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ORIGINAL PAPER

Development of Active and Nanotechnology-based Smart Edible Packaging Systems: Physical–chemical Characterization

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Abstract This work aims at characterising polysaccharide-based films without (GA) and with the incorporation of free natamycin (GA-NA) and natamycin-loaded in a smart delivery device consisting in poly(*N*-isopropylacrylamide) nanohydrogels (GA-PNIPA). Transport properties (water vapour, oxygen and carbon dioxide permeabilities), mechanical properties (tensile strength and elongation-at-break), opacity, water sensitivity (moisture content and contact angle) and thermal properties (differential scanning calorimetry and thermogravimetric analyses) were evaluated. Chemical interactions were studied by means of Fourier transform infrared spectroscopy and scanning electron microscopy was used to verify the presence of natamycin and nanohydrogel particles in the film matrix. The results show that natamycin and natamycin-loaded poly(*N*-isopropylacrylamide) (PNIPA) nanohydrogels can be successfully added to edible films without changing their main packaging properties. However, tensile strength decreased ($p<0.05$) when both natamycin and natamycin-loaded PNIPA nanohydrogels were incorporated (from 24.44 to 17.02 and 16.63 MPa, for GA-NA and GA-PNIPA, respectively). GA-NA and GA-PNIPA films are more opaque and showed to be more sensitive to water (i.e. higher values of moisture content and decrease of contact angle) than GA films. Scanning electron microscopy images confirmed the presence of natamycin and poly(*N*-isopropylacrylamide) nanohydrogels in the films' matrix. Since natamycin could be successfully released from polysaccharide-based films, the system could be used as active packaging ingredient when used free in the matrix or as smart packing when loaded with PNIPA nanohydrogels.

Keywords κ-Carragennan · Locust bean gum · Edible film · Nanotechnology · Poly(*N*-isopropylacrylamide)

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Introduction

The development of new formulations for antimicrobial agents release has attracted great attention due to the possibility of using such formulations in several applications (e.g. food packaging and surface treatments in biomedical devices) (Karlsson et al. 2010; Kuorwel et al. 2011). Moreover, edible packaging, using edible and biodegradable biopolymers, has been stated as one of the promises in packaging science (e.g. fresh-cut products, cheese, fruits, fish) (Cerqueira et al. 2009a; Lima et al. 2010; Rojas-Graü et al. 2007; Souza et al. 2010a). Active packaging presents great potential for applications on food packaging in order to enhance the ability to maintain food quality and safety (Han et al. 2005; Martins et al. 2010).

The application of active agents onto food surfaces, by dipping, brushing or spraying, could be inefficient, leading to the rapid diffusion of the active substances resulting in partial inactivation of the active substance and favouring the occurrence of microbial resistances (Fuciños et al. 2012; Reps et al. 2002). The design of active edible films to be used in food preservation is one of the solutions for this problem, controlling the release rate of the active agents from the film to the food surface. However, the release of antimicrobial substances from these biopolymers is generally achieved through passive diffusion mechanisms, so that there is no real effective control of the process (Fajardo et al. 2010; Ouattara et al. 2000). Therefore, it is necessary to

design smart edible packaging systems that allow the antimicrobial to be released from the biopolymers on demand only when it is necessary and as a response to environmental changes that would put the safety and stability of the food at risk.

The behaviour of active compounds in a film matrix will change with the physical, chemical and biological properties of the structure, which also will depend on the size of the structures (i.e. macro- to nano-scale). In the same way, properties of film matrix could be modified incorporating active compounds. Some authors showed the influence of nanostructures in edible films properties showing in some cases their antimicrobial effect (Imran et al. 2012; Martins et al. 2012a). Despite not having antimicrobial properties, some nanostructures can be used as carriers of antimicrobials in food packaging. It has been demonstrated that poly(*N*-isopropylacrylamide) (PNIPA) nanohydrogels can protect natamycin from hostile environments and allow its smart release utilising environmental temperature stimuli (Fuciños et al. 2012). The PNIPA nanohydrogels mode of action allows incorporated drugs to be squeezed out of the polymer when the temperature is raised above a critical temperature.

Natamycin, also know has pimaricin, is a natural antimycotic agent produced by *Streptomyces natelensis* and is used as a surface treatment on foods to control fungal growth. It was approved as generally recognised as safe by the Food and Drug Administration (FDA) in the USA and as a natural preservative in the European Union (E235). However, in both cases, it has low limits of application (i.e. 20 mg/kg on the finished products and less that 1 mg/dm^2 on cheese surface, respectively) (Regulation (CE) no. 1333 2008; FDA 2012). Being limited, the amount of the natamycin incorporated into food should be at the lowest possible level that is necessary to achieve the desired effect. The low solubility of natamycin requires the utilisation of a matrix (usually in commercial products synthetic polymers such as polyvinyl acetate) in order to be applied in food surfaces, providing the good distribution (Cerqueira et al. 2009a; Reps et al. 2002).

It has been shown that the mixture of polysaccharides such as locust bean gum galactomannan and κ-carrageenan have a synergistic behaviour that affects the films' physico–chemical properties (Martins et al. 2012b); these properties were mainly unchanged when functional compounds where incorporated (Martins et al. 2012a; Ruiz et al. 2013). Despite edible packaging materials with the incorporation of natamycin have been extensively reported, the incorporation of natamycin and natamycin-loaded nanohydrogels into locust bean gum and κ-carrageenan-based films has still not been studied. Moreover, to the best of our knowledge, no work has been reported with the incorporation of smart nanohydrogels in edible packaging and the evaluation of their properties. Therefore, this study aimed at evaluating the effect of incorporating free natamycin and natamycin-loaded PNIPA nanohydrogels in edible films' physical and chemical properties.

Materials and Methods

Materials

κ-Carrageenan (Gelcarin DX5253) and locust bean gum (Genu gum type RL-200) were purchased from FMC Biopolymer (Norway) and CP Kelco (USA), respectively. Glycerol 87 % was obtained from Panreac (Spain). Natamycin (pimaricin) containing 50 % lactose and 50 % natamycin was provided by VGP Pharmachem (Barcelona, Spain). In this work, unless otherwise indicated, natamycin is expressed as levels of impure natamycin (with 50 % lactose). The materials used for the PNIPA nanohydrogels synthesis were *N*-isopropylacrylamide (99 %) from Acros Organics (Geel, Belgium), isoparaffinic synthetic hydrocarbon (Isopar^{TM} M, 98 %) from Esso Chemie (Cologne, Germany), sorbitan sesquiolate (Span^{TM} 83, 98 %) from Uniquema (Wilmington, DE, USA), PEG-40 sorbitol hexaoleate (Atlas^{TM} G-1086, 98 %) from Uniquema (Wilmington, DE, USA), sodium bisulfite ($NaHSO_3$) for analysis from Merck (Darmstadt, Germany) and chloroform and diethyl ether from Panreac (Barcelona, Spain).

Preparation of Natamycin-Loaded PNIPA Nanohydrogels

The synthesis of *N*-isopropylacrylamide nanohydrogels and the preparation of natamycin-loaded PNIPA nanohydrogels was performed as presented by Fuciños et al. (2012). For the synthesis, a water in oil microemulsion formed by 58 % of aqueous phase (AP—80 % water and 20 % monomer), 17 % of oil phase (OP—Isopar^{TM}) and 25 % of surfactant (Atlas^{TM} G-1086 and Span^{TM} 83) was prepared. The phases (AP and OP) were solubilized and mixed in a 100-mL reactor equipped with mechanical stirring and thermostatised at 25 °C. Reaction medium was purged by bubbling nitrogen to eliminate oxygen. The polymerization was began by adding the initiator at a ratio $m_{NaHSO3}/m_{monomer}=0.01$ and monitored by the temperature increase inside the glass reactor. Chloroform and diethyl ether were used to selectively purify the obtained polymer. The pure polymer was then dried overnight in an oven (50 °C) and then ground in a colloid mill (IKA Werke GmbH & Co. KG, Staufen, Germany).

Natamycin-loaded PNIPA nanohydrogels were prepared by dispersing, with agitation, the PNIPA nanohydrogel powder in distilled water for 3 h at room temperature to allow the nanoparticles to swell properly. This suspension was then mixed with a natamycin water solution to obtain final concentrations of 12.5 mg/mL of nanohydrogel and 0.4 mg/mL of

natamycin. The mixture was then stirred 12 h at 25 °C to guarantee the incorporation of natamycin into the nanohydrogel particles.

Preparation of κ-Carrageenan and Locust Bean Gum (κ-car/LBG) Films

Polysaccharide film-forming solutions were prepared using the method described by Martins et al. (2012b). Briefly, 0.4 and 0.6 % (*w*/*v*) of κ-carrageenan (κ-car) and locust bean gum (LBG), respectively, were suspended in distilled water under agitation during 1 h at 25 °C; after that, 0.3 % of glycerol is added to the solution. Then, the film-forming solutions were homogenised at 80 °C under agitation during 30 min. Natamycin and the natamycin-loaded PNIPA nanohydrogels were added after the decrease of the temperature to 30 °C in order to avoid the nanohydrogel collapse. A free natamycin concentration of 0.5 mg/mL was chosen based on the published work by Fajardo et al. (2010). A PNIPA concentration of 0.2 % was added to films, chosen based in preliminary experiments in order to have a good miscibility of the polymers and processability of the films. The compounds were added and stirred until a homogeneous solution was obtained. Then, 28 mL of solution was cast into polystyrene Petri dishes and dried at 25 °C during 48 h. Films were conditioned in desiccators containing a saturated solution of $Mg(NO_3)_2{\cdot}6H_2O$ at 53 % relative humidity (RH) and 20 °C. Films without the incorporation of any compound are mentioned as GA films, and with the addition of free natamycin and natamycin-loaded PNIPA nanohydrogels as GA-NA and GA-PNIPA films, respectively.

Films Morphology—Scanning Electron Microscopy

The κ-car/LBG films were mounted on scanning electron microscopy (SEM) stubs with double-sided adhesive tape. Then, film samples were coated with a thin layer of gold. A Nova NanoSEM 200 (Netherlands) instrument was used to observe the morphologies of the film cross sections and surface at an accelerating voltage from 10 to 15 kV.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectra of the films were recorded with a PerkinElmer 16 PC (Boston, USA) FTIR spectrometer in the wavelength range 4,000–400 cm^{-1} at a resolution of 4 cm^{-1}, using attenuated total reflection mode. The absorbance of each FTIR spectrum was normalised between 0 and 1. The measurements were performed in triplicate.

Moisture Content

To determine the moisture content (MC) of films, about 50 mg of film was dried at 105 °C during 24 h (until the equilibrium weight). The weight loss of the sample was determined, from which the moisture content was calculated.

Surface Hydrophobicity

The sessile drop method, based on optical contact angle method, was used to estimate surface hydrophobicity of the test films. Contact angle (θ) was determined with a face contact angle meter (OCA 20, DataPhysics, Germany) by the sessile drop method (Kwok and Newmann 1999) in which a droplet ultra-pure water was deposited on the film surface with a 500-μL precision syringe (Hamilton, Switzerland). The drop image was recorded by a video camera, and the profile of the droplet was numerically solved and fitted to Laplace–Young equation. Five measurements were replicated for each film and three independent measurements per sample. All measurements were done on the "air-side" of the films (face in contact with the air during film drying) at 22.2±0.8 °C.

Permeability to Gases (Water Vapour, Oxygen and Carbon Dioxide)

Water vapour permeability (WVP) was measured based on the methodology described by Casariego et al. (2009). Films samples were sealed on cups containing distilled water, and afterwards they were placed inside a desiccators containing silica gel (0 % RH; 20 °C). To maintain uniform conditions to all samples, a fan was used inside the desiccator. Periodical cup weightings (2 h) were performed to monitor the weight loss over time until steady state was reach. Finally, water vapour transmission rate was calculated by dividing the slope of linear regression of weight loss by film area, and WVP (in gram per metre per second per pascal) as follows:

$$\mathrm{WVP} = \frac{\mathrm{WVTR} \cdot \mathrm{L}}{\Delta \mathrm{P}} \tag{1}$$

where L is film thickness (in meter) and ΔP is water vapour partial pressure difference (in pascal) across the two sides of the film. For each measurement, at least three replicates were made for each type of film.

Films thickness was measured at ten different points using a digital micrometer (no. 293–5, Mitutoyo, Japan) with ±0.001-mm accuracy.

Oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) measurements were conducted based on Cerqueira et al. (2009b). In brief, tests were determined in a gas permeation cell consisted of two chambers and a test film was placed between the two chambers. Measurements were conducted at 20 °C. A permeation gas (oxygen or dioxide carbon) flowed continuously through the lower

chamber, and nitrogen (as a sweep gas) was passed through the upper chamber. When the steady state was reached, the penetrated gas O_2 or CO_2 in the sweep gas stream was analysed by a gas chromatograph (Chrompack 9001, Middelburg, Netherlands) at 110 °C with a column Porapak Q 80/100 mesh 2 m × 1/8 in. × 2 mm SS equipped with a hydrogen flame ion detector. Helium was used as carrier gas at 23 mL min^{-1}. A standard mixture containing 10 % CO_2, 20 % O_2 and 70 % N_2 was used for calibration. For each measurement, at least three replicas were made for each type of film.

Mechanical Properties

The mechanical properties of the films were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation) in accordance to Casariego et al. (2009). Samples (50×20 mm^2) were clamped between grips with an initial distance of 30 mm. The force and deformation were recorded during extension at 5 mm min^{-1}. Tensile strength (TS) and elongation-at-break (EB) were expressed in megapascal and percentage, respectively. For each measurement, at least five replicates were made for each type of film.

Differential Scanning Calorimetry and Thermogravimetric Analyses

Differential scanning calorimetry (DSC) measurements were performed in a Shimadzu DSC-50 (Shimadzu Corporation, Kyoto, Japan) calibrated with indium as standard. Approximately 10 mg of the sample was placed in aluminium DSC pans (Al crimp Pan C.201-52943). The measurements were performed between 20 and 200 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere. Thermogravimetric analyses (TGA) were completed with a Shimadzu TGA-50 (Shimadzu Corporation, Kyoto, Japan). Samples were placed in the balance system and heated from 20 to 580 °C at a heating rate of 10 °C min^{-1} under a nitrogen atmosphere. For each measurement, at least two repetitions were made for each type of film.

Opacity and Colour

The opacity of the samples was determined according to the Hunter lab method, as the relationship between the opacity of each sample on a black standard (Y_b) and the opacity of each sample on a white standard (Y_w) (Eq. 2). The measurements were repeated three times for each film.

$$\text{Opacity} = \frac{Y_b}{Y_w} \cdot 100 \quad (2)$$

The colour of the films was determined with a Minolta colorimeter (Cr 400; Minolta, Japan). A white standard colour plate (Y=93.9, x=0.3158, y=0.3321) for the instruments' calibration was used as a background for colour measurements of the films, and the L^*, a^* and b^* values of each film were evaluated by reflectance measurements. In this system, L^* indicates the lightness (ranging from black to white), and the horizontal axes, indicated by a^* and b^*, are the chromatic coordinates (ranging from $-a^*$ (greenness), $-b^*$ (blueness) to + a^* (redness), $+b^*$ (yellowness)). The values of a^* and b^* approach 0 for neutral colours and increase as the colour becomes more chromatic and more saturated.

Statistical Analysis

Statistical analysis was performed using the analysis of variance procedure with SigmaPlot 11.0 software for Windows (free trial). Tukey's test was applied to detect differences of means, and $p<0.01$ was considered to be statistically significant.

Results and Discussion

One of the most important and difficult tasks in the incorporation of active compounds and new materials in a matrix is understanding if they will be miscible with the matrix and if they will not influence the processability and their structure. In this study, the preliminary experiments allowed the determination of free natamycin and natamycin-loaded PNIPA nanohydrogels concentrations that when added to the polysaccharide-based matrix do not affect significantly their processability. Natamycin had been used in concentrations below the values allowed by the legal regulations (Regulation (CE) no. 1333, 2008; Fajardo et al. 2010; FDA 2012). For natamycin-loaded PNIPA nanohydrogels, different concentrations were tested, and it has been shown that for increasing concentrations the films became more brittle and unmanageable, being impossible to remove them from the support after the casting process without breaking (results not shown). Films with 0.2 % of natamycin-loaded PNIPA nanohydrogels have been chosen for the tests; the obtained films were homogeneous, transparent and flexible. At this point, one of the objectives in this work was accomplished, and the study of the physical–chemical properties of the films was used to confirm visual observations.

SEM was used to characterise the topography and morphology of the films before and after incorporation of free natamycin and natamycin-loaded PNIPA nanohydrogels. Figure 1 shows SEM images of the films' surfaces without and with the addition of free natamycin and of natamycin-loaded PNIPA nanohydrogels. All samples show the homogeneity of the films, without the presence of pores or/and cracks. It is clear that the roughness of the films surface increase when natamycin-loaded PNIPA nanohydrogels

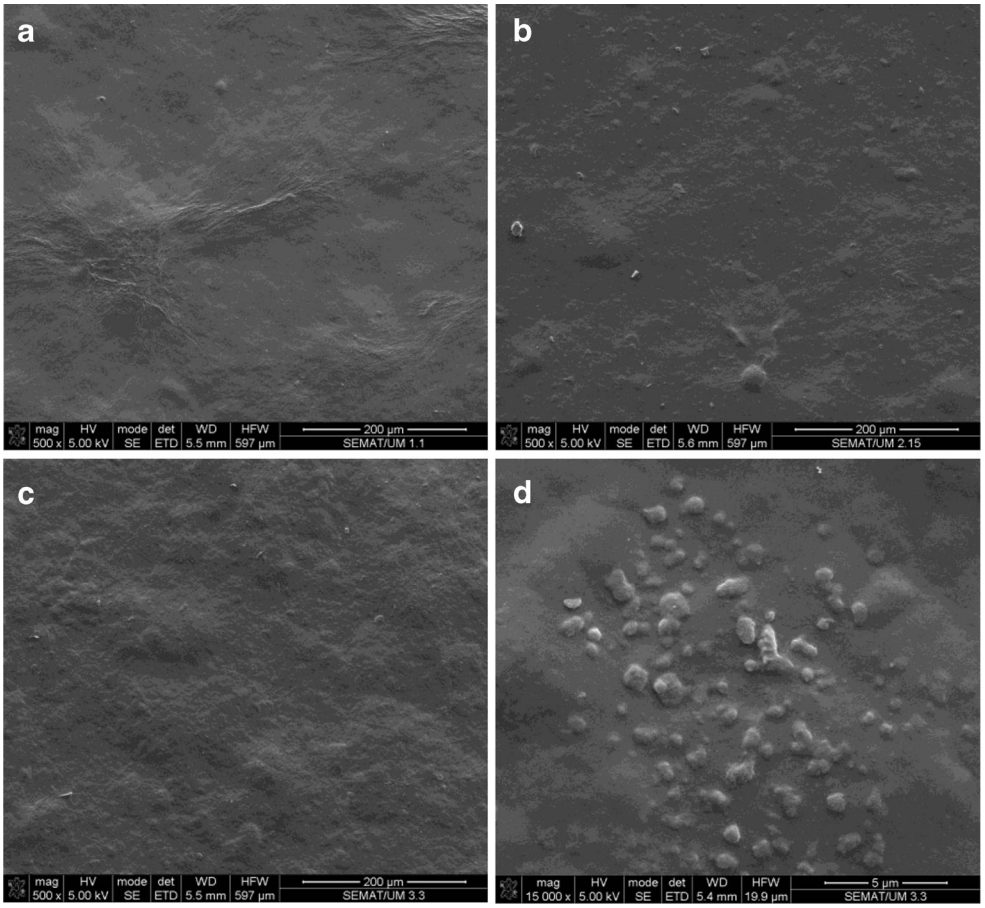

Fig. 1 SEM images of the polysaccharide films surface: without natamycin (**a**) with free natamycin (**b**), and with natamycin-loaded PNIPA at different magnifications: ×500 (**c**) and×15,000 (**d**)

were added to the films (Fig. 1c, d). Figure 1d shows the presence of PNIPA nanohydrogels with sizes in the range reported by Fuciños et al. (2012).

Fourier Transform Infrared Spectroscopy

The effect of the addition of natamycin and natamycin-loaded PNIPA nanohydrogels in films was initially evaluated by FTIR analyses. When different compounds are mixed, physical bonds and chemical interactions are reflected by changes in characteristic spectra peaks; these changes can reflect polymer miscibility (e.g. shifting of absorption bands).

Figure 2 shows FTIR spectra of film samples before and after the addition of free natamycin and natamycin-loaded PNIPA nanohydrogels. The results are in good agreement with the results presented by Martins et al. (2012a, b), where peaks and characteristic bands of the κ-car/LBG films (GA) are discussed. The broad peak between 3,600 and 3,200 cm^{-1} is related with the hydroxyl group of –OH stretching vibrations; the region between 3,000 and 2,800 cm^{-1} is representative of the –CH stretching vibration. The band in the region of 750–1,300 cm^{-1} corresponds to the carbohydrates region, called fingerprint region, where bands are specific for each polysaccharide (Cerqueira et al. 2012). The incorporation of free natamycin in the film matrix (GA-NA) leads to the presence of new peaks; however, the main broad peaks are maintained being the spectra of the films with natamycin similar to GA films (Fig. 2). However, the appearance of new peaks in the region of –CH vibration 2,853 cm^{-1} is related to symmetric vibrations of the CH_3 group (Jaiswal et al. 2010), mainly resulting from the presence of natamycin. A peak related with the vibration of a conjugated ester of natamycin at 1,743 cm^{-1} appears in the GA-NA spectrum (Atta et al. 2012; Cevher et al. 2008). This peak corresponds to the C=O stretching vibration indicating the presence of the carbonyl radical in the ester functional group.

Figure 2 also shows the representative FTIR spectra of polysaccharide films with natamycin-loaded PNIPA nanohydrogels (GA-PNIPA). The differences between FTIR spectra of GA and GA-PNIPA are clear. This can be representative of the chemical interaction between the film matrix and their components (i.e. polysaccharide and glycerol) and the natamycin-loaded PNIPA nanohydrogels. Changes in the region between 4,000 and 2,700 cm^{-1} and between 1,900 and 800 cm^{-1} in the "fingerprint" region indicate interactions between components. The strong vibration band attributed to the hydroxyl groups of polysaccharides is shifted from 3,300 to 3,355, presenting a lower intensity when natamycin-loaded PNIPA nanohydrogels are added to the films. The differences observed in the region of –CH

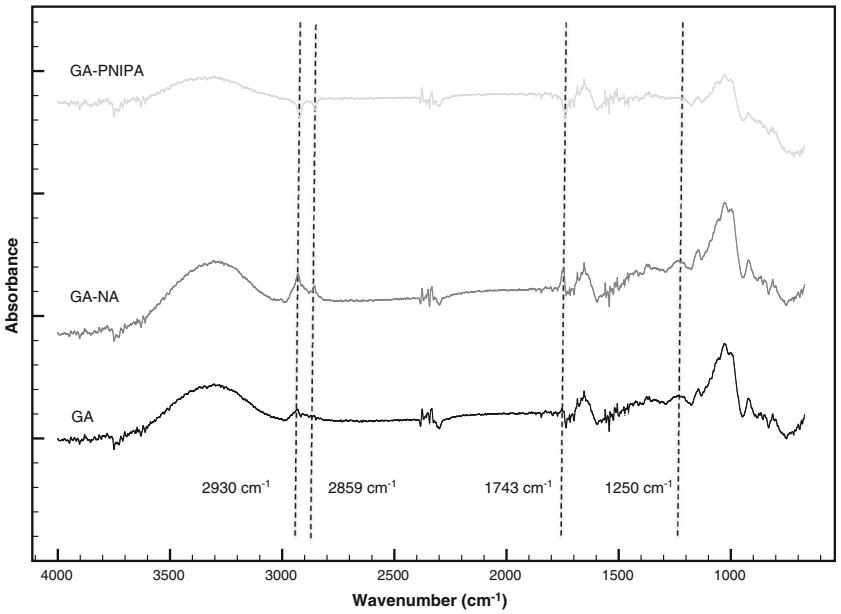

Fig. 2 FTIR spectra of the films without incorporation of compounds (GA), films with free natamycin (GA-NA) and films with natamycin-loaded PNIPA nanohydrogels (GA-PNIPA)

vibrations with changes in peak intensity are related with stretching vibrations (Jaiswal et al. 2010; Dumitriu et al. 2011). The ester sulphate groups (−SO) disappear (Martins et al. 2012b) and the strong broad band at 1,029 cm^{-1} (C–O stretching of alcoholic groups) has a considerably lower intensity, probably due to the influence of PNIPA in the film polysaccharide matrix.

Moisture Content and Surface Hydrophobicity

MC and surface hydrophobicity were studied in order to understand how the incorporation of free natamycin (GA-NA) and natamycin-loaded PNIPA nanohydrogels (GA-PNIPA) in the films influence the matrix affinity to water. Table 1 shows film moisture content, showing that incorporation of free natamycin in films leads to the increase ($p<0.01$) of MC values. Similar results were observed for GA-PNIPA films, where the addition of natamycin-loaded PNIPA nanohydrogels leads to the increase ($p<0.01$) of the MC from 17.72 to 19.16 %. Contact angle (θ) of water with the film surface was used to evaluate the surface hydrophobicity of the film. The differentiation between hydrophobic and hydrophilic surfaces is based on water contact angle, where a surface with a contact angle value higher than 65–70° represents a hydrophobic surface (Carneiro-da-Cunha et al. 2010; Pereira et al. 2010). Therefore, studied films can be considered to have hydrophobic surfaces, presenting θ values higher than 74.48°. Moreover, results from contact angle measurements suggest that surface hydrophilicity of films is increased ($p<0.01$) by the incorporation of natamycin-loaded PNIPA nanohydrogels.

These values are in agreement with those presented by other authors for polysaccharide-based films with natamycin incorporation (Bierhalz et al. 2012), being the values of moisture content in the range of those obtained for other polysaccharide-based films (Cerqueira et al. 2012).

Overall, results show that the addition of natamycin and the presence of natamycin-loaded PNIPA nanohydrogels led to an increase of water affinity of edible films. In the case of free natamycin, the results can be explained by the presence of lactose in the commercial natamycin preparations that changes the properties of the films structure because the increase of crystal domains leads to a more hydrophilic behaviour (see "Thermal Properties" section). Since natamycin (and lactose) are encapsulated in PNIPA, in the case of natamycin-loaded PNIPA nanohydrogels, the behaviour should be related with

Table 1 Values of moisture content, contact angle, water vapour, oxygen and carbon dioxide permeabilities (WVP, O_2P and CO_2P, respectively) of the films without the incorporation of compounds (GA), with natamycin (GA-NA) and with natamycin-loaded PNIPA nanohydrogels. Values reported are the means ± standard deviations. Different letters in the same column indicate a statistically significant difference ($p<0.01$)

Film samples	Moisture content (%)	Contact angle (θ)	WVP×10^{-11} (g (m s Pa)$^{-1}$)	O_2P×10^{-13} (g (m s Pa)$^{-1}$)	CO_2P×10^{-13} (g (m s Pa)$^{-1}$)
GA	17.72±0.07a	112.36±6.40a	5.94±0.82a	5.84±0.87a	1.97±0.48ab
GA-NA	19.69±0.48b	108.95±7.28a	7.13±0.40b	8.46±2.02b	2.27±0.18a
GA-PNIPA	19.16±0.27b	74.48±2.09b	6.48±0.91ab	6.98±2.29ab	1.82±0.18b

the capacity of the PNIPA to bond water due the presence of high numbers of hydroxyl groups at the surface of the film. In fact, the hydrophilic/hydrophobic balance of PNIPA structure is known and the hydrogen bonds formed between water molecules and hydrophilic groups form a stable shell around the hydrophobic groups. This behaviour is favoured below the lower critical solution temperature increasing the solubility of PNIPA nanohydrogel in water (Fuciños et al. 2012).

Permeability to Gases (Water Vapour, Oxygen and Carbon Dioxide)

The study of gas permeabilities allows understanding how changes in film structure (e.g. incorporation of compounds) can influence parameters such as sorption, solubility and diffusion of the matrix. Natamycin and natamycin-loaded PNIPA nanohydrogels were added to the films not with the objective of improving films properties (e.g. decrease permeability to water vapour), but with the main purpose of creating an active and smart packaging and evaluating the effect of incorporated compounds in edible films' properties.

Table 1 presents the values of permeability to water vapour (WVP), oxygen (O_2P) and carbon dioxide (CO_2P) of the studied films. The polysaccharide matrix is changed by the incorporation of natamycin (GA-NA) and natamycin-loaded PNIPA nanohydrogels (GA-PNIPA) apparently decreasing the barrier to water vapour; however, only the increase of WVP values for the GA-NA films is statistically significant ($p<0.01$). It has been shown above, through moisture content and surface hydrophobicity determinations, that the films GA-NA and GA-PNIPA present a higher water affinity when compared with GA. This behaviour will influence WVP increasing the adsorption of water molecules and thus increasing the permeabilities of the films.

The same behaviour was observed for O_2P, leading to a significant increase ($p<0.01$) of the values only for GA-NA films when these are compared with GA films. The presence of natamycin-loaded nanohydrogels in the film matrix does not change significantly ($p>0.01$) the permeabilities to O_2 and CO_2 when compared to GA films. Nevertheless, the utilisation of natamycin-loaded nanohydrogels avoids at a significant level the increase of CO_2P observed in the GA-NA series. This could be due to both the presence of a lower natamycin concentration and its encapsulation in GA-PNIPA films, and its ability minimises the influence of natamycin in films' transport properties.

Other authors observed similar results when free natamycin was incorporated in other polysaccharides films (Da Silva et al. 2012; Fajardo et al. 2010; Martins et al. 2010). The presence of free natamycin lead to the presence of crystals in the film matrix, thus affecting permeability values (Da Silva et al. 2012). This fact was confirmed in thermal analyses where the enthalpy of melting of the films increased in the presence of natamycin (see "Thermal Properties" section). Free natamycin possibly created additional sites, associated with the increase of the free volume of the film, favouring the dissolution of oxygen and increasing the mobility of water, oxygen and carbon dioxide molecules through the film (Fajardo et al. 2010).

Mechanical Properties

TS and EB of a material are used to study their resistance to tensile stress and to determine the percentage of increase in length that occurs before the sample breaks. Additionally, it can provide interesting information related with the structural organisation of the film matrix and also allows to evaluate the effect of the addition of active compounds.

Table 2 shows the values of EB and TS for the studied films. Incorporation of free natamycin (GA-NA) and natamycin-loaded PNIPA nanohydrogels in films influences their mechanical properties, decreasing ($p<0.01$) the values of EB and TS. The values for the films without the addition (GA) of free natamycin and natamycin-loaded PNIPA nanohydrogels are in good agreement with the values reported by Martins et al. (2012a, b). The incorporation of compounds in film matrix increases polymer mobility, explained by the higher hydrophilicity of the films, as shown by the values of moisture content (Table 1), leading to a decrease of TS values. Similar results were observed by others (Caner et al. 1998; Cerqueira et al. 2012; Ziani et al. 2008) who shown that the increase of polymer mobility leads to a decrease of TS and at same time an increase of EB. However, in this case, the increase of EB was not observed and the presence of the free natamycin and natamycin-loaded PNIPA nanohydrogels lead to a decrease ($p<0.01$) of the EB values. The chemical structure of the film was influenced by the presence of the free natamycin and natamycin-loaded PNIPA nanohydrogels, suggesting

Table 2 Values of elongation-at-break, tensile strength, opacity, melting temperature and enthalpy energy of the films without the incorporation of compounds (GA), with natamycin (GA-NA) and with natamycin-loaded PNIPA nanohydrogels (GA-PNIPA)

Film sample	Elongation-at-break (%)[a]	Tensile strength (MPa)[a]	T_m (°C)	ΔH_m(J/g)
GA	15.58±3.88a	24.44±1.85a	146.6	151.1
GA-NA	12.01±1.23b	17.02±3.48b	148.8	203.0
GA-PNIPA	11.91±1.23b	16.63±3.08b	148.7	183.1

Different letters in the same column indicate a statistically significant difference ($p<0.01$)

[a] Values reported are the means ± standard deviations

the decrease of the intermolecular forces of the polysaccharide-based films resulting in a ductile and less flexible material. Changes in the chemical structure were confirmed by FTIR analyses (see "Fourier Transform Infrared Spectroscopy" section). The same behaviour was observed by Imran et al. (2012) where the incorporation nano-scale liposomes lead to the decrease of TS and EB of hydroxypropyl methylcellulose films, attributed to discontinuities in the polymer matrix and alterations in polymer chain interactions.

Thermal Properties

DSC was used to evaluate the influence of free natamycin and natamycin-loaded PNIPA nanohydrogel in the enthalpy of melting (ΔH_m) and melting temperature (T_m) of the films.

Figure 3 shows the thermograms between 20 and 200 °C for the studied films. The presence of an endothermic peak is related with the melting transition of the films. T_m values are close for all the samples, around 150 °C (Table 2), and are in agreement with the reported values for other polysaccharide-based films (Cerqueira et al. 2012; Souza et al. 2010b). Despite that both parameters, ΔH_m and T_m, can be associated with the crystallinity of the samples (Cerqueira et al. 2012; Souza et al. 2010b; Sperling 2006), only the values of ΔH_m show variations. The presence of free natamycin (GA-NA) and natamycin-loaded PNIPA nanohydrogel (GA-PNIPA) leads to the increase of ΔH_m explained by the increase of the crystallinity of the films. As explained in "Moisture Content and Surface Hydrophobicity" and "Permeability to Gases (Water Vapour, Oxygen and Carbon Dioxide)" sections, the presence of natamycin leads to the presence of crystal domains in the film matrix (Da Silva et al. 2012); the occurrence of such crystal domains may also be explained by the increase of moisture content that influences the intensification of films' crystallinity (Chen et al. 2008).

Thermogravimetric analyses evaluate the changes in weight of film samples with the increase of temperature. Figure 4 shows thermal events related with the weight loss of films that are in agreement with the results observed for other polysaccharide-based films (Martins et al. 2012a, b; Ruiz et al. 2013).

TGA thermograms show similar behaviours for all studied films, with the presence of four thermal events. The first event (maximum peak from 65 to 70 °C) is attributed to water evaporation, the second (maximum peak from 215 to 225 °C) is attributed to chemisorbed water through hydrogen bonds due to the presence of glycerol (Quijada-Garrido et al. 2007), the third (maximum peak from 260 to 270 °C) is related to dehydration, depolymerization and pyrolytic decomposition of the polysaccharide backbone (Zohuriaan and Shokrolahi 2004) and the last thermal event is related with the decomposition of the samples.

Peak events show that natamycin presence (GA-NA) leads to a more significant weight loss associated with two thermal events: water evaporation (peak 1) and chemisorbed water (peak 2) when compared with the GA films, increasing from 11.26 to 13.77 % and from 11.85 to 17.22 %, respectively. These differences were also observed for the moisture content analysis and are related with a higher hydrophilic behaviour of GA-NA films. It is important to mention that the sums of the mass losses of these two events were higher for GA-NA films (30.99 %), followed by GA-PNIPA (27.51 %) and GA films (23.11 %) (results not shown), values that are in agreement with moisture content results.

Opacity and Colour

Colour parameters and opacity of films are extremely important in food packaging for consumer acceptance. Good optical properties of films are achievable through the control of the incidence of light in the food product or/and ensuring that the consumer can clearly see the food product.

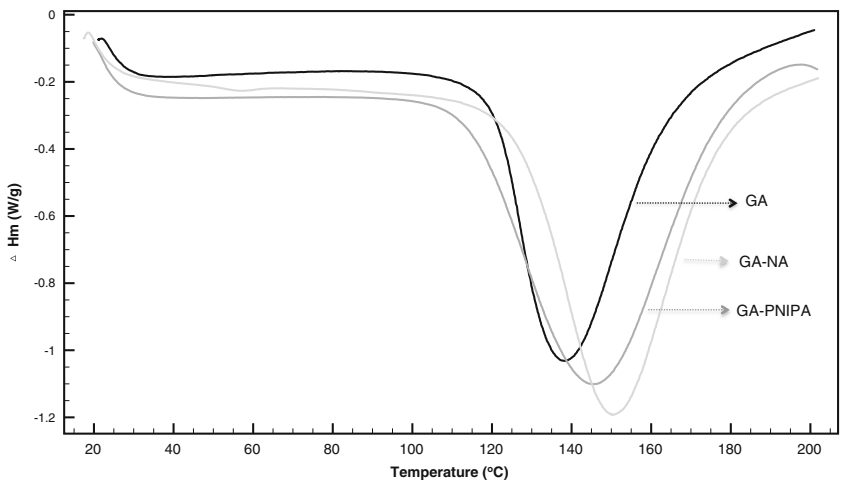

Fig. 3 DSC thermogram of films without the incorporation of compounds (GA), films with free natamycin (GA-NA) and films with natamycin-loaded PNIPA nanohydrogels (GA-PNIPA)

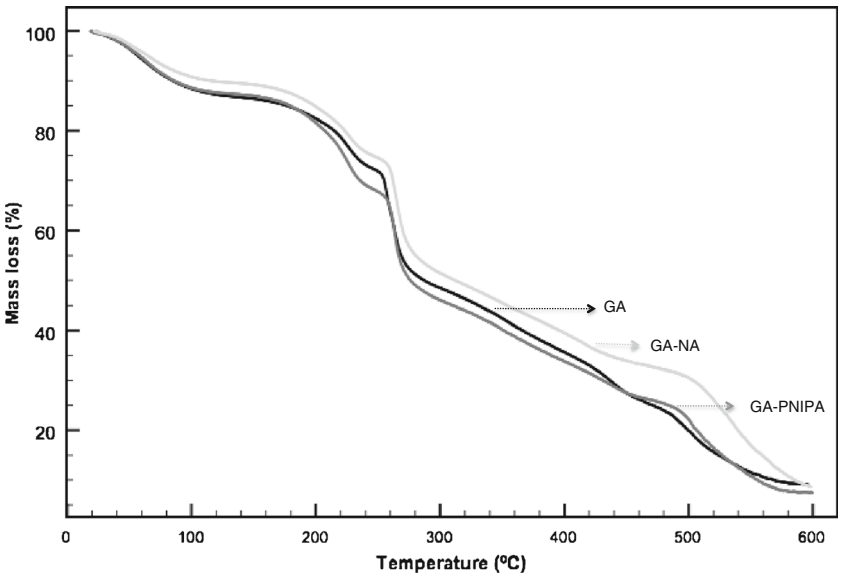

Fig. 4 TGA thermogram of films without the incorporation of compounds (GA), films with free natamycin (GA-NA) and films with natamycin-loaded PNIPA nanohydrogels (GA-PNIPA)

Table 3 shows the values obtained for opacity and colour parameters L^*, a^* and b^*. The presence of free natamycin (GA-NA) and natamycin-loaded PNIPA nanohydrogels (GA-PNIPA) in the films lead to the increase ($p<0.01$) of opacity values when compared with films without compounds (GA). Opacity values of the films GA-NA and GA-PNIPA are not statistically different from each other ($p>0.01$).

Regarding the colour parameters L^*, a^* and b^*, the incorporation of free natamycin (GA-NA) has statistical influence ($p<0.01$) in a^* and b^*. On the other hand, the presence of natamycin-loaded PNIPA nanohydrogel (GA-PNIPA) does not influence ($p>0.01$) those two parameters when comparing with the films without the active compounds (GA). The presence of free natamycin decreases a^*, meaning a decrease of red and an increase of green colour that is, in this case, very close to 0. The presence of free natamycin lead to an increase of b^* (meaning that films become more yellowish, which is characteristic of natamycin and lactose). Equally, the presence of natamycin in the films is the most influent parameter influencing opacity and colour, with GA-NA films presenting higher values of opacity, a^* and b^* than GA-PNIPA films.

Table 3 Values of opacity, L^*, a^* and b^* of the films without the incorporation of compounds (GA), with natamycin (GA-NA) and with natamycin-loaded PNIPA nanohydrogels (GA-PNIPA)

Film sample	Opacity	L^*	a^*	b^*
GA	8.34±2.14a	96.78±0.34a	0.17±0.05a	2.80±0.16a
GA-NA	12.19±0.48b	96.49±0.14a	−0.06±0.08b	4.49±0.16b
GA-PNIPA	11.08±0.70b	97.09±0.18a	0.13±0.03a	3.12±0.22a

Values reported are the means ± standard deviations. Different letters in the same column indicate a statistically significant difference ($p<0.01$)

Conclusion

Polysaccharide-based films have been mentioned as good vehicles for the incorporation of active compounds in order to improve stability, efficiency and release of those molecules. However, the incorporation through nanostructures has been less studied and the incorporation of natamycin-loaded nanohydrogels in the film was never evaluated. This work shows that natamycin-loaded poly(*N*-isopropylacrylamide) nanohydrogels can be successfully added to edible films without changing their main properties, despite the influence in colour and mechanical properties. Also, free natamycin was tested but highly influenced the films properties. The incorporation of active agents into the film matrix, besides adding new functionalities, can also cause changes in the structure and physical properties of films that will influence the release behaviour of the active compound. Further studies should be done in order to evaluate the different release behaviour of free natamycin and natamycin-loaded nanostructures from the films into food model systems.

This work presents a new concept of edible active and smart packaging containing natamycin-loaded nanohydrogels that may help in the controlled release of active compounds and can be a guide for the development of new materials.

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