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Antibacterial chitosan-based blends with ethylene–vinyl alcohol copolymer

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ABSTRACT

This study reports for the first time about the formulation, morphology, water barrier and the antimicrobial activity of high and low molecular weight chitosonium-acetate based solvent-cast blends with ethylene–vinyl alcohol (EVOH) copolymers. The blends based on the low molecular weight chitosan grade showed enhanced phase morphology, transparency, enhanced water barrier properties, up to 86% water permeability reduction compared to pure chitosonium-acetate films, as well as excellent antimicrobial activity. When the fraction of low molecular weight chitosan exceeded the phase inversion in the blend, phase segregation became noticeable but good interfacial adhesion was still observed. On the other hand, the blends with the high molecular weight chitosan grade were translucent, even when this component was in the dispersed phase, and exhibited clearly separated phase morphology but also presented antimicrobial performance. In both cases, and in accordance with previous works in our laboratory, the release from the blends of protonated glucosamine groups (so-called active species) correlated well with the antimicrobial phenomenology of the developed materials. The study also showed that EVOH copolymers can also be made antimicrobial by a water sorption-induced release mechanism, if acetic acid is incorporated into the polymer formulation before casting from solution. On the overall, antimicrobial chitosan-based blends with EVOH copolymers, when low molecular weight chitosan was used as the dispersed phase in the blend, exhibited optimum performance in terms of optical properties, water resistance, enhanced water barrier and, therefore, excellent application outlook in antimicrobial applications.

1. Introduction

There is a growing interest in the development of new formulations of antimicrobial agents in various applications, including food coating and packaging, surfaces and biomedical applications. However, the direct application of antimicrobial agents onto, for instance, food surfaces, by dipping or spraying, could be inefficient, owing to the rapid diffusion of the active substances within the bulk of the product (Siragusa & Dickson, 1992; Torres, Motoki, & Takano, 1985). Alternatively, the use of plastic packaging materials containing active antimicrobial agents could result in more efficient vehicles, by controlling the release, i.e. the so-called intended migration, of these agents from the film to the food surface, thus helping maintain high concentrations where required (Ouattara, Simard, Piette, Bégin, & Holley, 2000).

Since antimicrobial compounds are now designed to migrate onto critical substrates such as foods, natural biocide agents and technologies are currently being investigated and implemented. Natural antimicrobial agents include enzymes (lactoperoxidase, lactoferrin, avidin, lysozyme), microbial preservatives produced from starter cultures (nisin, lactacin/lactococin/lacticin, natamycin, variacin), and plant sources (herbs, spices, extracts, essential oils and their isolated components) (Davidson, 1997). Special attention has been given to chitosan polysaccharide based systems, not only due to their intrinsic antimicrobial capacity but also due to their excellent film forming properties. This would in principle allow the use of this polysaccharide as a direct coating on foods or as packaging constituents. Chitosan derives from chitin, the second most abundant polysaccharide on earth after cellulose. This biopolymer is mostly available from waste products in the shellfish industry, and therefore, abundant commercial supplies are currently offered. It can also be obtained from the chitin component of fungal cell walls. Several studies have already demonstrated the antibacterial and antifungal action of this compound for both bioactive preservative and bioactive packaging applications (Chung et al., 2004; Coma et al., 2002; Fernandez-Saiz, Ocio, & Lagaron, 2006; Moller, Grelier, Pardon, & Coma, 2004; Shahidi & Abuzaytoun, 2005). Systematic research has also been reported on the effect of plasticizers concentrations, storage time (Butler, Vergano, Testin, Bunn, & Wiles, 2006), acid types and concentrations (Caner, Vergano, & Wiles, 1998), molecular weight (Park, Marsh, & Rhim, 2002) and degree of deacetylation of chitosan (Wiles, Vergano, Barron, Bunn, & Testin, 2000) on the physical properties of chitosan films. Concerning the mode of action of chitosan films, it has already been well demonstrated that its

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biocide effect is generally related to the release from the biopolymer of protonated glucosamine fractions into the culture medium (Fernandez-Saiz, Lagaron, Hernandez-Muñoz, & Ocio, 2008; Fernandez-Saiz et al., 2006). As a result, the higher the water dispersability that the film presents, the higher the antimicrobial action it exhibits. However, the high water sensitivity of this polysaccharide leads to a reduction in barrier properties or even the complete solubilization into foods with high water activities, restricting its industrial application. To improve this potential drawback, the use of physical or chemical treatments has been previously considered. Some examples are the incorporation of cross-linking agents such as glutaraldehyde, glyoxal or epichlorohydrin to hold the film structure (Suto & Ui, 1996; Tual, Espuche, Escoubes, & Domard, 2000). Alternatively, blending chitosan with other polymers may also be a convenient and effective method. To that end, several investigations entailing the use of blends of chitosan with other polymers, i.e. keratine, starch, cellulose, konjac glucomannan, poly-lactic acid, gluten gliadins, etc., have attracted much attention in recent years (Fernandez-Saiz et al., 2008; Li, Peng, Yie, & Xie, 2006; Moller et al., 2004; Sebti, Chollet, Degraeve, Noel, & Peyrol, 2007; Suyatma, Copinet, Tighzert, & Coma, 2004; Tanabe, Okitsu, & Tachinaba, 2002; Zhai, Zhao, Yoshii, & Kume, 2004). The similarity of cellulose and chitosan in primary structures hypothesized that they could form homogeneous composite films. Consequently, many works have been conducted by blending both components with the aim of improving the mechanical properties of chitosan after wetting (Wu et al., 2004) or reducing the cost of materials, as cellulose and derivatives are inexpensive biopolymers to produce (Moller et al., 2004). Nevertheless, the incorporation of chitosan within any of the above mentioned matrices does not necessary imply an improvement in water resistance. In fact, many of the published studies on this matter generated biocomposite materials with optimum antimicrobial activity but poor water barrier properties (Moller et al., 2004; Park, Daeschel, & Zhao, 2004; Park et al., 2002). Therefore, if the aim was to obtain water-resistant antimicrobial materials, the choice of a more suitable water-resistant polymer to form the blend is a crucial factor to be considered. A previous work performed by Tanabe et al. (2002) achieved this purpose by adding chitosan as an additive to keratin films to reinforce the mechanical properties, obtaining antibacterial blends which showed remarkably improved waterproof characteristics. Other possibility is to design chitosan-based systems in which the biopolymer active species release is controlled by a material such as a porous or a highly plasticizable polymer or biopolymer water-resistant coating.

The use of water swellable materials for the release of active and bioactive compounds has been widely known for many years. These materials can have a synthetic nature or be isolated from biomass resources. In these systems, the release of desired components occurs mainly by counter-transport of the solvent "in" and diffusion "out" of the component through the swollen polymer. In general, these materials are in a "glassy" state at room temperature and as the solvent penetrates, it causes stresses which are accommodated by an increase in the radius of gyration and end-to-end distances, which lead to macroscopic swelling and T_g drops to below room temperature. Thus, the solvent molecules move into the glassy polymer matrix with a well-defined front at a particular rate and, simultaneously, the thickness of the rubbery or swollen region increases with time in the opposite direction (Colombo, 1993; Ranga Rao & Padmalatha, 1988).

Ethylene–vinyl alcohol (EVOH) copolymers have become, due to their low permeability to gases and organic vapors, one of the most widely used high barrier family of materials (Lagaron, Catala, & Gavara, 2004). EVOH properties are known to be strongly related to ethylene content, as a result, commercial grades vary from high to low contents depending on the end application. Thus, copolymers with higher ethylene contents have higher oxygen permeability, but are less affected by water sorption and vice versa.

Also, the use of EVOH as an antimicrobial carrier with swelling-induced controlled release capacity could be of great interest in many applications within fields such as biomedical, health care and active packaging. Thus, a previous study reported the water sorption-induced release of isopropanol as a function of EVOH polymer composition (Cava, Sammon, & Lagaron, 2007).

The premises of this first work were to develop novel coating formulations in which the release of glucosamine active species onto the surface would occur from within a more water-resistant food contact permitted polymeric matrix used in gas and aroma barrier packaging applications as a food contact layer, i.e. EVOH copolymers. Thus, the antimicrobial properties of various chitosan grades when this biopolymer compound was incorporated into EVOH copolymers were evaluated. The morphology and the water barrier properties of the novel blends were also studied in this first work. Additionally, the samples were obtained at different casting temperatures in an attempt to determine the more appropriate forming conditions to obtain better antimicrobial and water-resistant materials. Finally, the biocide properties observed in this work were correlated with glucosamine migration assays.

2. Materials and methods

2.1. Samples preparation

Two different commercial ethylene–vinyl alcohol copolymer grades (Soarnol) supplied by the Nippon Synthetic Chemical Industry (NIPPON GOSHEI) were analyzed: EVOH29 and EVOH32, where the number indicates the mole percentage of ethylene in the copolymer composition. These materials were dissolved at 4% (w/v) into mixtures of isopropanol/water (70/30 v/v). Chitosan polysaccharides of both high molecular weight (so-called csHMW and with 83.3% degree of deacetylation and viscosity of 1700 cps at 1% in 1% acetic acid as stated by the manufacturer) and low molecular weight (so-called csLMW, 90.9% degree of deacetylation and viscosity of 185 cps at 1% in 1% acetic acid as stated by the manufacturer) were all purchased from Sigma–Aldrich (Spain). Chitosan polysaccharide dispersions were prepared in 2% (v/v) acetic acid to a final concentration of 3% (w/v) and stirred at 37 °C for approximately 3 h. The chitosonium-acetate solutions were filtered through polyester cloth to remove residues of insoluble particles and then autoclaved before its further use for blending. Pure chitosonium-acetate films were obtained by casting at 37, 80 and 120 °C. In addition, pure EVOH films were obtained by the same method at 80 °C. Composite films of EVOH29/LMW-chitosan with high ratios of polysaccharide (i.e. 50 and 80 wt%) were obtained under various temperature conditions (37, 80 and 120 °C). Blends of EVOH with lower contents of chitosan, did not form good films but 80/20 (wt%) EVOH/chitosonium-acetate blends could be finally obtained by adding an excess of glacial acetic acid to the EVOH solution to a final concentration of 2% (w/v). Nevertheless, the higher chitosan concentration blends, i.e. 50 and 80 wt%, were also obtained using the same solution of EVOH and acetic acid for comparison purposes. Typical pure and composite films with a thickness of ca. 50 μm were obtained. A detailed list of all the samples prepared in this study is given in Table 1.

2.2. SEM measurements

For scanning electron microscopy (SEM) observation, pure and some of the composite samples were cryofractured in liquid nitrogen and mounted on bevel sample holders. The fracture surface of the different samples was sputtered with Au/Pd in a vacuum. The

Table 1
Samples composition, casting temperatures and sample masses used in this study for the antimicrobial tests.

Sample	EVOH/chitosan ratio (wt%)	Casting temperature (°C)	Film mass (mg)	EVOH/chitosan mass (mg)
csLMW	0/100	37, 80, 120	24	0/24
csLMW	0/100	37, 80, 120	60	0/60
csLMW	0/100	37, 80, 120	96	0/96
csHMW	0/100	80	24	0/24
EVOH32	100/0	80	96	96/0
EVOH29	100/0	80	96	96/0
EVOH32/LMW	80/20	80	120	96/24
EVOH29/LMW	80/20	80	120	96/24
EVOH29/HMW	80/20	80	120	96/24
EVOH29/LMW	50/50	37, 80, 120	120	60/60
EVOH29/LMW	20/80	37, 80, 120	120	24/96

SEM pictures (Hitachi S4100 from Hitachi High Technologies Co., Wokingham, UK) were taken with an accelerating voltage of 10 keV on the sample thickness.

2.3. Water permeability

Direct water permeability was determined from the slope of weight gain versus time experiments at 24 °C and 0% RH. The films were sandwiched between the aluminium top (open O-ring) and bottom parts of aluminium permeability cells. A Viton rubber O-ring was placed between the film and the aluminium top of the cell to enhance sealability. Then the bottom part of the cell was filled with silica to guarantee dry conditions and the pinhole secured with a rubber O-ring and a screw. Finally, the cell was placed at 75% RH and the solvent weight gain through a film area of 0.001 m^2 was monitored and plotted as a function of time. The samples were preconditioned at the testing conditions for 24 h, and permeability was determined from the linear part of the weight gain data after steady state was reached. Cells with aluminium films (with thickness of ca. 13 μm) were used as control samples to estimate the water permeability through the sealing. The permeability sensibility of the permeation cells was determined to be of ca. 0.086×10^{-16} kg m/s m^2 Pa based on the weight gain measurements of the aluminium cells. Water weight gain was calculated as the total cell gain minus the gain through the sealing. The tests were done in duplicate.

2.4. Bacterial strain and growth conditions

Samonella spp. CECT 554 and *S. aureus* CECT 86 were obtained from the Spanish Type Culture Collection (Valencia, Spain). The strains were stored in Tryptone Soy Broth (TSB) with 20% glycerol at −80 °C until needed. For experimental use, the stock cultures were maintained by regular subculture on agar Tryptone Soy Agar (TSA) slants at 4 °C and transferred monthly. A loopful of bacteria was added to 10 mL of TSB and incubated at 37 °C overnight. A 100 μl aliquot from the overnight culture was again transferred to TSB and grown at 37 °C to the mid-exponential phase of growth. This culture served as the inoculums in the susceptibility study, starting with approximately 10^5 CFU/mL in the tests tubes. These CFU counts were accurately and reproducibly obtained by inoculation of 0.1 mL of the culture having an absorbance value of 0.2, as determined by optical density at 600 nm by UV/vis spectroscopy.

2.5. Antimicrobial tests

To assess the effectiveness of the antimicrobial polymer film formulations, the bacterial growth was evaluated according to the macro-dilution method described by the National Committee of Clinical Laboratory Standards (1999) with some modifications (NCCLS, 1999; Paulson, 1999). For this assay, the film specimens indicated in Table 1 were introduced in test tubes containing 10 mL of sterile Mueller Hinton Broth (MHB) at pH 6.2. After 15 min, tubes were inoculated with 10^5 cells/mL of *Salmonella* spp. or *S. aureus* in mid-exponential phase and incubated at 37 °C for 24 h. Then, a 0.1 mL aliquot of MHB from each tube was subcultivated on TSA plates. Finally, plates were read after overnight incubation at 37 °C. These results were expressed as CFU/mL and compared with a control sample without film. The antimicrobial properties of samples containing MHB and titrated with different concentrations of acetic acid were also evaluated by the same methodology. Three replicate experiments per condition were performed.

2.6. Glucosamine release to the nutrient Broth

A standardized spectrophotometric method based on reaction with ninhydrin was used to determine the release and dissolution of chitosan from the blends to the MHB medium. The exact methodology for this assay has been thoroughly developed and described in a previous work (Fernandez-Saiz et al., 2008). For this assay, pure LMW-chitosonium-acetate films and the blends EVOH29/LMW-chitosan were analyzed in triplicate. The results were expressed in mg of released chitosan per 10 mL of nutrient Broth.

2.7. ATR–FTIR measurements

ATR–FTIR spectra were collected at 24 °C and 40% RH coupling the ATR accessory GoldenGate of Specac Ltd. (Orpington, UK) to a Bruker (Rheinstetten, Germany) FTIR Tensor 37 equipment. Time resolved experiments were collected by averaging five scans at 4 cm^{-1} resolution at predefined time intervals.

2.8. Statistical analysis

The statistical significance of differences in water permeability, antimicrobial capacity and chitosan release between the test samples was determined using Xlstat-Pro (win) 7.5.3 (Addinsoft, NY). Data were ranked and statistical differences were evaluated on the ranks with a one-way analysis of variance (ANOVA) and Fisher's multiple comparison test. In all cases, a value of $p < .05$ was considered significant. The data were expressed as mean ± *SD*.

3. Results and discussion

3.1. Morphology

Simple naked eye examination of the blends indicated that while the pure samples and blends with LMW-chitosan, particularly those with low contents of chitosan, yielded transparent films, the blends with HMW-chitosan were are all translucent and with a slight brownish color. To observe the morphology at

the micron and submicron level, SEM observations were carried out in the samples (see Fig. 1).

In general, the SEM examination reveals very little phase details in samples of EVOH with LMW-chitosan, particularly when the latter polymer is a minor phase constituent, suggesting that a good dispersion and/or phase interaction takes place between the two polymers (Fig. 1E and F) in the compositional range. On the other hand, the sample in Fig. 1G exhibits strong phase separation suggesting that blending EVOH with HMW-chitosan does not lead to phase continuous materials under the conditions applied in the study. The reason for this behavior is not known but could related to molecular weight differences and to the lower degree of deacetylation shown by the HMW-chitosan which could lead to more difficult mixing and interpolymer interaction. Regarding LMW-chitosan, when the fraction of this polymer was increased (Fig. 1H and I), the compatibility of the two materials seemed to also be reduced. Nevertheless, in all the EVOH29/LMW-chitosan obtained blends the EVOH microdomains were homogenously dispersed within the chitosan matrix in the blend films with apparent interfacial adhesion. Compatibility reduction in the latter blends was not improved when the blends were obtained by adding excess of glacial acetic, as used in the formulation of the blend EVOH/Cs (80/20 wt/wt) (see Fig. 1J and K). This indicates that adding an excess of the organic acid is not apparently responsible for the different morphology observed across the different blends.

3.2. Water barrier properties

Water resistance and water barrier are important properties, which can determine the application of plastics in various applications including food packaging and other areas. Thus, films with lower water sensitivity are necessary for the protection of foodstuffs of high water activity. In the food area, high barrier packaging, vacuum packaging and dehydrated products also required in general high barrier to water vapor. The high sensitivity of chitosan films to water, which can lead to polymer dissolution at the highest water activity, is widely known and, therefore, this property needs be enhanced to make feasible the use of this material as a packaging constituent. The direct permeability results obtained on the films are plotted in Figs. 2 and 3. Additionally, Table 2 shows the water permeability drop (in percentage) in the blends when compared to the corresponding pure chitosan films.

As expected, the EVOH pure samples presented lower water permeability values (i.e. EVOH29: 4.57×10^{-16} kg m/s m^2 Pa) than those obtained in pure chitosan films (i.e. csLMW: 7.76×10^{-14} kg m/s m^2 Pa). For the pure chitosan samples formed at 80 °C (i.e. HMW- and LMW-chitosan matrices), the value of permeability for HMW-chitosan was slightly lower than that of LMW-chitosan. These results tend to agree with a previous study performed by Chen and Hwa (1996) which showed lower permeability values from membranes prepared from high molecular weight chitosan. In contrast, another work by Park et al. (2002), in which the effect of changing the molecular weight on the water permeability of chitosan films did not result in significant changes. When the polysaccharide was incorporated into EVOH as a dispersed phase, i.e. 80/20 (wt%) (EVOH/chitosan), the water permeability of the new blend diminished to a considerable extent compared to that of the pure chitosan.

Thus, Table 2 reveals significant barrier improvements of ca. 69–86%, being the blend EVOH29/LMW-chitosan the one with

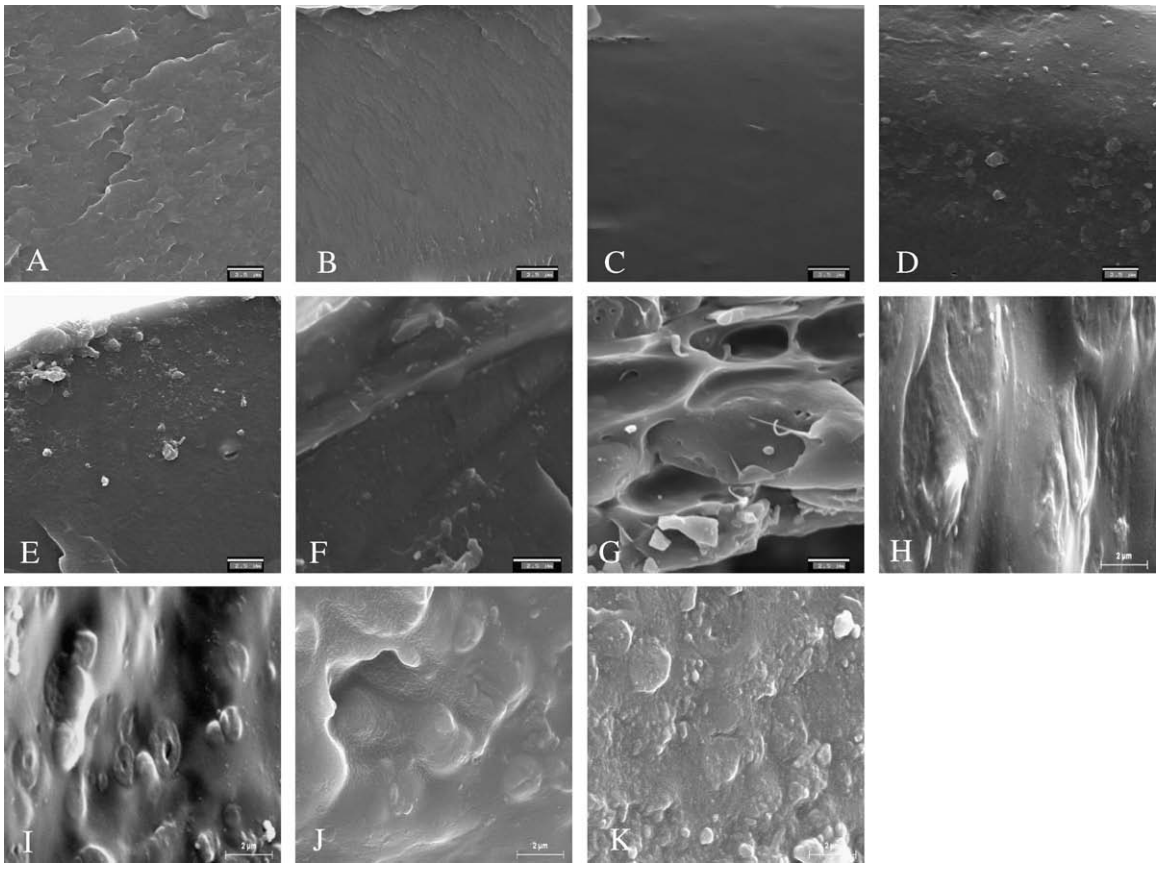

Fig. 1. Scanning electron micrographs of the crosssection of: (A) EVOH29 pure film; (B) EVOH32 pure film; (C) HMW chitosonium-acetate film; (D) LMW chitosonium-acetate film; (E) EVOH29/LMW (80/20) (wt%); (F) EVOH32/LMW (80/20) (wt%); (G) EVOH29/HMW (80/20) (wt%); (H) EVOH29/LMW (50/50) (wt%); (I) EVOH29/LMW (20/80) (wt%); (J) EVOH29/LMW (50/50) (wt%) acetic acid was also added to the EVOH solution; (K) EVOH29/LMW (20/80) (wt%) acetic acid was also added to the EVOH solution. For samples (A)–(G) the scale marker is 2.5 μm and for the samples (H)–(K) is 2 μm.

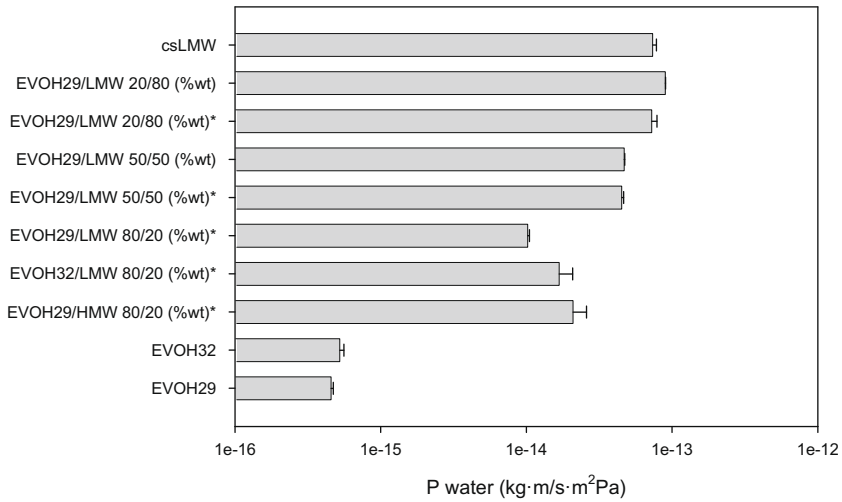

Fig. 2. Water permeability of pure chitosonium-acetate films, EVOH films and their blends, cast at 80 °C. The ∗ means that glacial acetic acid was added to the EVOH solution.

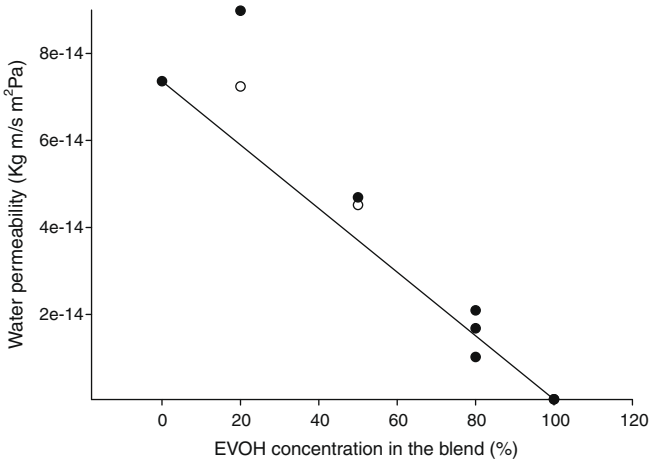

Fig. 3. Plot of water permeability versus EVOH29 content in EVOH29/chitosan blends formed at 80 °C. The open symbols correspond to the blends obtained with EVOH containing glacial acetic acid. Note that the concentration of 80% also shows the one data point obtained with EVOH32 and reported in Table 2 with a 77.2 permeability drop.

Table 2
Water permeability decreased (%) in the samples studied. Permeability decrease for each blend is referred to the corresponding pure chitosan film obtained under similar conditions.

Sample	Casting temperature (°C)	Water permeability decrease[b] (%)
[a]EVOH29/HMW 80/20	80	69.1*
[a]EVOH32/LMW 80/20	80	77.2*
[a]EVOH29/LMW 80/20	80	86.1*
EVOH29/LMW 50/50	80	36.3*
EVOH29/LMW 20/80	80	−22.0*
[a]EVOH29/LMW 50/50	80	38.6*
[a]EVOH29/LMW 20/80	80	1.6
EVOH29/LMW 50/50	37	27.1
EVOH29/LMW 20/80	37	− 45.6*
EVOH29/LMW 50/50	120	63.3*
EVOH29/LMW 20/80	120	38.8

* Statistical differences of water permeability with respect to the matrix of reference in each case.
[a] Glacial acetic acid was added to the EVOH solution.
[b] Water permeability decrease (%): 100 − (water permeability of the sample × 100/water permeability of chitosan matrix formed at the same temperature).

the highest barrier (i.e. 1.02×10^{-14} kg m/s m^2 Pa). Moreover, when the ratio of chitosan in this particular blend formed at 80 °C was increased to 50% it still presented a reduction in permeability of 36% compared to the pure chitosan film. On the contrary, the 20/80 EVOH29/LMW-chitosan blend resulted in poorer barrier with respect to the corresponding pure chitosan sample. For this latter blend, when obtained in excess of acetic acid (using EVOH solutions containing acetic acid) the permeability was, however, found to be somewhat better but only similar to neat chitosan.

For a better understanding of the permeability results, the water vapor permeability of the samples EVOH29/LMW-chitosan cast at 80 °C was plotted versus EVOH content (see Fig. 3).

In this figure, a positive deviation from the simple additive rule is observed for the just mentioned samples with 50 and 80 wt% of chitosan, suggesting lack of morphological synergy for the cited blends. The deviation is much more acute for the films with 80% of chitosan. SEM observations in Fig. 1 indicated that the latter blends showed clear phase segregation. In contrast, the blends with 80 wt% of EVOH seemed to follow more closely the rule of mixtures and even the sample EVOH29/LMW-chitosan presented a negative deviation indicating synergies in regard to this property and therefore good blend constituents compatibility. Previous works on the subject also concluded that lower water vapor permeability values were found when the homopolymer polyvinyl alcohol (PVOH) and chitosan blends cast from acetic acid solutions were studied (Park, Jun, & Marsh, 2001). Another study performed by Suyatma et al. (2004) on the mechanical and barrier properties of biodegradable films made from chitosan and poly-lactic acid (PLA) blends demonstrated that the water sensitivity of chitosan decreased with increasing PLA contents in the blend because of the higher hydrophobicity of the latter constituent. In another work, the incorporation of chitosan on a corn starch matrix led to biodegradable blends with a homogeneous matrix in which the polysaccharide improved markedly the water vapor barrier properties of pure starch films (Garcia, Pinotti, & Zaritzky, 2006). However, the latter work does not agree with a more recent study performed by Bourtoom and Chinnan (2008) in which water vapor permeability of rice starch–chitosan biodegradable blend films was found to be lower than chitosan pure specimens.

Fig. 4 shows the effect of casting temperature on the water permeability values of pure chitosan matrices and of some of the EVOH29/LMW-chitosan blends. Lower chitosan content blends or pure EVOH films were not possible to obtain at 37 °C or at 120 °C, due to extensive hazing or whitening of the EVOH phase

due to crystallization or to the formation of microvoids due to rapid solidification of the surface, respectively. From the results, Fig. 4 shows only small differences between pure LMW-chitosan samples formed under the three different temperature conditions, albeit the sample cast at 120 °C presented 14.8% lower permeability than the films cast at 80 °C. Nevertheless, this difference was not statistically significant (data not shown). Concerning the composite films, they all presented a similar behavior when formed at 37 °C or at 80 °C. However, similarly as with pure chitosan, the films of the blends formed at 120 °C showed enhanced barrier properties. Thus, the blends with 50 wt% of chitosan showed a great improvement of the water barrier (i.e. 63.3%), while those with 80 wt% of polysaccharide were also better barrier. The enhanced barrier at 120 °C could be associated to molecular crosslinking and/or amidation processes as a result of the elevated temperature applied during the casting period. This hypothesis is in accordance with other previous works performed on the forming conditions of chitosan films (Srinivasa, Ramesh, Kumar, & Tharanathan, 2004; Zotkin, Vikhoreva, & Kechek'yan, 2004; Zotkin, Vikhoreva, Smotrina, & Derbenev, 2004). For that reason, susceptibility tests were performed also for these particular samples to discern the influence of such chemical changes on the biocide properties of the samples.

3.3. Susceptibility tests

The antimicrobial capacity of pure chitosonium-acetate and EVOH films and their subsequent blends was determined against the growth of *Salmonella* spp. and *S. aureus* as described in the experimental section. Table 3 shows the results found when 120 mg of each blend were tested. The bacterial reduction obtained when the same amount of each component of the blend was tested as a pure matrix is also indicated in this table.

The pH of the nutrient Broth after film immersion and just before bacterial inoculation was measured for all cases and indicated in the table. Finally, the effect of the addition of different volumes of acetic acid into the Broth on the growth of these strains was studied in an attempt to analyze the influence of the reduction of the pH in the antimicrobial activity of the samples (see Table 4).

As it can be seen from observation of Table 3, the blends EVOH/chitosan (80/20 wt%) presented a bactericidal effect in all cases for both strains since bacterial counts diminished by at least 2 log-units from the initial inoculum size. When the same amount of each component of the blend was evaluated as a pure film, the chitosonium-acetate matrix showed an inhibitory effect against *S. aureus* but, nevertheless, no detectable effect was observed for *Salmonella* spp. at the used amount (i.e. 24 mg). This result corroborates the higher vulnerability of *S. aureus* to chitosan observed in previous works (Fernandez-Saiz, Lagaron, & Ocio, 2009a, 2009b). Furthermore, whereas the pure cast EVOH films did not present a detectable biocide capacity, samples cast from the EVOH solution with acetic acid showed and inhibitory effect against the growth of both bacterial strains (log CFU/mL: 5.19–6), which may be probably due to a pH decrease of the nutrient medium (4.99–5.18) (see later). It should be noted that to obtain EVOH/chitosan blends acetic acid was added to the chitosan solution but for the case of 80/20 blend some excess of this component was also added to the EVOH fraction to be able to form the film.

When the blends EVOH29/LMW-chitosan formed at 80 °C with higher ratios of polysaccharide (i.e. 50 and 80 wt%) were tested an inhibitory effect was obtained on the growth of *Salmonella* spp. while a bactericidal effect was observed against *S. aureus*. It is worth noting that these composite matrices showed a similar antimicrobial effect regardless of the relative amount of chitosan. This phenomenon may be due to the particular morphology of these blends with segregated phases and possibly to the lower acetic acid needed in the formulation, which could have both influenced the glucosamine release from the film into the nutrient Broth medium and therefore the antimicrobial activity of the matrix. Curiously, these blends showed a lower antimicrobial activity than the EVOH/chitosan 80/20 (wt%) samples even though they presented higher quantities of chitosan in the formulation and a similar effect on the pH of the nutrient Broth.

To elucidate the influence of medium pH on the antimicrobial effects of each sample, the biocide properties of the acetic acid itself were studied. The results are indicated in Table 4. From this table, it can be observed that the lethal effect of the acetic acid on the development of *Samonella* spp. starts to be detectable below a pH of approximately 5.1 (log CFU/mL: 6.48). For the case of *S. aureus*, even though the inhibition begins at a higher pH, the microbial growth presents a gradual drop and only from a pH < 5 a bacteriostatic effect was detectable (log CFU/mL: 5.64). Therefore, from the data in Table 4 it can be derived that *Salmonella* spp. presents a slightly higher sensitivity to acetic acid. The pH values recorded in this assay when an inhibitory effect was seen were very similar to those obtained when the EVOH samples containing acetic acid were studied and are, therefore, in accordance with their microbial reduction values. In fact, a somewhat higher antimicrobial effect was obtained for *Salmonella* spp. than for *S. aureus* in line with the just mentioned findings. Therefore, it is confirmed that the

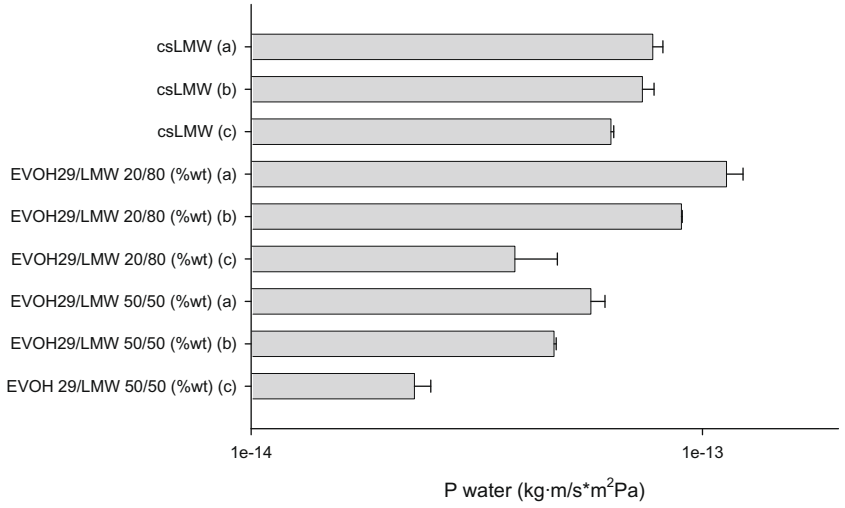

Fig. 4. Water permeability of pure chitosonium-acetate films, EVOH films and their blends. Films cast at 37 °C (a), 80 °C (b) and 120 °C (c).

Table 3
Antimicrobial properties of chitosonium-acetate films, EVOH films and of their blends against the growth of *Salmonella* spp. and *S. aureus*.

Sample	Mass (mg)	Casting temperature (°C)	*Salmonella* spp. (log CFU/mL)	*S. aureus* (log CFU/mL)	pH MHB
Control without film	–	–	8.67 (0.07)[b] ABC	8.24 (0.20) A	6.2
csLMW	24	80	8.08 (0.27) ABCD	5 (0.25) D	5.88
csHMW	24	80	8.53 (0.32) ABC	5.5 (0.11) CD	6.06
csLMW	60	80	6.03 (0.01) EFG	3.14 (0.22) EF	5.70
csLMW	96	80	n.d.g.[c] J	n.d.g. I	5.50
EVOH32	96	80	9.06 (0.18) A	8.32 (0.28) A	6.2
EVOH29	96	80	8.75 (0.19) AB	8.36 (0.26) A	6.2
EVOH32[a]	96	80	5.30 (0.52) FGH	5.94 (0.32) BC	4.99
EVOH29[a]	96	80	5.19 (0.18) GH	6.02 (0.13) BC	5.03
EVOH32/LMW (80/20)[a]	120	80	2.36 (0.14) I	3.32 (0.48) EF	5.50
EVOH29/LMW (80/20)[a]	120	80	2.57 (0.15) I	n.d.g. I	5.32
EVOH29/HMW (80/20)[a]	120	80	3.31 (0.30) I	n.d.g. I	5.51
EVOH29/LMW (50/50)	120	80	6.33 (0.21) EFG	2.19(0.44)GH	5.44
EVOH29/LMW (20/80)	120	80	6.30 (0.21) EFG	2.08 (0.09) GH	5.65
EVOH29/LMW (50/50)[a]	120	80	3.01 (0.59) I	3.74 (1.3) DEF	5.32
EVOH29/LMW (20/80)[a]	120	80	1.64 (1.49) IJ	1.84 (0.01) H	5.22
csLMW	60	37	5.88 (0.98) EFG	2.82 (0.35) FG	5.66
csLMW	96	37	n.d.g. J	n.d.g. I	5.45
EVOH29/LMW (50/50)	120	37	6.25 (0.07) EFG	2.01 (0.35) H	5.49
EVOH29/LMW (20/80)	120	37	5.84 (0.48) EFG	n.d.g. I	5.30
csLMW	60	120	7.35 (0.10) ABCDE	6.4 (0.4) B	6.07
csLMW	96	120	6.5 (0.3) DEFG	5.6 (0.5) BCD	5.98
EVOH29/LMW (50/50)	120	120	7.16 (0.10) BCDE	6.01 (0.26) BC	6.25
EVOH29/HMW (20/80)	120	120	7.02 (0.03) CDEF	6.21 (0.39) BC	6.30

[a] Glacial acetic acid was added to the EVOH solution.
[b] Standard deviation.
[c] n.d.g., no detectable growth. Different letters in the same column indicate significantly different groups determined by Fisher's test ($p < .05$).

Table 4
Antimicrobial properties of acetic acid on the growth of *Salmonella* spp. and *S. aureus*.

Acetic acid (mM)	*Salmonella* spp. (log CFU/mL)	*S. aureus* (log CFU/mL)	pH MHB
2.06	8.57 (0.11) A	8.20 (0.12) A	–
4.15	8.78 (0.01) A	7.84 (0.05) AB	5.50
6.14	8.36 (0.88) A	7.75 (0.14) AB	5.25
8	6.48 (0.02) B	7.42 (0.30) B	5.1
12.4	4.02 (0.33) C	6.52 (0.21) C	4.9
17	2.97 (0.55) C	5.64 (0.26) D	4.8

The standard deviation is given between brackets. Different letters in the same column indicate significantly different groups determined by Fisher's test ($p < .05$).

Table 5
Protonated glucosamine groups release from the pure chitosonium-acetate films and EVOH/chitosonium-acetate blends to the nutrient Broth after incubation at 37 °C for 24 h.

Sample	Film mass added (mg/10 mL)	Casting temperature (°C)	Released chitosan (mg/10 mL)
csLMW	24	80	5.67 (1.0)* E
csLMW	60	80	38.44 (5.0) CD
csLMW	96	80	85.2 (4.4) B
csLMW	120	80	115.35 (5.3) A
EVOH29/LMW (80/20)	120	80	5.30 (1.24) E
EVOH29/LMW (50/50)	120	37	43.70 (2.4) C
EVOH29/LMW (50/50)	120	80	33.90 (4.7) D
EVOH29/LMW (50/50)	120	120	6.64 (0.9) E
EVOH29/LMW (20/80)	120	37	40.38 (3.9) CD
EVOH29/LMW (20/80)	120	80	33.23 (2.7) D
EVOH29/HMW (20/80)	120	120	10.63 (1.7) E

* The standard deviation is given between brackets. Different letters indicate significantly different groups determined by Fisher's test ($p < .05$).

antimicrobial effect of these particular EVOH films was almost certainly due to the pH drop of the medium as a result of the acid addition. Regarding the blends EVOH/chitosan 80/20 (wt%), the final pH of the nutrient Broth is higher than expected even though the acetic acid fraction used is the same than in the pure EVOH films. Probably, the acetic acid in the EVOH solution interacts more efficiently with the amino groups from chitosan during blending and before casting, leading to composite films with more cationic species but lower acidic character than the EVOH–acetic acid samples. This suggestion could help explain the fact that the blends EVOH/chitosan 80/20 (wt%) showed better biocide properties than those of EVOH/chitosan 50/50 and 20/80 (wt%). Thus, despite the lower amount of polysaccharide in the 80/20 blend, the glucosamine groups of the chitosan chains must be more extensively protonated (since higher acetic acid overall concentration is present for this particular blend) in the former case and therefore, act more effectively against bacterial cells. Since the morphology study in

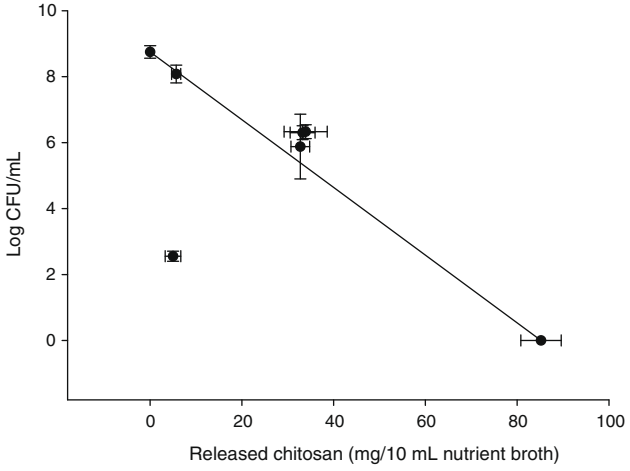

Fig. 5. Antimicrobial performance versus the release of protonated glucosamine fractions against *Salmonella* spp.

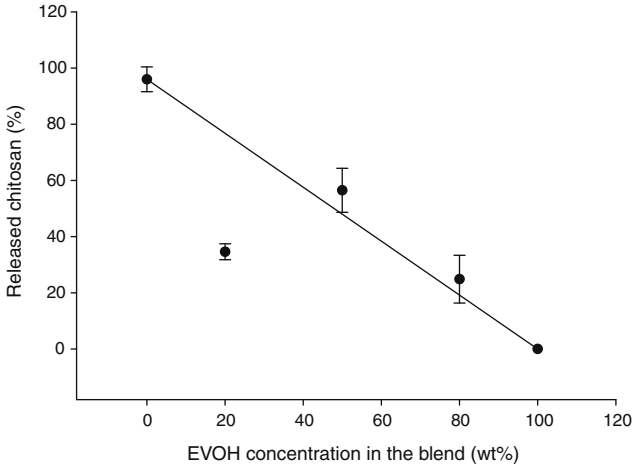

Fig. 6. EVOH concentration (wt%) in the blends EVOH29/LMW versus their corresponding released glucosamine fractions.

Fig. 1 also indicates that the chitosan fraction in this particular blend is more homogeneously dispersed it could also facilitate the migration into the culture solution and hence enhance biocide properties. The blends of EVOH/chitosan 50/50 and 20/80 formed with an excess of acetic acid were also tested and showed no significant differences in the pH reduction but better antimicrobial properties than the same blends formed with lower acetic acid. This finding corroborates the acetic acid playing a significant role in the protonation of chitosan and yielding higher antimicrobial performance.

The antimicrobial properties of some of the blends of EVOH29/LMW-chitosan with 50 and 80 wt% of chitosan content and cast at 37 °C and 120 °C and of the pure chitosan film were also analyzed in order to ascertain the effect of casting temperature on the biocide properties of the studied systems. The results are also indicated in Table 3. From the values obtained, it can be observed that, in line with the results related to water permeability, the samples formed at 37 °C showed the same behavior than the above mentioned samples cast at 80 °C. Thus, a bactericidal effect was obtained against *S. aureus* for all these cases. In the same way, and in relation to the growth of *Salmonella* spp., an inhibitory effect was obtained, except for the pure chitosonium-acetate film of 96 mg which was able to reach a bactericidal effect. Nevertheless, the matrices formed at 120 °C presented poorer biocide properties when compared to equivalent films specimens formed at 37 or 80 °C. Thus, such samples produced only an inhibitory effect on the growth of *S. aureus* whereas for *Salmonella* spp. only the pure chitosan sample of 96 mg exhibited a significant antimicrobial action. The change in behavior observed in the films cast at 120 °C matches with the significant improvement in water barrier properties detected for these particular samples. Thus, the casting method at such high temperature could probably lead to chemical changes such as amidation or browning reactions which would reduce the solubility of chitosan into the nutrient medium (Srinivasa et al., 2004; Zotkin, Vikhoreva, & Kechek'yan, 2004; Zotkin, Vikhoreva, Smotrina, et al., 2004), and, as a consequence, the biocide properties. This finding also agrees with a previous work

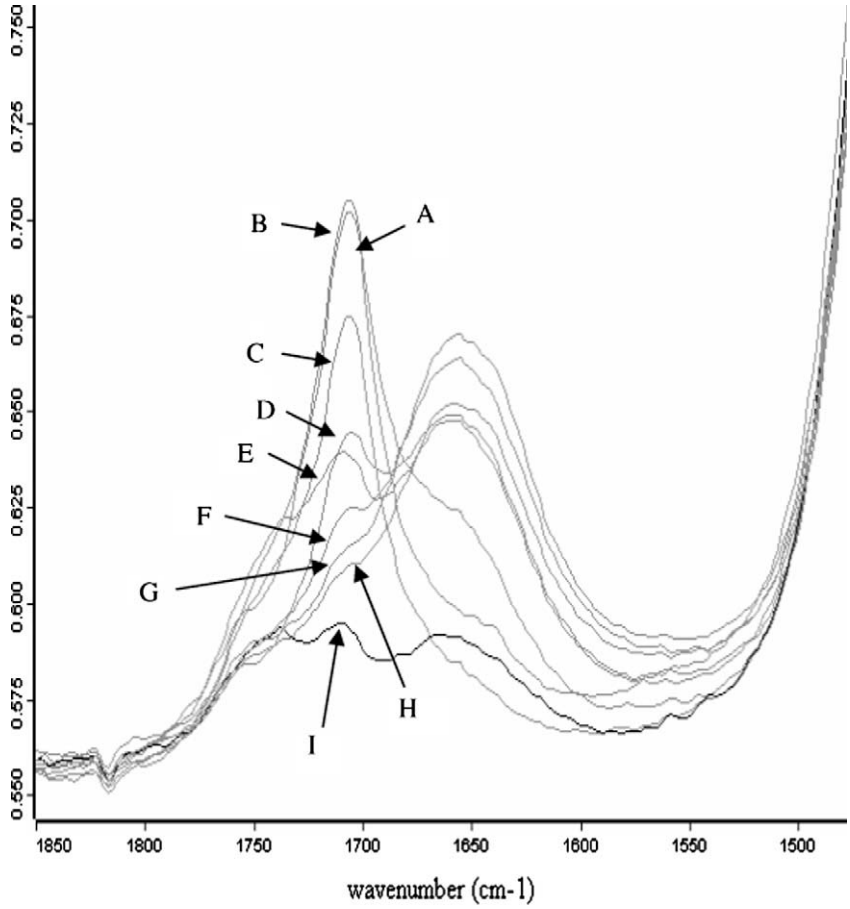

Fig. 7. ATR–FTIR spectra in the range from 1500 to 1850 cm^{-1} taken in EVOH films containing acetic acid after different conditionings. (A) Just formed film. (B) Film stored at 40% RH (23 °C) for 1 month. (C) Film stored at 0% RH (23 °C) for 1 month. (D) Film maintained at 75% RH for 24 h. (E) Film maintained at 75% RH for 48 h. (F) Film maintained at 100% RH for 24 h. (G) Film immersed in water for 24 h. (H) Film maintained at 100% RH for 48 h. (I) Film immersed in water for 24 h and then placed at 0% RH for 24 h.

performed in our laboratory on the biocide properties of chitosan-based films cast under different temperature conditions (Fernandez-Saiz et al., 2009a, 2009b).

3.4. Glucosamine release tests

The glucosamine migration was quantified for pure chitosonium-acetate matrices cast at 80 °C and the blends EVOH29/LMW-chitosan in an attempt to correlate this with the observed antimicrobial effects. To carry out these tests the ninhydrin method was used. The results obtained in these assays are indicated in Table 5.

From these results, a general trend can be observed in which the chitosan release was in general higher in the samples which showed better antimicrobial properties. This finding agrees with previous work in which a direct correlation between the chitosan release and the antimicrobial properties was demonstrated when pure chitosan films and composite matrices gliadins/chitosonium-acetate films were evaluated (Fernandez-Saiz et al., 2008). For a better understanding of the migration values obtained in the current work, the data of the samples obtained at 80 °C was plotted versus the final microbial counts of *Salmonella* spp. (see Fig. 5). In this figure, a good linear relation between variables is seen, except for the blend EVOH29/chitosan 80/20 (wt%), where the migration value (5.3 mg) was lower than expected for that microbial reduction. This blend as mentioned before was done in excess of acetic acid and had more homogeneous phase morphology hence providing better release efficiency.

Additionally, the percentage of released chitosan versus the EVOH content in the blend was also plotted (see Fig. 6). From this figure, a lineal relation between both variables can also be observed except now for the composite sample with 20 wt% of EVOH (80 wt% of chitosan), where the % of migration is reproducibly lower than expected from the linear plot. This reduction in the chitosan release corresponds with a concomitant reduction in antimicrobial properties. The precise reason why this sample presented this particular behavior is unknown but as mentioned above it could be related to the poor phase morphology presented and/or to the particular distribution of phases which could difficult migration or hindered the protonation of the chitosan fraction.

3.5. Antimicrobial EVOH

The susceptibility tests performed in the present work showed a bactericidal and an inhibitory effect against the growth of *S. aureus* and *Salmonella* spp. when the composite EVOH/chitosan 80/20 (wt%) films and EVOH films containing acetic acid were tested, respectively. According to the results, the biocide properties of these blends are enhanced as a consequence of the combined action of the release of higher protonated glucosamine groups and/or of entrapped acetic acid. The preservation of the antimicrobial properties of pure chitosonium-acetate films over time has been thoroughly investigated in our laboratory in a recent work (Fernandez-Saiz et al., 2009b). In this study, very low % RH or dry environments and mild temperatures (4–23 °C) proved to be the optimum storage conditions for this specific antimicrobial material. Nevertheless, the hypothesized presence of acetic acid inside the pure EVOH films at the various conditionings applied and over time is not known. For this reason, the presence of this acid in the pure

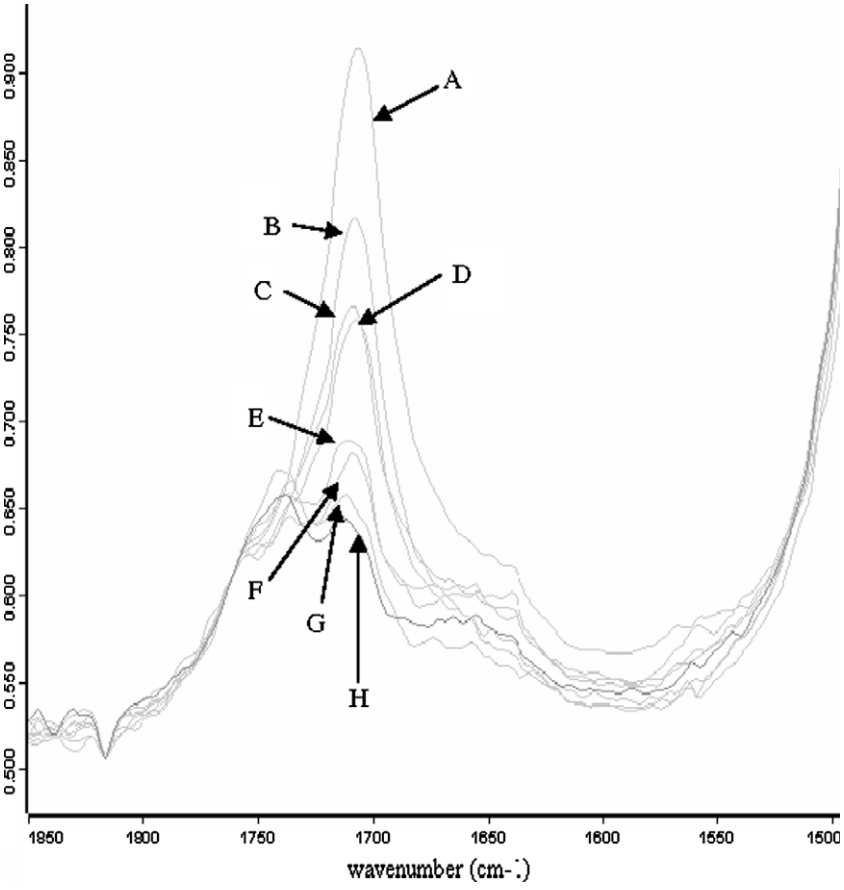

Fig. 8. ATR–FTIR spectra in the range from 1500 to 1850 cm^{-1} taken in EVOH films containing acetic acid after different conditionings. (A) Just formed film. (B) Film maintained at 80 °C for 2 h. (C) Film maintained at 120 °C for 20 min. (D) Film maintained at 80 °C for 8 h. (E) Film maintained at 80 °C for 24 h. (F) Film maintained at 120 °C for 40 min. (G) Film maintained at 80 °C for 48 h. (H) Film maintained at 120 °C for 4 h.

EVOH matrices was studied by ATR–FTIR spectroscopy after conditioning at various temperatures and/or humidity conditions. The intensity of the "free" acetic acid band centered at 1706 cm^{-1} (C=O stretching mode) in each sample was compared with that of the just formed film. The results are shown in Figs. 7 and 8. In the former figure, the influence of humidity conditions and a constant temperature (i.e. 23 °C) was observed. Thus, when the samples were stored at 0% or 40% RH the acid was kept entrapped within the EVOH network even after a month of storage. On the other hand, the band intensity vanished when the film was stored at high moisture conditions (75% and 100% RH) due to a moisture plasticization-induced release mechanism 30. Thus, simultaneous to the acid band intensity reduction, there is an increase in the 1650 cm^{-1} water band (O–H bending) in the material as a result of the sorption of this solvent with increasing relative humidity. The sample I was maintained immersed in water for a day and stored at 0% RH for a day before analysis by the spectroscopic technique. The I spectrum matched closely that of the similarly conditioned EVOH film not formed with acetic acid, showing the combined signal of remnant water and that from the incomplete hydrolysis of the ethylene–vinyl acetate copolymer precursor of the EVOH (result not shown). These results demonstrates that the acid present within the EVOH film will be released in the presence of high humidity products, being thus able to exert some antibacterial activity. Fig. 8 shows the evolution of the acid band intensity after conditioning at high temperatures. From this figure it can be observed that the acetic acid band vanished after 4 and 48 h when the acetic acid–EVOH film was maintained at 120 and 80 °C, respectively.

From the above assays, it is demonstrated that sufficient acetic acid to render bacteriostatic properties can be retained inside EVOH films when stored at mild temperatures and low humidity conditions. In any case, more experiments are currently underway in order to tailor the biocide properties of this material when cast in the presence of various biocides which will be reported elsewhere.

4. Conclusions

This study reports for the first time about the formulation of water barrier novel blends of chitosan with EVOH copolymers by solution casting from water/isopropanol solutions of acetic acid, which exhibit antimicrobial performance by the release of protonated glucosamine fractions and in some cases by the synergistic mild release in terms of pH drop of entrapped acetic acid. The latter mechanism occurs primarily in pure EVOH films cast in the presence of acetic acid, which renders the material bacteriostatic. Optimum performance in terms of morphology, optical properties, water barrier and biocide activity was found to be for the blends of LMW-chitosan, in which this component is the dispersed phase in the composition. Further studies which aim at tailoring biocide performance and at the characterization of other relevant morphological and physical properties such as mechanical properties are being currently carried out. The results from this work could potentially help the design of novel biobased coatings making use of chitosan salts and EVOH, which can retain the transparency and the dimensional stability in the presence of humidity of EVOH, but with enhanced water barrier compared to chitosan and with excellent biocide performance by controlled release of protonated glucosamine species.

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