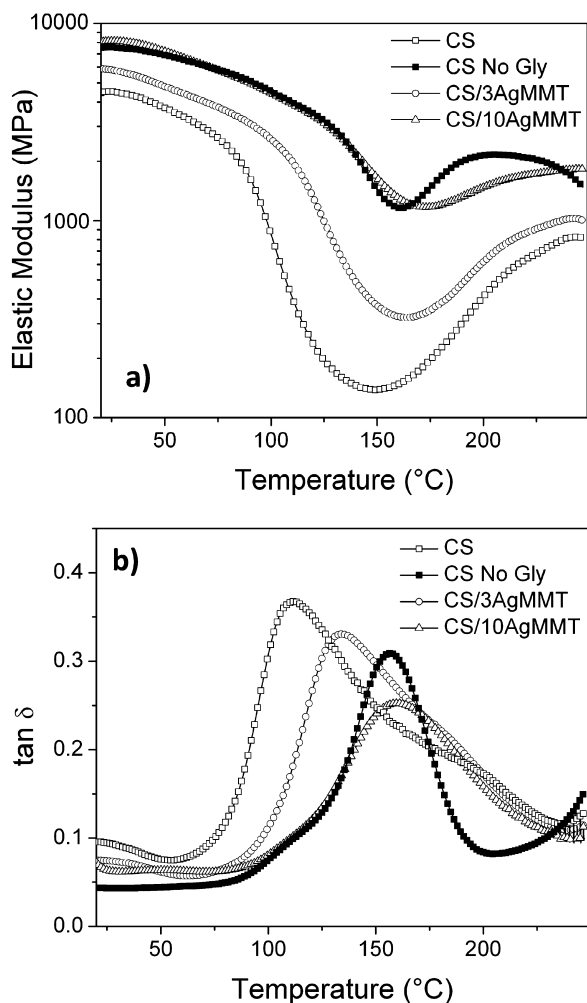


Fig. 6. DMA curves including storage modulus (a) and $\tan \delta$ (b) as a function of temperature for chitosan and its nanocomposites. (□) CS, (■) CS No GLY, (○) CS/3AgMMT and (△) CS/10AgMMT.

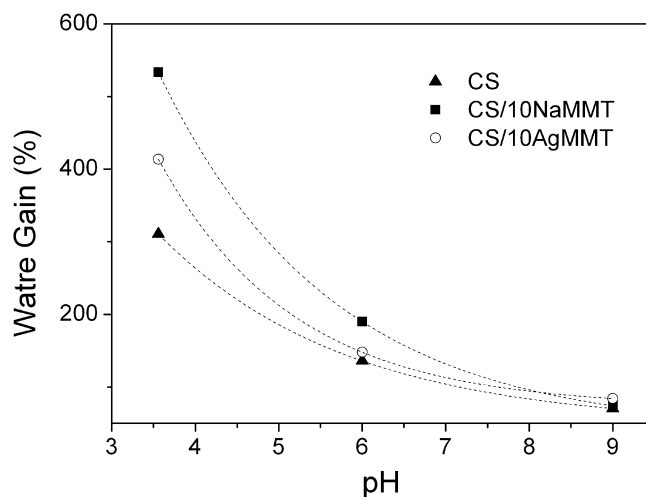


Fig. 7. Water sorption values at equilibrium at 25 °C of film (▲) CS, (■) CS/NaMMT and (○) CS/AgMMT at various pHs.

Table 2Antimicrobial activity of films recorded by in vitro test on a mix of two strains of *Pseudomonas* spp.

Time [h]	CNT		CS		CS-NaMMT		CS-AgMMT	
	Log CFU mL ⁻¹	SD	Log CFU mL ⁻¹	SD	Log CFU mL ⁻¹	SD	Log CFU mL ⁻¹	SD
0	2.39	0.12	2.39	0.12	2.39	0.12	2.39	0.12
24	8.41	0.45	7.96	0.05	8.45	0.17	5.10	0.03
48	8.67	0.19	8.48	0.04	8.25	0.21	8.21	0.16
72	8.87	0.14	8.43	0.07	8.43	0.13	8.46	0.49

rial a higher stability in liquid water. A similar behavior has been reported by Reicha et al. (2012) who showed that the maximum swelling values decreased significantly with increasing the metallic Ag content in the chitosan-Ag based films upon increasing both the time of the electrochemical process and the UV irradiation time. The reduction in swelling values may be attributed to the acting of the metallic silver nanoparticles as crosslinkers between chitosan chains. These crosslinking points increase with increasing the Ag content in the matrices which reduces the mobility of the chitosan chains and consequently reduces the swelling extent of the films.

Fig. 8(a) shows the amount of Ag ions released in water from nanocomposites containing 3 and 10 wt% of Ag-MMT. From the data, it can be inferred that a burst release process, often reported in the literature for the systems containing not-supported silver nanoparticles, does not occur for the material under investigation; on the contrary, silver ions are released in a steady and prolonged manner. This shows that most of released Ag⁺ comes from the dissolution of Ag₂O nanoparticles layer and to the oxidation of the metallic surface as the water diffuses in the material (Yang et al., 2012). As expected, the amount of silver ions released after a certain immersion time increases as the concentration of the silver particles in the polymer increases. Moreover, it is worth noting that after an immersion time of about 20 days, an increase of the ions release is still measurable indicating that the silver reservoir is not yet depleted. A similar slow release of silver ions (up until one month) has been found by Triebel et al. (2011) for TPU/silver nanocomposites. The steady Ag release can be a useful property for tailoring the antimicrobial activity of the bionanocomposite films and to design

materials whose function in preventing food degradation must be preferably exerted over a long range of time.

The effectiveness of silver nanoparticles immobilized in chitosan has been tested against *Pseudomonas* spp. because these microorganisms are considered food-borne bacteria (Bishop & White, 1986). The analyses were performed for 72 h to be sure that microorganisms attained the stationary phase. In Table 2 results obtained from the in vitro tests are reported.

As can be seen from data, a significant delay in microbial growth was obtained in presence of the active chitosan films. In particular, after 24 h a microbial load substantially lower than the control samples was found for the films containing AgMMT. The picture in Fig. 8(b) better highlights the different microbial population grew in the various samples. After 48 h in all the broths the bacteria reached the maximum load. The same result was confirmed after 72 h of monitoring. The effects of silver-montmorillonite nanoparticles was also demonstrated in other recent works of the scientific literature (Costa, Conte, Buonocore & Del Nobile, 2011; Gammariello, Conte, Buonocore & Del Nobile, 2011; Incoronato et al., 2010; Incoronato, Conte, Buonocore & Del Nobile, 2011) where nanoparticles were incorporated in different polymeric matrices than chitosan and also applied to fresh food products. The hydrophilic cell wall structure of Gram-negative bacteria as *Pseudomonas* spp. is essentially constituted of lipopolysaccharides that attract toward the weak positive charge available on silver ions, thus allowing the silver-based antimicrobial nanoparticles to be effective (Bezic, Skocibusic, Dinkic, & Radonic, 2003). The effect recorded after 24 h was followed by an increasing cell loads despite the Ag⁺ released is still increasing over time. This is ascribable to a reversible stress of the cells. It is widely recognized that silver nanocomposite can cause reversible damages to the outer membrane that compromise microbial proliferation but within a prolonged stress condition, the cell would repair the damages, re-acquiring the growth ability (Bezic et al., 2003; Johnston & Brown, 2002). Although previous experimental evidence demonstrated that silver ions release is governed by water transport properties of the film, by nature and quantity of the silver particles and their distribution within the matrix, further work is still necessary to better correlate silver diffusion mechanism to its antimicrobial activity.

4. Conclusion

Silver-montmorillonite (Ag-MMT) active nanoparticles were obtained by allowing silver ions from nitrate solutions to replace the Na⁺ ions of natural montmorillonite and to be reduced by thermal treatment. It has been proved that both Ag metallic and AgO/Ag₂O nanoparticles are mainly located on the surface of MMT platelets with a preferential location on the edges.

The obtained nanoparticles were embedded into chitosan matrix and successfully used to prepare bionanocomposites film exhibiting multifunctional properties. The ultrasound-assisted procedure resulted in an efficient tool for the chitosan macromolecules to intercalate into the silicate galleries. The achievement of the intercalation as well as the interaction between chitosan and silver nanoparticles lead to an enhancement of the thermal stability of the bionanocomposites and to an improvement of their

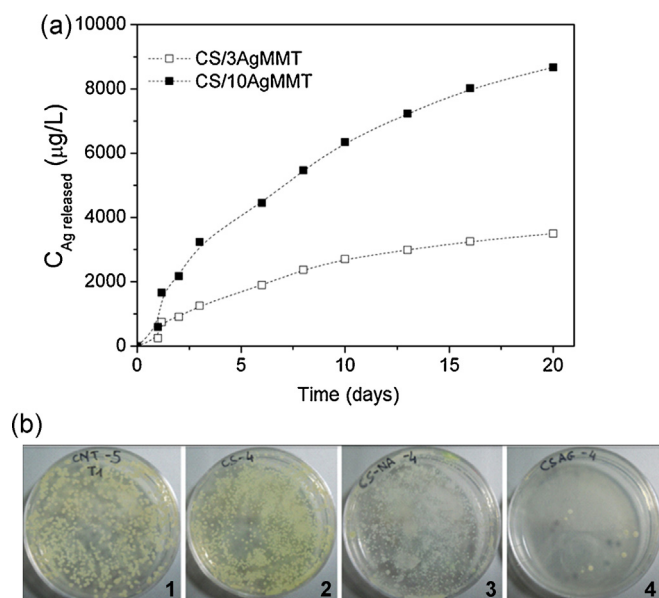


Fig. 8. (a) Ag release kinetics from active nanocomposites in aqueous solution at $T=25\text{ }^{\circ}\text{C}$ ($\text{pH}=7$) and (b) microbial population growth after 24 h in the various samples: (1) control (CNT); (2) neat chitosan (CS); (3) CS/10NaMMT (CS-NaMMT) and (4) CS/10AgMMT (CS-AgMMT).

mechanical strengths mainly due to a better load transfer between matrix and fillers.

Results show that silver ions are released in a steady and prolonged manner, thus showing that most of the released Ag^+ comes from the oxidation of the metallic surface as the water diffuses into the material. Antimicrobial tests showed that after 24 h a significant delay in microbial growth was obtained with the Ag-MMT chitosan film, while reaching, after 48 h, the maximum load of bacteria, probably due to a reversible stress of the cells.

Moreover, it has been proved that the presence of Ag-MMT particles allows a reduction of the liquid water uptake in comparison to the Na-MMT bionanocomposite. This is due to the effect of Ag ions or Ag metallic surface which increases the network extent of chemical and physical cross-linking between the chitosan macromolecules conferring to the obtained material a higher stability in liquid water. This result is extremely significant because one of the limitations to chitosan commercialization is mainly related to its high water liquid uptake which compromises the sample dimensional stability and consequently its applicative properties.

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