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Functional Characterisation and Antimicrobial Efficiency Assessment of Smart Nanohydrogels Containing Natamycin Incorporated into Polysaccharide-Based Films

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Abstract The potential application of polysaccharide-based films containing smart nanohydrogels for the controlled release of food preservatives is demonstrated here. Smart active packaging is the most promising alternative to traditional packaging as it provides a controlled antimicrobial effect, which allows reducing the amount of preservatives in the food bulk, releasing them only on demand. This work evaluates the usefulness of smart thermosensitive poly(N-isopropylacrylamide) (PNIPA) nanohydrogels with or without acrylic acid (AA) incorporated into polysaccharide-based films (GA) to transport natamycin and release it as a response to environmental triggers. Release kinetics in liquid medium from GA films containing PNIPA/AA nanohydrogels (GA-PNIPA(5) and GA-PNIPA-20AA(5)) presented a characteristic feature regarding the films without nanohydrogels that was the appearance of a lag time in natamycin release, able to reach values of around 35 h. Another important feature of natamycin release kinetics was the fact that the release from GA-PNIPA/AA films only occurred when temperature was increased, so that the natamycin release was restricted to when there is a risk of growth of microorganisms that cause food spoilage or the development of pathogenic microorganisms. Additionally, it could be observed that the relative fraction of natamycin released from GA-PNIPA/AA films was significantly ($p < 0.05$) higher than that released from GA films loaded with the same amount of free natamycin. It can be hypothesised that the encapsulation of natamycin into nanohydrogels helped it to be released from GA films, creating reservoirs of natamycin into the films and, therefore, facilitating its diffusion through the film matrix when the nanohydrogel collapses. In a solid medium, the low water availability limited natamycin release from GA-PNIPA/AA films restricting the on/off release mechanism of PNIPA/AA nanohydrogels and favouring the hydrophobic interactions between natamycin and polymer chains at high temperatures. Despite the low natamycin release in solid media, antimicrobial efficiency of GA-PNIPA(5) films containing natamycin in acidified agar plates was higher than that obtained with GA films without natamycin and GA films with free natamycin, probably due to the protecting effect against degradation when natamycin was included in the nanohydrogels, allowing its release only when the temperature increased.

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Nomenclature

AA	Acrylic acid
AP	Aqueous phase
Atlas™ G-1086	PEG-40 sorbitol hexaoleate
DL	Detection limit
F_t	Fraction of natamycin released from films towards agar plates
GA-NA	GA films containing free natamycin

GA-PNIPA-20AA(5)	Polysaccharide-based films containing PNIPA-20AA(5) nanohydrogel
GA-PNIPA-20AA(5)+NA(8)	GA films containing PNIPA-20AA(5) nanohydrogels loaded with 7.92 μg of natamycin
GA-PNIPA(5)	Polysaccharide-based films containing PNIPA(5) nanohydrogel
GA-PNIPA(5)+NA(8)	GA films containing PNIPA(5) nanohydrogels loaded with 7.92 μg of natamycin
GA-PNIPA/AA	Polysaccharide-based films containing poly (N-isopropylacrylamide) nanohydrogel with or without copolymerised acrylic acid
GA	Polysaccharide-based films
GA+NA(564)	GA films containing 564.24 μg of free natamycin
GA+NA(8)	GA films containing 7.92 μg of free natamycin
Isopar™ M	Isoparaffinic synthetic hydrocarbon
LCST	Lower critical solution temperature
MIC	Minimum inhibitory concentrations
n	The diffusional exponent that depends on both the geometry of the delivery system and the physical mechanisms involved
NIPA	N-isopropylacrylamide
NMBA	N,N′-methylenebisacrylamide
OP	Oil phase
P 1.01	Penicillium commune
PDA	Potato dextrose agar
PNIPA-20AA(5)	Poly(N-isopropylacrylamide) nanohydrogel copolymerised with 20 % (w/w) of acrylic acid using 5 % (w/w) of crosslinking
PNIPA	Poly(N-isopropylacrylamide)
PNIPA(5)	Poly(N-isopropylacrylamide) nanohydrogel with 5 % (w/w) of crosslinking
PNIPA/AA	Poly(N-isopropylacrylamide) nanohydrogel with or without copolymerised acrylic acid
RP-HPLC	Reverse phase high-performance liquid chromatography
Sc 1.02	Saccharomyces cerevisiae
Span™ 83	Sorbitan sesquiolate
ST	Surfactant
v_{max}	Maximum release rate (h^{-1})
γF_{max}	Maximum relative fraction of natamycin released from films
γF_t	Relative fraction of natamycin released from films
λ	The lag time for the natamycin release, expressed in hours (h)

Introduction

In recent years, considerable importance has been given to the development of new materials for food packaging applications. One of the main objectives in the food industry is to replace the traditional food packaging, designed to protect food against the effects of the environment (e.g. microorganisms, oxygen, off-odours and light), where the key safety objective is for it to be as inert as possible (i.e. there should be a minimum of interaction between the food and the packaging) (Dainelli et al. 2008) by multifunctional packaging (e.g. active and smart packaging) (Han 2005; Han et al. 2005; Rooney 2005). The most innovative developments in the area of food packaging propose a multifunctional packaging based on the interaction between the package materials, the food and the environment, leading to a dynamic role in food preservation and quality and consumers safety (Brody et al. 2008; Dainelli et al. 2008). This group of multifunctional packaging can comprise two types of packaging: (a) active packaging with the purpose of extending the shelf life of food whilst maintaining (or even improving) its quality (Vermeiren et al. 1999) and (b) smart packaging which responds to environmental conditions (e.g. temperature and pH) increasing food protection (e.g. by releasing an antimicrobial) or alerting the consumers to contamination and/or the presence of pathogens (Sekhon 2010).

Natamycin (pimaricin) is a polyene antifungal compound produced by Streptomyces natalensis and related species. It was approved as Generally Recognised As Safe (GRAS) by the Food and Drug Administration (FDA) in the USA and as a natural preservative in the European Union (E235). It is largely used in the food industry as a preservative on the surface of cheese, fruit and other non-sterile products, such as meat and sausages (Farid et al. 2000; Vanden Bossche et al. 2003). However, natamycin presents some technological problems associated with its use as food preservative: low solubility in water and low chemical stability in acidic conditions and under light radiation (Koontz et al. 2003; Stark and Tan 2003).

In previous works (Fuciños et al. 2012; Fuciños et al. 2014b), the limitations of active packaging systems for food applications have been addressed. Most of these active packaging systems include passive diffusion systems (Türe et al.

2009; Fajardo et al. 2010; Hanušová et al. 2010; Bierhalz et al. 2012) in which an antimicrobial compound release is controlled by the concentration difference between the food, the surrounding medium and packaging materials, thus resulting in an uncontrolled release of antimicrobial compounds. In Fuciños et al. (2012, 2014b), the use of smart poly(*N*-isopropylacrylamide) (PNIPA) nanohydrogels has been proposed as an alternative to passive diffusion mechanism, which allows a controlled release of bioactive molecules (i.e. natamycin) as a response to environmental changes (temperature and/or pH). Thus, the amount of preservative in the food could be reduced. These smart PNIPA nanohydrogels have a hydrophilic/hydrophobic balance in the side chains that allows the formation of hydrogen bonds between water molecules and hydrophilic groups of the hydrogel. Below its lower critical solution temperature (LCST), nanohydrogels remain soluble and swelled and, in this state, can be loaded with preservative. The value of LCST for PNIPA polymers is around 33 °C (Schild 1992; Zhang et al. 2002; Schmaljohann 2006). When the temperature increases above the LCST, the hydrophobic groups are strengthened dramatically leading to the collapse of the polymer chains (Schild 1992; Zhang et al. 2002; Cordeiro et al. 2009; Fuciños et al. 2014a), and the release of the preservative occurs. By varying the composition of PNIPA nanohydrogels, it is possible to easily modulate the polymer collapse pattern. Fuciños et al. (2014a) showed how the copolymerisation of PNIPA with acrylic acid (AA) imparts different structural properties to nanohydrogels affecting the particle size and swelling capacity. In the same manner, natamycin release kinetics were very different, depending on the nanohydrogel composition (Fuciños et al. 2014b). PNIPA nanohydrogel copolymerised with AA allowed a slower but more continuous release of natamycin. Thus, the antimicrobial efficiency of natamycin was improved when encapsulated in PNIPA/AA nanohydrogels due to the protection that the nanohydrogel gives against environmental degradation whilst also lowering the presence of preservatives in food bulk (Fuciños et al. 2012). But to apply them to the food surface, a carrier or support of loaded nanoparticles such as an edible film or a coating may be helpful.

Considering food packaging as one of the main potential applications of these smart nanohydrogels, in Cerqueira et al. (2014), natamycin-loaded nanohydrogels were successfully incorporated into polysaccharide-based films without influencing their main physical properties. So it seems that this packaging system could allow improving by, on the one hand, applying active agents onto the food surface by film formation and, on the other hand, by achieving a controlled release of the preservative only when it is necessary, due to nanohydrogel behaviour under temperature changes.

In this work, natamycin release kinetics from nanohydrogels incorporated into polysaccharide-based films (GA-PNIPA/AA)

were evaluated, under different temperatures and media, in free state or when encapsulated in nanohydrogels. Additionally, the antimicrobial efficiency of these new active and smart films was assessed in a food model system.

Materials and Methods

Preparation of Natamycin-Loaded PNIPA/AA Nanohydrogels

The synthesis of PNIPA/AA nanohydrogels and the preparation of natamycin-loaded PNIPA/AA nanohydrogels were performed as presented by Fuciños et al. (2012). For the synthesis of nanohydrogels, a water in oil (W/O) microemulsion formed by 58 % (*w/w*) of aqueous phase (AP—80 %, *w/w*, water and 20 %, *w/w*, monomer), 17 % (*w/w*) of oil phase (OP—isoparaffinic synthetic hydrocarbon: Isopar™, 98 %, *v/v*, Quimidroga, S.A., Barcelona, Spain) and 25 % (*w/w*) of surfactant (ST—PEG-40 sorbitol hexaoleate: Atlas™ G-1086 and sorbitan sesquiolate: Span™ 83, Croda Ibérica, S.A., Barcelona, Spain) was prepared. For pure PNIPA nanohydrogels (PNIPA(5)), the ratio (*w/w*) of *N*-isopropylacrylamide (NIPA, 99 %, *w/w*, Acros Organics, Geel, Belgium) based on the monomers ($m_{NIPA}/m_{monomer}$) was 1. For NIPA copolymerised with AA (stabilised with hydroquinone monomethyl ether, for synthesis, Merck, Darmstadt, Germany) (PNIPA-20AA(5)) nanohydrogels, $m_{NIPA}/m_{monomer}$ was 0.80 and $m_{AA}/m_{monomer}$ was 0.20. To preserve the shape and size of the particles during handling, we used the *N,N′*-methylenebisacrylamide (NMBA) crosslinking agent for synthesis (Merck, Darmstadt, Germany) at a ratio $m_{NMBA}/m_{monomer}=0.05$.

The phases (AP and OP) were solubilised and mixed in a 100-mL reactor equipped with mechanical stirring and thermostatised at 25 °C. The reaction medium was purged by bubbling nitrogen to eliminate oxygen. Polymerisation was triggered by adding the initiator (NaHSO₃ for analysis, Merck, Darmstadt, Germany) at a ratio $m_{NaHSO3}/m_{monomer}=0.01$ and monitored by the temperature increase inside the glass reactor. Chloroform (Panreac, Barcelona, Spain) was used to selectively precipitate inverse micelles formed during microemulsion, which contain synthesised nanoparticles. This mixture was poured over diethyl ether (Panreac, Barcelona, Spain) to dissolve and remove any residual impurities. The pure polymer was separated from diethyl ether by decantation and then it was dried overnight in an oven (50 °C) and then ground in a colloid mill (IKA-Werke GmbH & Co. KG, Staufen, Germany).

Natamycin-loaded PNIPA/AA nanohydrogels were prepared by dispersing, with agitation, the PNIPA/AA 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 (50 % (w/w) lactose, VGP Pharmachem, Barcelona, Spain) water solution to obtain final concentrations of 12.5 mg mL^{-1} of nanohydrogel and 0.4 mg of commercial natamycin powder per mL (21.93 μg mL^{-1} of solubilised pure natamycin). The mixture was then stirred for 12 h at 25 °C to guarantee the incorporation of natamycin into the nanohydrogel particles (Fuciños et al. 2014b).

Preparation of Natamycin-Loaded PNIPA/AA Nanohydrogels Incorporated into κ-Carrageenan and Locust Bean Gum (GA) Films

Polysaccharide film-forming solutions were prepared as described in Cerqueira et al. (2014). Briefly, 0.4 and 0.6 % (w/v) of κ-carrageenan (Gelcarin DX5253, FMC Biopolymer, Norway) and locust bean gum (Genu gum type RL-200, CP Kelco, USA), respectively, were suspended in distilled water under agitation during 1 h at 25 °C, after which 0.3 % (w/v) of glycerol (87 %, v/v, Panreac, Spain) was 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/AA nanohydrogels were added after the decrease of the temperature to 30 °C in order to avoid the nanohydrogel collapse.

Two different concentrations of free natamycin were evaluated: 500 μg mL^{-1}, based on the published work by Fajardo et al. (2010), and 21.93 μg mL^{-1}, based on the concentration of solubilised pure natamycin charged into the nanohydrogels. Based on these concentrations and assuming that natamycin was distributed uniformly in the film, the final amount of natamycin in the films (with 35 mm of diameter) was estimated to be 564.24 and 7.92 μg, respectively (films prepared with free natamycin). These films are mentioned as GA+NA(564) and GA+NA(8), respectively. Natamycin-loaded nanohydrogels, prepared as explained in the section "Preparation of Natamycin-Loaded PNIPA/AA Nanohydrogels", were added to films to a final concentration of 0.2 % (v/v). The final amount of natamycin in each film with 35 mm of diameter was estimated to be 7.92 μg. These films are mentioned as GA-PNIPA(5)+NA(8) and GA-PNIPA-20AA(5)+NA(8) for the two different nanohydrogels used in this work.

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, resulting in films with 0.045±0.002 mm of thickness. In the same manner, films without the incorporation of any compound were prepared and used as control and mentioned as GA films. All films were conditioned in desiccators containing a saturated solution of Mg(NO$_3$)$_2$·6H$_2$O at 53 % relative humidity (RH) and 20 °C.

Natamycin Release from PNIPA/AA Nanohydrogels Incorporated into GA Films

Liquid Medium

The procedure for studying the natamycin release from nanohydrogels in a liquid medium was based on that described in Fuciños et al. (2012). GA films with 35 mm diameter submerged in 3 mL of distilled water were placed in a dialysis membrane (MWCO 3500 g mol^{-1}, SnakeSkin^{TM}, Pierce, Rockford, IL, USA) and dialyzed against 12 mL of distilled water at 10 and 37 °C, with constant stirring. Samples of 1 mL were taken at intervals from the solution in order to determine the natamycin concentration. The volume removed from the probe was returned after measurements had been made in order to maintain a constant volume. Two replicates of each film were evaluated for each temperature.

In order to evaluate natamycin availability in the liquid medium, a control experiment was performed, quantifying natamycin amount in distilled water, initially added with an aqueous solutions of commercial natamycin (with the same amounts of natamycin employed to be incorporated into the films), and incubated at 10 and 37 °C (below and above, respectively, the LCST of nanohydrogel to allow its swollen and collapse, respectively). Aliquots were removed at different time intervals and natamycin amount was determined in the same way as natamycin released from films in the liquid medium (see "In Distilled Water" section). Three replicates were evaluated for each temperature.

Solid Medium

GA films with 35 mm diameter were placed on Petri dishes having the same diameter, which contained 2 mL of agar (bacteriological European type, Cultimed, Panreac, Barcelona, Spain) (12 mg mL^{-1}). Then, Petri dishes were incubated at two different temperatures, 5 and 37 °C, and 55 % RH. At each sampling time, films were removed from the agar surface using a clamp. Agar samples were then sliced, frozen and then lyophilised to extract and quantify the natamycin released from GA films as explained in the section "In Agar Plates". Four replicates of each film were evaluated for each temperature.

In the same way as in the liquid medium, a control experiment was performed quantifying natamycin availability in agar plates initially added with an aqueous solution of commercial natamycin to obtain final concentrations in agar from 0.1 to 2.5 μg mL^{-1}, and incubated at 5 and 37 °C and 55 % RH. Natamycin was extracted and quantified at different time intervals in the same way as natamycin released from films in the solid medium (see "In Agar Plates" section). Three replicates were evaluated for each temperature.

Natamycin Quantification

In Distilled Water

The natamycin concentration in the dialysate (distilled water) was determined spectrophotometrically at 319 nm (Beckman Coulter Inc., Brea, CA, USA) as described in Fuciños et al. (2012) by the molar absorption coefficient (ε) calculated for natamycin (0.077 ± 0.006 mL μg^{-1} cm^{-1}).

In Agar Plates

Samples with 2 mL of agar media containing the natamycin released from GA films were sliced, frozen and then lyophilised. After lyophilisation, samples were embedded in 2 mL of methanol (CHROMASOLV® for HPLC, ≥99.9 %, v/v, Sigma-Aldrich, Munich, Germany) during 1 h under agitation at 25 °C in order to extract the natamycin without any additional dilution. Then, samples were filtered (0.45 μm) in order to remove any remaining agar. In these extracts, natamycin was quantified by reverse phase high-performance liquid chromatography (RP-HPLC), following the method of Roberts et al. (2011), with some modifications. RP-HPLC was performed using an Agilent 1200 system (Agilent Technologies, Palo Alto, CA, USA). Separation was performed employing an ACE® C18 column (4.6 mm× 150 mm, 5 μm) with the column oven kept at 30 °C. The flow rate was 1.0 mL min^{-1}, the injection volume was 20 μL and the detection wavelength was 319 nm. Mobile phase A consisted in Milli-Q water/acetic acid (puriss., *p.a. ACS* reagent, ≥99.8 %, v/v, Sigma-Aldrich, Munich, Germany) (97:3, v/v) and mobile phase B consisted of methanol/acetic acid (97:3, v/v). Mobile phase A was maintained at 90 % (v/v) for the first 6 min and then decreased linearly to 10 % (v/v) over 25 min. Finally, mobile phase A was increased to 90 % (v/v) and the column was re-equilibrated for a further 9 min.

The accuracy of natamycin extraction and quantification by RP-HPLC was determined by adding 2 mL agar samples with 100 μL of a pure natamycin (≥95 %, w/w, HPLC, Sigma-Aldrich, Munich, Germany) solution to obtain final concentrations in agar from 0.1 to 2.5 μg mL^{-1}. Table 1 shows the natamycin rate recovery for each concentration tested.

Table 1 Accuracy of the natamycin extraction and quantification by RP-HPLC

Natamycin concentration in agar plates (μg mL^{-1})	% Accuracy
2.50	78.80±2.83
1.00	79.00±1.41
0.50	96.00±11.31
0.25	120.00±11.31
0.10	207.00±18.38

The results of natamycin extraction and detection, shown in Table 1, are acceptable for natamycin concentrations above 0.25 μg mL^{-1}. This detection limit (DL) was considered sufficient to address the natamycin release assays from the films with PNIPA/AA nanohydrogels, since minimum inhibitory concentrations (MIC) of natamycin are usually between 1 and 10 μg mL^{-1} or even higher for some moulds that, in general, are less sensitive than yeasts (Stark and Tan 2003; Delves-Broughton et al. 2010).

Microbial Assay

Strains and Growth Conditions

Stock cultures of *Penicillium commune* (P 1.01) were grown on potato dextrose agar (PDA, specific for cultivation of fungi, Difco™, Detroit, MI, USA) acidified to pH 3.5 with 10 % (w/v) L(+)-tartaric acid (Panreac, Barcelona, Spain) at 25 °C and maintained on slants of this media at 4 °C. The inoculum of this strain was prepared as explained in Fajardo et al. (2010) by overlaying mature slants with sterile Tween 80, 0.05 % (w/v), and gently scraping the surface with a Drigalski spatula. The mould culture suspensions were filtered through sterile gauze pads to remove the mycelium, and spore count was determined using a Neubauer chamber. Necessary dilutions were made with Tween 80, 0.05 % (w/v), to obtain 10^{6} spores per mL counts.

Stock cultures of *Saccharomyces cerevisiae* (Sc 1.02) were grown in YPD medium (yeast extract 10 g L^{-1}; peptone 20 g L^{-1}; D(+)-glucose 20 g L^{-1}, all from Panreac, Barcelona, Spain) at 25 °C and maintained at −40 °C in the same media, supplemented with 15 % (w/w) glycerol (87 %, v/v, Panreac, Spain). The inoculum was prepared with a 12-h culture after three steps, containing 26×10^{6} colony-forming units (CFU)mL^{-1}. Counts were determined by standard plate count with PDA acidified to pH 3.5 with 10 % (w/v) L(+)-tartaric acid.

Inhibition Studies Employing a Food Model System

Following the procedure described by Fuciños et al. (2012), a food model system consisting of Petri dishes with 5 mL of PDA acidified (pH=3) with 10 % (w/v) L(+)-tartaric acid and plate storage under fluorescent lighting was used, in order to simulate unfavourable conditions for the chemical stability of natamycin. Three replicates of each film, prepared as explained in "Preparation of Natamycin-Loaded PNIPA/AA Nanohydrogels Incorporated into κ-Carrageenan and Locust Bean Gum (GA) Films", were evaluated.

P. commune (P 1.01) and *S. cerevisiae* (Sc 1.02) were used as indicator microorganisms. An aliquot of 25 μL of a 12-h culture of Sc 1.02 (26×10^{6} CFU mL^{-1}) and 10 μL of P 1.01 (26×10^{6} spores mL^{-1}) was inoculated in triplicate into the

Petri dishes. After 1 h in the fridge to allow the inoculated culture to be absorbed properly, films (GA, GA+NA(564), GA+NA(8), GA-PNIPA(5)+NA(8) and GA-PNIPA-20AA(5)+NA(8)) were placed covering the entire surface of the Petri dishes. All plates were incubated at 5 °C for 3 days and then at 37 °C, until the end of the experiment (8 days).

Data Fitting and Statistical Analyses

Student's t test ($\alpha=0.05$) was used to analyse differences between two samples for a single variable. One-way analysis of variance (ANOVA) followed by Tukey post-tests was performed for multiple comparisons. Both analyses were performed by using GraphPad Prism™ 5 (GraphPad Software Inc., San Diego, CA, USA).

Plotting was also performed by using GraphPad Prism™ 5. Data fitting, parametric estimation and significance tests, both for parameters and models, were performed with SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA, USA).

Results and Discussion

Natamycin Release from PNIPA/AA Nanohydrogels Incorporated into GA Films

Initially, natamycin diffusion from films to a liquid (distilled water) or solid medium (agar) was evaluated to determine the maximum release efficiency of the active compound from the system according to the application.

Liquid Medium

Taking into account that the natamycin release, during the dialysis process, will stop when the equilibrium concentration is reached, not all the natamycin loaded into the films could be recovered in the dialysis medium. That is why it has been considered that the maximum amount of natamycin released from the films is the amount of natamycin released in dialysis experiments carried out with free natamycin (Fuciños et al. 2014b). The relative fraction of natamycin released (γF_t) is defined by the following expression:

$$\gamma F_t = \frac{C_{t\ \text{GA-NA}}}{C_{\text{max NA}}} \tag{1}$$

where $C_{t\,\text{GA-NA}}$ is the amount of natamycin (µg) released from the film to the dialysis medium (passing through the dialysis membrane) and $C_{\text{max NA}}$ is the amount of natamycin (µg) under the steady-state conditions determined in the dialysis experiments with free natamycin (without film) for a given temperature. The amount of natamycin released through the dialysis membrane was not affected by temperature (data not

shown) and was calculated with the equation developed in Fuciños et al. (2014b):

$$C_t = C_{\text{max}} + (C_0 - C_{\text{max}})e^{-m\left(1+\sum_{i=1}^{k}\beta_i \cdot X_i\right)t} \tag{2}$$

where C_t is the concentration of the natamycin released (µg mL^{-1}) at time t (min), C_0 is the initial natamycin concentration in the medium (µg mL^{-1}), C_{max} is the maximum achievable concentration of the released natamycin (µg mL^{-1}), m is the maximum achievable release rate of natamycin (min^{-1}), k is the number of variables involved in a particular release system, β_i is the coefficient which influences the m and reflects the linear effect and X_i represents the independent variable (temperature).

Figure 1 shows the natamycin release profiles obtained from GA films. The two most characteristic features of the natamycin release from GA films containing PNIPA/AA nanohydrogels (GA-PNIPA(5) and GA-PNIPA-20AA(5)) were that the release was controlled by the temperature and the kinetic shape was not hyperbolic, as expected from a simple diffusion process such as that observed for GA-NA cases (Fig. 1a, b). The lag time in Fig. 1c, d corresponds to the beginning of natamycin release. In addition, it seems that the temperature influences natamycin release from GA films, which increased when natamycin was incorporated into GA films by encapsulation into PNIPA/AA nanohydrogels (explained by the nanohydrogel collapse when the temperature rises above its lower critical solution temperature). This restricts natamycin release when the temperature is increased, i.e. when there is a risk of contamination/growth of microorganisms that cause food spoilage or the development of pathogenic microorganisms.

For a better analysis, experimental data were fitted with the reparametrised logistic model proposed by Vázquez et al. (2008) to make explicit the lag time:

$$\gamma F_t = \frac{\gamma F_{\text{max}}}{1 + e^{2 + \frac{4 \cdot v_{\text{max}}}{\gamma F_{\text{max}}}(\lambda - t)}} \tag{3}$$

where γF_{max} is the maximum relative fraction of natamycin released from the GA films through the dialysis membrane, v_{max} is the maximum release rate (h^{-1}) and λ and t are the lag time for the natamycin release and the sampling time, respectively, both expressed in hours (h).

Equation 3 provided a good fit of the experimental data and the parameters generated are shown in Table 2. Natamycin release from GA films when it was not nanoencapsulated (GA-NA(564)) appears to be governed only by molecular diffusion and by the polymer relaxation, as presented by Fajardo et al. (2010), and both phenomena will be influenced by temperature increase. The early time release data (~60 %) of the natamycin release from the GA films studied were also fitted with the

Fig. 1 Variation of the relative fraction of natamycin released (γF) with time from GA films loaded with free natamycin: GA+NA(564) and GA+NA(8) (**a** and **b**, respectively); and natamycin encapsulated into PNIPA/AA nanohydrogels: GA-PNIPA(5)+NA(8) and GA-PNIPA-20AA(5)+NA(8) (**c, d**), through a 3500-g·mol^{-1} cut-off dialysis membrane in distilled water at 10 °C (*empty circle*) and 37 °C (*filled circle*). The *lines* are the fitting curves generated from Eq. 3
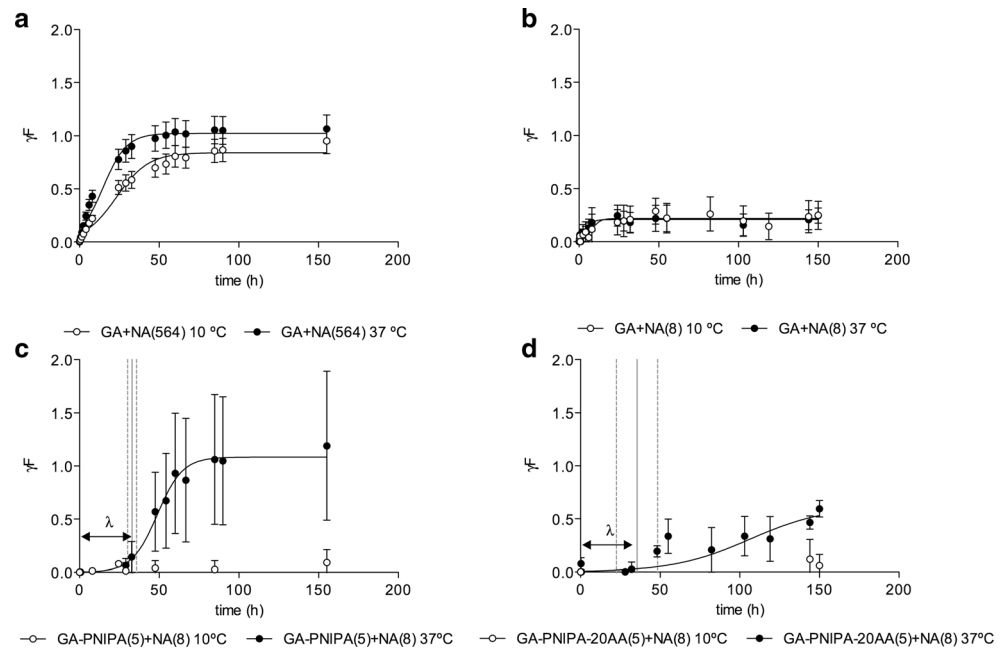
following well-known mathematical model (Ahuja et al. 2007; Blanco et al. 2008; Fuciños et al. 2014b):

$$\gamma F_t = k \cdot t^n \tag{4}$$

where γF_t is the relative fraction of natamycin released at time t, k is a release kinetic constant that depends on the biomolecule-polymer system characteristics and n is the diffusional exponent that depends on both the geometry of the delivery system and the physical mechanisms involved (Ritger and Peppas 1987). Thus, according to the n value, the release from a thin film can be catalogued into one of the following diffusion mechanisms (Siepmann and Siepmann 2008):

– $n=0.5$: Fickian diffusion (Case I). Biomolecule release is controlled only by diffusion into the release medium.

– $n=1$: Case II transport. Biomolecule release is controlled by the polymer swelling and relaxation mechanism.

– $0.5<n<1$: Non-Fickian transport or anomalous transport. Biomolecule release is controlled by both mechanisms.

The values of diffusion exponent n were 0.80 ± 0.04 and 0.66 ± 0.04 for natamycin release from GA film (GA-NA(564)) at 10 and 37 °C, respectively, indicating that natamycin release from GA films was governed by polymer relaxation and natamycin diffusion into the release medium. The differences between these values are not significant ($p>0.05$). However, due to the increase of the polymer relaxation rate with increasing temperatures (Bourbon et al. 2010), the natamycin release is favoured when temperature increases from 10 to 37 °C, showing significant ($p<0.05$) differences in the values of v_{max} and γF_{max} (Table 2).

Table 2 Natamycin release parameters from different GA films and temperatures, calculated with Eq. 3, in liquid medium

Film	T (°C)	γF_{max}	v_{max} (h^{-1})	λ (h)	r^2
GA-NA(564)	10	0.841 ± 0.027a, α	0.021 ± 0.002a, α	2.941 ± 2.326n.s., a, α	0.9716
	37	1.024 ± 0.028b, α	0.035 ± 0.004b, α	-0.032 ± 1.576n.s., a, α	0.9712
GA+NA(8)	10	0.218 ± 0.014a, β	0.021 ± 0.011n.s., a, α	2.735 ± 2.048n.s., a, α	0.8348
	37	0.209 ± 0.011a, β	0.023 ± 0.006a, α	-0.145 ± 1.093n.s., a, α	0.8765
GA-PNIPA(5)+NA(8)	10	–	–	–	–
	37	$1.084\pm0.040\alpha$	$0.034\pm0.005\alpha$	$32.914\pm2.797\beta$	0.9798
GA-PNIPA-20AA(5)+NA(8)	10	–	–	–	–
	37	$0.582\pm0.142\gamma$	$0.005\pm0.001\beta$	$35.438\pm12.942\beta$	0.8364

Values reported are the means±standard deviation ($n=2$). a, b: statistically significant differences ($p<0.05$) between different temperatures for the same film; α, β, γ: statistically significant differences ($p<0.05$) between different films for the same temperature

n.s. parameter not significant ($p>0.05$)

Unlike GA-NA(564) films, when the concentration of natamycin was reduced to the same values as in the films with nanohydrogels (GA+NA(8)), it was not possible to extract all the natamycin loaded. This could be explained by a fixed amount of natamycin that always remains trapped in the films, for all the tested natamycin concentrations (Fig. 2). Contrary, if the initial amount of natamycin is very high, the fraction of natamycin remaining entrapped is imperceptible. Around 75 % (w/w) of the natamycin initially charged remained trapped in the GA+ NA(8) film. However, this amount only accounts for around 1 % (w/w) of the natamycin charged into the GA+NA(564) film.

The values of the diffusion exponent n for GA+NA(8) film were 0.75±0.34 and 0.62±0.23, at 10 and 37 °C, respectively. In the same manner as for the GA+NA(564) films, there were no significant ($p > 0.05$) differences between the n values at both temperatures assayed, showing that natamycin release from the films was governed by the polymer relaxation and natamycin diffusion into the release medium.
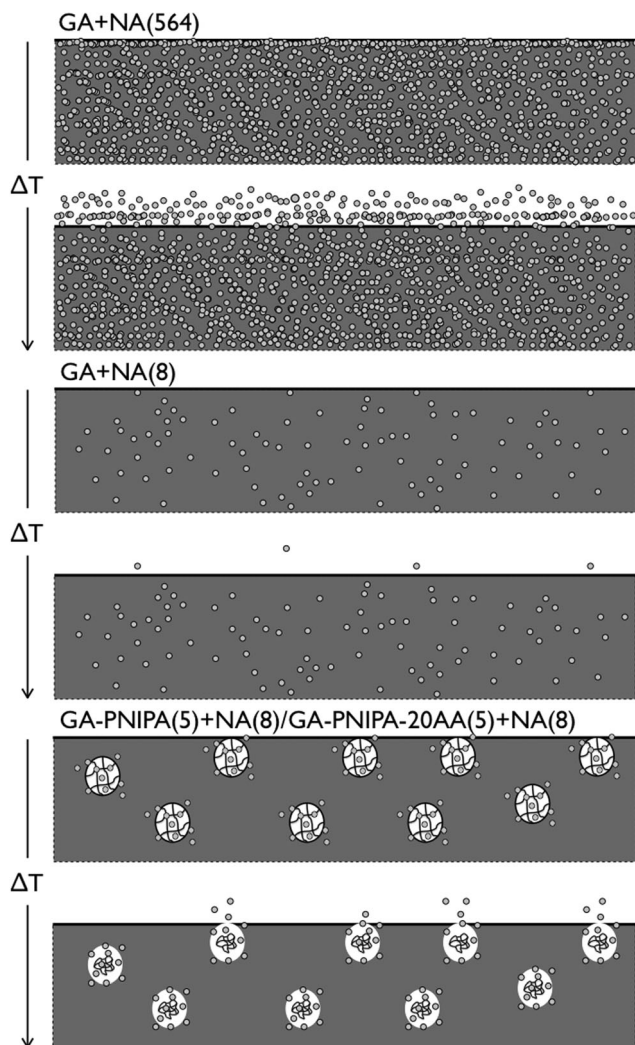

Fig. 2 Scheme of hypothetical behaviours to natamycin release, from different polymeric matrix, after a temperature rise

As mentioned above, the effect of temperature in natamycin release is more obvious when the natamycin is loaded into PNIPA/AA nanohydrogels and then incorporated into GA films. At 10 °C, the release practically did not occur for both nanohydrogels assayed (Fig. 1), and for that reason, it was not possible to fit the experimental data (Table 2). The release was increased when the temperature was raised to 37 °C. These results are consistent with PNIPA behaviour, widely described in literature (Zhang et al. 2002; Huang and Lowe 2005; Fuciños et al. 2014a), where it is shown that when the temperature increases above the LCST, at ca. 33 °C, hydrophobic interactions amongst PNIPA chains strengthen dramatically, leading to the collapse of the hydrogel and, consequently, to the release of the biomolecule.

The γF_{max} value for the natamycin release at 37 °C from GA-PNIPA(5)+NA(8) films was approximately 2-fold ($p < 0.05$) the value for the natamycin release from GA-PNIPA-20AA(5)+NA(8) films (Table 2). In addition, the v_{max} value was statistically different ($p < 0.05$) for both films with nanohydrogels. However, the v_{max} value for the GA-PNIPA(5)+NA(8) films was not statistically different ($p < 0.05$) from films with free natamycin (i.e. GA-NA(564) and GA+NA(8)) (Table 2). This could be explained by the natamycin location near the surface or on the surface of nanohydrogel particles in the case of the nanohydrogel without AA (PNIPA(5)); when the collapse occurs at 37 °C, a fast release of natamycin occurs, leading to the burst effect (Fig. 1c). In contrast, natamycin could be more included into the most hydrophilic nanohydrogel matrix (PNIPA-20AA(5)). This behaviour is consistent with the results shown in Fuciños et al. (2012, 2014b), where it has been shown that when the nanoparticle collapses (with the increase of temperature), natamycin is released from inside the polymer matrix at a slower rate than when it remained near the polymer surface, justifying the slower and continuous release of natamycin from GA-PNIPA-20AA(5) films. The intense collapse, which occurred in GA-PNIPA(5) films when temperature increased, resulted in a rapid increase of natamycin release leading to a higher γF_{max} value (Table 2) than that obtained with GA-PNIPA-20AA(5) films, despite the release having stopped earlier (Fig. 1).

As explained before, there is a clear difference between the kinetic release of natamycin from GA films with and without nanohydrogels (Fig. 1). This fact was reflected in the statistical ($p < 0.05$) differences between their lag times (Table 2), increasing from values close to 0 h (i.e. the natamycin release began immediately) to values around 35 h when natamycin is added to the films loaded in the nanohydrogels. There were no significant differences between the lag time (λ) with GA+ NA(564) and GA+NA(8), and also there were no significant ($p > 0.05$) differences between the values of λ with GA-PNIPA(5)+NA(8) and GA-PNIPA-20AA(5)+NA(8). In Fuciños et al. (2014a), results showed that in a basic release

medium (pH>pK_a of AA), it is difficult to find differences between the LCST value for the nanohydrogels with and without AA due to the presence of ionised –COO⁻ groups, which prevent the collapse of nanohydrogels with AA. So, it is not strange that natamycin release due to the collapse of the nanohydrogel begins at the same time for both nanohydrogels, with and without AA, despite the more hydrophilic nanohydrogel collapse being less intense.

Finally, it is important to mention that the encapsulation of natamycin into nanohydrogels to be included into the GA films seems to help natamycin to be released when it was included in low quantities, since γF_{max} with GA-PNIPA(5)+NA(8) and GA-PNIPA-20AA(5)+NA(8) was significantly ($p<0.05$) higher than γF_{max} with GA+NA(8), at 37 °C. This behaviour can be explained by the scheme presented in Fig. 2, where the location of nanohydrogel particles near the film surface could favour natamycin release from films. Scanning electron microscopy (SEM) images of these films containing nanohydrogels, shown in a previous work (Cerqueira et al. 2014), seem to confirm the location of nanoparticles at the surface of films. So the encapsulation of natamycin into nanohydrogels (GA-PNIPA(5)+NA(8) and GA-PNIPA-20AA(5)+NA(8)) allows for a more concentrated presence of natamycin at certain points of the matrix (i.e. surface) compared to natamycin incorporated alone into the films (GA+NA(8)), facilitating natamycin diffusion from film matrix.

Solid Medium

In order to evaluate the amount of natamycin released from GA films with a different composition into a solid medium, i.e. agar, the fraction of natamycin released (F_t) was calculated as the amount of natamycin extracted from the agar samples (as explained in "In Agar Plates"), in relation with the total amount of natamycin initially added to the film. Table 3 shows the F_t values from different GA films and temperatures.

Lower fractions of natamycin were released to agar plates as the diffusion process in a solid medium is indeed far more complex than in a simple liquid medium. However, the release study in this matrix could provide a more realistic evaluation of the diffusion process in a food matrix.

In the same manner as in the liquid medium, the natamycin release from GA-NA(564) films increased and stabilised quickly for both temperatures (Table 3). However, in general, the F_t values in the solid medium were statistically ($p<0.05$) lower when temperature is increased to 37 °C, so it seems that natamycin release from these films was lower at higher temperatures, contrary to the results observed in a liquid medium. The behaviour was similar for the natamycin release from GA+NA(8) films; however, the differences are not statistically significant ($p>0.05$). In general, as in the liquid medium, lower F_t values were obtained from this film compared with those F_t

values obtained with GA films initially loaded with high amounts of natamycin (Table 3).

Regarding natamycin release from PNIPA/AA nanohydrogels embedded into GA films, in general, F_t values with GA-PNIPA(5)+NA(8) films were significantly ($p<0.05$) higher than those obtained with GA-PNIPA-20AA(5)+NA(8) films, and as explained for the release kinetics in the liquid medium, this was probably due to a higher exposure of natamycin near the surface of nanohydrogel particles, as was concluded by Fuciños et al. (2012, 2014b). Nevertheless, in contrast to what happened in the liquid medium, it seems that the release only occurred at 5 °C.

A control experiment was performed to check the availability of natamycin, both in distilled water (liquid medium) and agar plates (solid medium), during the storage time at temperatures used in natamycin release experiments. The results obtained (data not shown) indicate that the degradation or entrapment of natamycin in agar is not enough to explain the low natamycin release from films at 37 °C. There were no significant ($p>0.05$) differences of natamycin concentration between samples stored at 10 and 37 °C in water. And although significant ($p<0.05$) differences appeared between samples stored at 10 and 37 °C, differences only appeared over prolonged periods of time, in the experiment of natamycin availability in agar. However, low natamycin values were observed from 1 h of storage at 37 °C (Table 3), in the experiments of natamycin release from films in agar. Therefore, the low water availability in the solid medium was proposed as the main cause of low natamycin release, slowing its diffusion from films. On the one hand, the absence of water reduces polymer relaxation, and therefore, the on/off release mechanism of PNIPA nanohydrogels could be limited; on the other hand, the absence of water at high temperatures could favour the formation of hydrophobic interactions between natamycin and polymer chains, as noted in Fuciños et al. (2014b) to explain a great natamycin entrapment in PNIPA nanohydrogels when the nanoparticles expelled the water contained in them.

Antimicrobial Efficiency of Natamycin-Loaded PNIP A/AA Nanohydrogels Incorporated into GA Films

In Fuciños et al. (2012), the protection capacity of PNIPA/AA nanohydrogels has been demonstrated, which allowed natamycin to remain active under conditions that were unfavourable for its chemical stability, thus improving its antimicrobial effect. That is why, in this work, we also evaluated the antimicrobial effectiveness of GA films containing natamycin-loaded nanohydrogels under the same unfavourable conditions for natamycin stability employed in Fuciños et al. (2012), i.e. acidified PDA plates and fluorescent lighting.

In plates inoculated with the indicator mould (P 1.01), growth appears to be limited by the coating itself,

Table 3 Fraction of natamycin released (F_t) from different GA films and temperatures in solid medium

Storage time (h)	T (°C)	Film			
		GA+NA(564)	GA+NA(8)	GA-PNIPA(5)+NA(8)	GA-PNIPA-20AA(5)+NA(8)
1	5	0.029±0.006a, α	0.025±0.016a, α	0.053±0.015a, β	< DL
	37	0.049±0.011b, α	0.009±0.013a, β	< DL	< DL
2	5	0.068±0.015a, α	0.025±0.029a, α, β	0.071±0.033a, α	< DL
	37	0.038±0.008b, α	0.020±0.023a, α, β	< DL	< DL
4	5	0.045±0.009a, α	0.029±0.035a, α, β	0.058±0.012a, α	< DL
	37	0.049±0.012a, α	0.006±0.011a, β	< DL	< DL
8	5	0.057±0.007a, α	< DL	0.036±0.012a, γ	< DL
	37	0.033±0.003b, α	0.008±0.015a, β	< DL	< DL
22	5	0.069±0.013a, α	0.027±0.053a, α, β	0.040±0.014a, α, β	< DL
	37	0.034±0.009b, α	0.008±0.016a, α	< DL	0.015±0.029a, α
30	5	0.068±0.007a, α	0.102±0.013a, β	0.058±0.018a, α	< DL
	37	0.044±0.010b, α	0.042±0.031b, α	< DL	< DL
46	5	0.060±0.013a, α	0.014±0.028a, β	0.060±0.012a, α	< DL
	37	0.023±0.008b, α	< DL	< DL	< DL
70	5	0.046±0.012a, α	0.015±0.018a, β	< DL	< DL
	37	0.031±0.016a, α	0.008±0.016a, α, β	< DL	< DL

Values reported are the means±standard deviation ($n=4$). a, b: statistically significant differences ($p<0.05$) between different temperatures for the same film; α, β, γ: statistically significant differences ($p<0.05$) between different films for the same temperature

since there is virtually no growth throughout the storage time and there were no noticeable differences between films with the antimicrobial treatments and controls (Fig. 3).

The plates inoculated with the indicator yeast (Sc 1.02) provide more revealing results that are consistent with the results observed in the release experiments into a solid medium (see "Solid Medium"). So, no growth was observed at
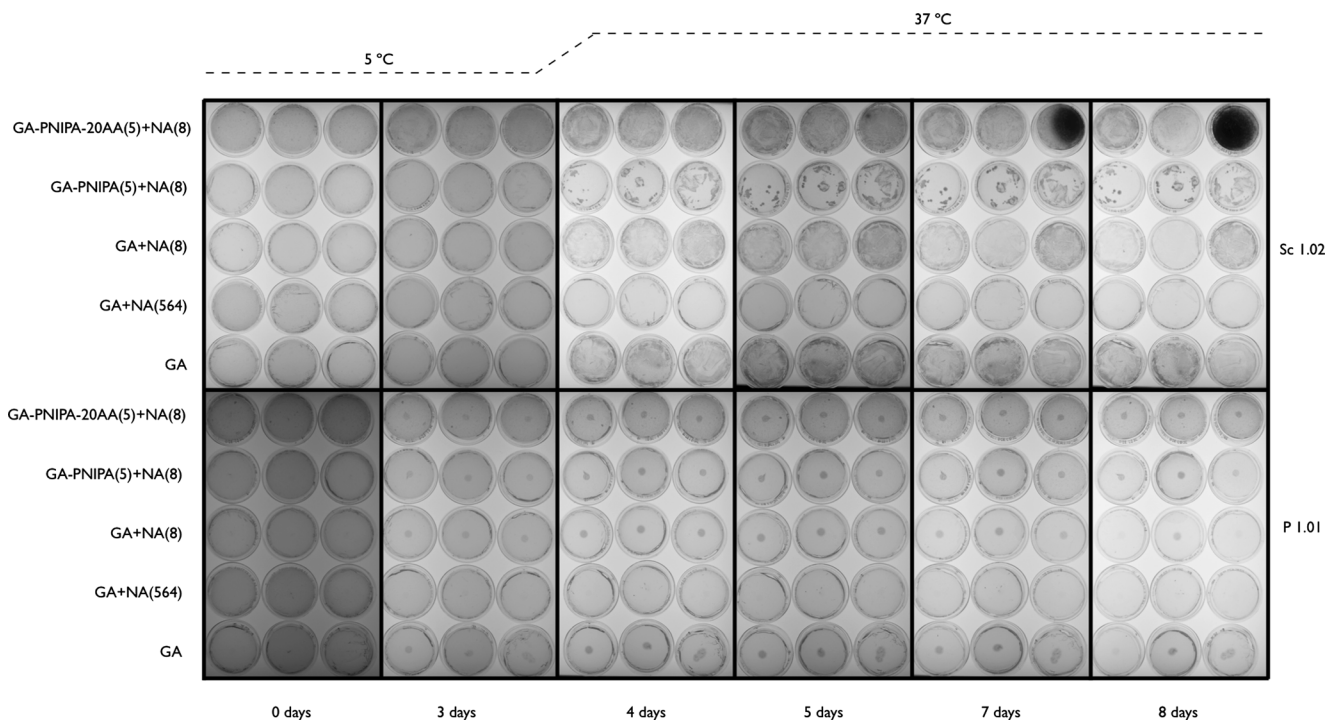

Fig. 3 Inhibition of *Saccharomyces cerevisiae* (Sc 1.02) and *Penicillium commune* (P 1.01) in acidified PDA agar plates by treatment with different GA films, under fluorescent lighting in order to simulate unfavourable conditions for the chemical stability of natamycin

5 °C and growth peaked rapidly when the temperature increased up to 37 °C in control plates treated with GA films without natamycin (GA) (Fig. 3). In plates treated with films that have the highest natamycin content (GA-NA(564)), inhibition was maintained until the end of the assay (8 days). By contrast, higher growth was observed in those plates treated with GA-PNIPA-20AA(5)+NA(8) films, according to the lower natamycin release observed from these films to agar plates (Table 3). Growth observed with the GA-PNIPA(5)+NA(8) and GA+NA(8) films was less intense, when compared with GA-PNIPA-20AA(5)+NA(8) films, especially for the first one (Fig. 3). Results showed plates with large inhibition zones when these were treated with GA-PNIPA(5)+NA(8) films when compared with those plates treated with GA and GA+NA(8) films. These differences could be due to the protection of natamycin against degradation conditions, i.e. under acidic conditions (Stark and Tan 2003) and fluorescent lighting (Koontz et al. 2003), when it was included into the nanohydrogels allowing its release only when the temperature increased (Fuciños et al. 2012). This makes it possible to maintain the inhibition for more days.

Conclusions

In view of the results observed in both systems—liquid and solid medium—it is necessary to continue working to improve the amount of natamycin released from the new smart films where it seems that the PNIPA/AA nanohydrogel swelling/collapse is limited once it was trapped into the GA films. In any case, this system makes it possible to reduce natamycin concentrations in food products whilst improving the antifungal effect in terms of that observed when natamycin was included alone in the GA film, by the same amount. We noted promising results that demonstrate the potential of polysaccharide-based films containing smart nanohydrogels for the controlled release of food preservatives.

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