Carbohydrate Polymers 117 (2015) 140–149

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Development of alginate microspheres as nystatin carriers for oral mucosa drug delivery

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ARTICLE INFO

Article history:
Received 8 June 2014
Received in revised form 22 August 2014
Accepted 1 September 2014
Available online 28 September 2014

Keywords:
Nystatin
Microspheres
Alginate
Chitosan
Buccal delivery

ABSTRACT

To develop more effective antifungal mucoadhesive systems for the treatment of oral candidiasis, three types of microspheres, alginate (AM1), chitosan coated (CCM) and hydrogel (AM2) containing nystatin (Nys) were successfully elaborated by emulsification/internal gelation method. Physicochemical properties of microspheres resulted in 85–135 μm mean sizes, spherical shaped with narrow distribution. Optimal encapsulation efficiency and negative zeta potentials were observed. AM2 showed a consistent decrease in viscosity with increasing shear rate (Herschel–Bulkley). Optimal mucoadhesive properties and swelling behaviour where evidenced. Nys release from AM1 and CCM followed a concentration gradient pattern, contrary AM2 followed a complex release mechanism. All systems exhibited a marked fungicidal activity against *Candida albicans* strains. *In vivo* studies demonstrated that Nys was not found in systemic circulation assuring the safety of the treatment. Nys amounts retained in the mucosa were more than enough to ensure an effective fungicidal action without tissue damage. Based on the obtained results, AM2 could be proposed as the vehicle with the best properties for the buccal vehiculization of Nys.

1. Introduction

Nystatin (Nys) is a polyene antifungal antibiotic, one of the oldest antifungal drug, produced by *Streptomyces noursei* strains (Kaur & Kakkar, 2010). Nys monomers selectively interact with ergosterol causing membrane disruption and eventual cell death (Coutinho & Prieto, 2003). Nys possesses a broad spectrum with both antifungal and fungistatic activity (Recamier, Hernandez-Gomez, Gonzalez-Damian, & Ortega-Blake, 2010) being effective against azole-resistant strains of *Candida* and amphotericin B-resistant strains of *Candida albicans* (Ellepola & Samaranayake, 1999). Nys is indicated for treatment of cutaneous and mucocutaneous fungal infections caused by *Candida* species, the main yeast capable of infecting the oral mucosa, being *C. albicans* the most common species isolated (Campos et al., 2012). Oral candidiasis is not a lethal disease in healthy patients. It is mainly caused by antibiotic or corticosteroid treatment and dental prosthesis. However, it must be treated to avoid chronic and systemic invasions of other tissues (Ship, Vissink, & Challacombe, 2007), especially among patients with diabetes mellitus, immunocompromised or under aggressive treatments (*e.g.* chemotherapy) to prevent opportunistic invasive fungal infections (Pemán & Salavert, 2012).

The presence of a large lactone ring with several double bonds renders it an amphiphilic and amphoteric molecule with poor solubility in aqueous media (360 mg/L at 24 °C), which reveals great formulation challenges (Croy & Kwon, 2004). This fact support that an increasingly important area of pharmaceutical research is focused on finding safe and effective methods of solubilizing poorly soluble drugs (Fernandez-Campos, Clares, Lopez, Alonso, & Calpena, 2013). In this way, micellar gels (Croy & Kwon, 2004), mucoadhesive devices for topical use (Sakeer, Al-Zein, Hassan, Desai, & Nokhodchi, 2010), liposomes (Ng, Wasan, & Lopez-Berestein, 2003), nanoemulsions (Fernandez-Campos et al., 2013), intralipids (Marín-Quintero et al., 2013), niosomes (El-Ridy, Abdelbary, Essam, El-Salam, & Kassem, 2011), microparticles (Martín-Villena et al., 2013), and pellets (Pál, Nagy, Bozó, Kocsis, & Dévay, 2013) have been developed for Nys vehiculization.

Oral cavity for drug delivery has attracted particular attention. It is composed of stratified squamous epithelium (outermost layer), the basement membrane, the lamina propria and the submucosa (innermost layer). Its permeability is estimated to be 4–4000 times greater than skin depending on the region (Galey, Lonsdale, &

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http://dx.doi.org/10.1016/j.carbpol.2014.09.032
0144-8617/

Nacht, 1976). Taking into account the special characteristics of the buccal mucosa, the general features expected of delivery systems for local drug delivery to the oral mucosa include biocompatibility, mucoadhesiveness, stability, non-immunogenicity and capability of sustained drug release to maintain therapeutic levels over an extended period of time (Aduba et al., 2013). The use of mucoadhesive microspheres would meet all those requirements as strategy to the buccal Nys delivery. Most of microspheres are based on natural or synthetic polymeric materials. The natural polymer alginate, an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, has been widely used due to its biodegradable nature with low toxicity, low cost and compatibility with the encapsulation of a wide range of drugs, with minimal use of organic solvents (Martins, Sarmento, Souto, & Ferreira, 2007). Alginate also exhibits optimal mucoadhesive properties to increase the contact time with absorptive sites enhancing the uptake of the encapsulated drug. On the other hand, chitosan, a cationic polysaccharide, produced by the deacetylation of chitin has been utilized as a membrane coating material to improve encapsulation efficiency and enhance stability with also good mucoadhesion capacity (Xiao & Sun, 2013).

Thus based on these considerations, and considering that no studies have been published about buccal Nys microspheres for the treatment of oral candidiasis, the major goal of this study was to get a more effective antifungal mucoadhesive system for the treatment of oral candidiasis. This system will provide a high spectrum of activity and a fungicidal activity against *C. albicans*. In this respect, in the present work we elaborated three types of Nys loaded microspheres. Their physicochemical properties, antimicrobiological effectiveness; *in vitro* release behaviour, and *in vivo* absorption were analysed.

2. Experimental

2.1. Materials

Nys and sodium alginate (65–75% guluronic and 25–35% mannuronic acid, ≈220 kDa) were provided by Fagron Iberica (Terrassa, Spain). Low molecular weight chitosan (≈50 kDa, 75–85% deacetylated) was from Sigma-Aldrich (Madrid, Spain). Calcium carbonate, calcium chloride, acetic acid, HPLC-grade methanol, *N*-dimethylformamide (DMF), acetonitrile and dimethilsulfoxide (DMSO) were obtained from Panreac (Barcelona, Spain). Soya oil, corn starch and Span® 80 were purchased from Guinama S.L.U. (Alboraya, Spain). Double distilled water was obtained from a Milli-Q® Gradinet A10 system apparatus (Millipore Iberica S.A.U.; Madrid, Spain). Polysulphone membranes were purchased from Iberlabo S.A. (Madrid, Spain). Sabouraud dextrose medium from Scharlab S.L. (Barcelona, Spain). Commercially available Nys, Mycostatin® oral suspension (Bristol-Myers Squibb Pharmaceutical Limited) was obtained from a local pharmacy. Phosphate buffered solutions (PBS) were elaborated according to European Pharmacopoeia.

2.2. Synthesis of alginate microspheres and coating procedure

The formulation of the Nys loaded microspheres was based on the emulsification/internal gelation method with modification (Martín-Villena et al., 2013). The W/O emulsion was performed with a sodium alginate aqueous solution, $CaCO_3$ and Nys as the internal phase and vegetable oil as the external phase. Briefly, 0.2 g of $CaCO_3$ were added to 40 mL of 5% (w/v) sodium alginate solution containing 474.55 ± 1.00 mg of Nys, and the resulted system stirred for 15 min at 450 rpm. After homogenization, the suspension was dispersed in 100 mL of vegetable oil (continuous phase) containing 2% (w/v) Span® 80. The mixture was stirred at 700 rpm for 10 min to form W/O emulsion. Under continuous stirring, 20 mL of vegetable oil containing 0.850 mL of glacial acetic acid were added to the W/O emulsion, to get the $CaCO_3$ solubilization. After 10 min under stirring, pregelified microspheres were separated from the oil dispersion by mixing with $CaCl_2$ solution 5% (w/v). The supernatant was discarded. The alginate microspheres (AM1) were centrifuged using an Eppendorf Centrifuge 5804 (Hamburg, Germany) at 1150 × *g* for 10 min, collected and washed using 100 mL distilled water by vacuum filtration and stored at 4 °C in Petri dishes.

Low molecular weight chitosan was selected as coating material for the microspheres at different concentrations. Coating procedures followed the method that had already been previously reported (Liu, Rauth, & Wu, 2007).

Then, 10 g uncoated microspheres (AM1) were immersed in a bath containing 100 mL 0.5% (w/v) chitosan acetic acid solution (1% v/v), and stirred at 300 rpm for 30 min on an orbital shaker for coating. The resulting chitosan-coated microspheres (CCM) were washed with 100 mL distilled water by vacuum filtration and kept at 4 °C. Finally a type of hydrogel microspheres (AM2) was prepared using a modification of the technique above described. 0.12 g of $CaCO_3$ were added to 40 mL sodium alginate solution 1.5% (w/v) containing 474.8 ± 0.30 mg of Nys. The suspension was dispersed in 100 mL vegetable oil containing Span 80® 3% (w/v), 0.4% (v/v) acetic acid under stirring at 760 rpm at room temperature for 10 min. Subsequently, pregelified microspheres were separated from the oil dispersion by mixing with $CaCl_2$ solution 0.08% (w/v). The supernatant was removed and the microspheres were centrifuged at 1150 × *g* for 10 min, collected and washed in 100 mL distilled water. Finally resulting AM2 were stored at 4 °C in Petri dishes.

Each type of microsphere without Nys (unloaded microspheres) was similarly elaborated for comparative studies.

2.3. Physicochemical characterization

2.3.1. Particle size and morphological analysis

Laser diffractometry (LD) was performed using the LS 13 320 laser particle counter (Beckman Coulter Inc., Brea, CA, USA), 0.02–2000 μm size range, yielding the volume distribution of the unloaded and Nys-loaded microspheres. Characterization parameters were the diameters $LD_{0.1}$, $LD_{0.5}$ and $LD_{0.9}$. A diameter $LD_{0.5}$ of 1 μm means that 50% of all particles possess a diameter of 1 μm or less. Equally, mean diameter over the volume distribution ($LD_{4.3}$) and polydispersity expressed as the Span factor were also calculated [$Span = LD_{0.9} - LD_{0.1}/LD_{0.5}$]. All measurements were repeated 24 h after preparation (t_0) and after 1–3 months stored at 4 °C. The shape and surface morphology were also examined by scanning electron microscopy (SEM). The samples were mounted on metal stubs, using double sided adhesive tape, gold coated under vacuum and then examined using a S510 electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

2.3.2. Zeta potential

Zeta potential measurements of diluted microspheres in PBS at 25 °C with a detection angle of 90° with a ZetaSizer® 2000 (Malvern Instruments Ltd., Malvern, UK). Samples were slowly injected through the cell, checking that all air bubbles under the sample port were removed. Nine measurements were done on the same sample at pH 5.5 and 7.5 after elaboration and each storage period at 4 °C.

2.3.3. Determination of percentage yield, loading content and encapsulation efficiency

The percentage yield (PY) was calculated as [PY = (Mass of microspheres/Mass of drug fed + Mass of polymer fed) × 100].

Drug loading content (LC) was calculated as [LC = Mass of Nys in microspheres/Mass of microspheres) × 100]. The encapsulation efficiency (EE) was determined for all samples after the manufacturing process, and repeated after 1–3 months stored in amber glass vials at 4 °C. Total Nys utilized was considered as the sum of encapsulated and non-encapsulated drug. Determination was performed as follows, 0.5 g of AM1 and AM2 at 25 °C was added to phosphate buffer (0.1 M, pH 7); the mixture was liquefied by gently shaking for 30 min at room temperature. 0.5 g of CCM was liquefied in 50 mL sodium citrate solution (0.1 M) by gently shaking at room temperature for 45 min. Thereafter, all samples were filtered through a 0. 22 μm filter and appropriate dilutions were carried out. Samples were analyzed in triplicate by a validated HPLC methodology described below. Equally, for determination of the amount of Nys adsorbed in the microsphere surface, 0.5 g of each type was added to a methanol:DMF:water solution (55:15:30, v/v/v) and maintained at 25.0 ± 0.5 °C under mechanical stirring (50 rpm) for 1 h. Nys remaining in the supernatant (after microsphere centrifugation, 1 h at 12,850 × *g*) was measured in triplicate by HPLC methodology. EE was calculated as [EE = (mass of Nys in microspheres/initial mass of Nys) × 100].

2.3.4. Rheological studies

Rheological properties of microspheres were evaluated at 25 °C using a rotational rheometer HAAKE Rheostress 1 (Thermo Fisher Scientific, Karlsruhe, Germany) equipped with a parallel plate geometry set-up with a fixed lower plate and an upper plate (Haake PP60 Ti, 6 cm diameter), and 0.5 mm separation. Viscosity curves and flow curves were recorded for 3 min during the ramp-up period (0–100 s^{-1}) 1 min at 100 s^{-1} (constant share rate period) and finally 3 min during the ramp-down period (100–0 s^{-1}). Adequate amounts of AM1 and CCM equivalent to recommended dose of Nys were previously diluted in water. Then sufficient amount of sample was applied to the surface of the plate reader following the removal of excess. AM2 measurement was possible without dispersion. All determinations were performed in triplicate. Data from the flow curves (when resulted to be non-Newtonian) were fitted to different mathematical models (Bingham, Ostwald de Wale, Herschel Bulkley, Casson and Cross) based on the correlation coefficient value (r^2) using the software Prism®, v. 3.00 software (GraphPad Software Inc., San Diego, CA, USA).

2.3.5. Evaluation of the swelling behaviour

The water absorbing capacity of microspheres was determined in artificial saliva medium (ASM) by a gravimetric method in triplicate. ASM was composed of sodium carboxymethyl cellulose 10 mg, sorbitol 30 mg, potassium chloride 0.12 mg, sodium chloride 0.84 mg, magnesium chloride hexahydrate 0.05 mg, calcium chloride anhydrous 0.15 mg and potassium dihydrogen phosphate 0.34 mg in 1000 mL of distilled water. The pH was adjusted to 5.5 with a solution of NaOH (2 N).

Briefly, 0.5 g samples of microspheres were placed in different Petri dishes containing 20 mL ASM at 37 °C. At various time intervals up to 6 h, hydrated microspheres were taken out from the incubation medium and filtered. The microspheres were then weighed, and the amount (%) of water uptake was determined [% = $(W_t - W_0/Wt) \times 100$)]; where W_t is the weight of the swollen polymer at a given time and W_0 is the weight in the dry state.

2.3.6. Evaluation of mucoadhesive force

To evaluate the mucoadhesive force, porcine buccal mucosa ($n = 3$) obtained from the *in vivo* studies were transferred from the animal facilities to the laboratory in containers filled with Hank's liquid (Amores et al., 2014). Tissues were then carefully separated from underlying tissue and cut into small pieces of 2.0 cm × 2.0 cm size. Tissue pieces were fixed on two same planks, respectively. One plank was fixed on a stainless steel base; the other was connected with a firm thread which fastened a light plastic beaker through a fixed little crown block. Formulations (1.5 g) were placed at between two pieces of substrates, and then slightly pressed upper plank using hand for 10 s. Next, water was dropped into the beaker at a speed of 1.0 mL/min until the two planks were pulled apart by the gravity of water. The beaker containing water was weighed and the mucoadhesive force (F) was calculated [$F = (W \times g)/A$], where W is the mass of water, g is the acceleration due to gravity and A is the surface area of the applied formulation. The results were then reported as mean values ± S.D. (mN/cm^2) and at least three replicate measurements for each sample were performed.

2.4. Drug release studies

The release studies were performed using vertical Franz diffusion cell (FDC 400, Crown Glass, Somerville, NJ) through polysulphone membranes as previously reported (Martín-Villena et al., 2013). The effective diffusional area was 2.54 cm^2 and 12 mL of receptor compartment capacity which was filled with methanol:DMF:water (55:15:30, v/v/v) what allowed keeping sink conditions in the whole experiment. The system was kept at 37 ± 0.5 °C by circulating water jacket and stirred continuously (600 rpm). Values are reported as the mean ± SD of five replicates. The obtained data were fitted to various kinetic equations (zero order, first order, Higuchi, Weibull and Korsmeyer-Peppas) to find out the mechanism of Nys release from microspheres. A nonlinear least-squares regression was performed using the WinNonLin® Professional edition software, V. 3.3 (Pharsight Corporation, Sunnyvale, CA, USA), and the model parameters calculated. Also the Akaike's Information Criterion (AIC) was determined for each model as it is an indicator of the model's suitability for a given dataset.

2.5. Antimicrobiological efficacy

Antifungal activity of microspheres was tested against the strain *C. albicans* ATCC 10231 obtained from the American Type Culture Collection (Manassas, VA, USA). The yeast strain was grown aerobically in Sabouraud Dextrose medium at 37 °C for 48 h. The inoculums were prepared by suspending colonies in sterile distilled water to achieve the desired density equivalent to the 2 McFarland standard. Growth kinetic of *C. albicans* was compared with the growth curves of the yeast cultured in presence of Nys in the following forms: (i) AM1, (ii) unloaded AM1, (iii) CCM, (iv) unloaded CCM, (v) AM2, (vi) unloaded AM2, commercial Nys suspension (vii), and ASM (viii). An appropriate amount of microspheres was weighted and diluted in sterile ASM at pH 5.5 to obtain a final Nys concentration of 20 μg/mL approximately. Then 10 μL of inoculum were added to 1 mL microspheres suspension in ASM. The resulting yeast loading was 10^6 colony forming units (CFU)/mL. A serial dilution of this first test tube were done in ASM to obtain final concentrations of 10^5, 10^4, 10^3 and 10^2 CFU/mL (dilution 1:10, 1:100, 1:1000 and 1:10000, respectively). This serial dilution allows obtaining countable isolated colonies in the plaque. Samples were incubated for 0, 2, 4, 8, 24 and 48 h at 37 °C under continuous shaking (6 rpm). After each time frame 10 μL of each sample were placed in Sabouraud plates and incubated at 37 °C for 48 h. Finally the colonies were counted by naked eye (at 24 and 48 h). Chloramphenicol was added to Sabouraud plates to prevent bacterial growth and does not affect the yeast growth (Marín-Quintero et al., 2013).

2.6. In vivo studies

The study was approved by the Ethics Committee of Animal Experimentation at the University of Barcelona in line with Helsinki

Declaration of 1975 (1983 revised), and performed under veterinary supervision. Six healthy female crossbred (Landrace × Large White) pigs, each weighing approximately 35 kg, were used. The following anaesthesia protocol was administered: ketamine 10 mg/kg i.m., xylazine 0.5 mg/kg i.m., and an i.v. propofol bolus as anaesthesia inducer; for maintenance isoflurane 5% in O_2 2 L/min was used, as required. Then the pigs were placed on surgery tables.

Adequate amounts of each type of microspheres and the commercially available Nys suspension, equivalent to 30 mg Nys were gently applied on buccal mucosa of one of the cheeks (9 cm^2 each one) with a paintbrush four times a day for two days. This applied dose corresponded to the recommended dosage for treatment of oral candidiasis.

For drug permeation studies, blood samples (2 mL/sample) from each pig were periodically collected from the femoral vein in heparinized tubes, prior to the experiment (control blood samples), and at 0.5, 1 and 1.5 h after treatment. Plasma was obtained by centrifugation. The plasma proteins were precipitated with acetonitrile:water (80:20, v/v) solution. This mixture was centrifuged (Digicen 21, Orto Alresa, Madrid, Spain) at 5000 × *g* for 15 min, the supernatant was filtered through a 0.45 μm filter and stored at −20 °C until Nys analysis by HPLC.

Pigs were sacrificed using an overdose of sodium thiopental anaesthesia at the end of the treatment. The condition of the mucosa was visually assessed in order to check that the treatments did not cause irritation on the tissue. Both cheeks were surgically removed, the treated cheek for analysis and the contra-lateral cheek as control. The tissues were cleaned with gauze soaked in a 0.05% solution of sodium dodecyl sulphate and washed in distilled water. Nys retained in the buccal mucosa was extracted with acetonitrile:water (80:20, v/v) mixture during 20 min under cold sonication in an ultrasound bath; the resulting samples were measured by HPLC. Mucosa permeation parameters were calculated if applicable.

2.7. Histological studies

Epithelial tissues were studied by freeze-substitution transmission electron microscopy (FSTEM). Samples were cut into approximately 2 × 1 mm pieces and fixed in 5% (w/v) glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2. Then, they were post-fixed in 0.2% RuO_4 (w/v) in a sodium cacodylate buffer at pH 6.8 with 0.25% $K_4Fe(CN)_6$ (w/v). After 1 h, the RuO_4 solution was replaced by fresh medium. After rinsing in the buffer, the tissue samples were cryofixed using the high pressure freezing (HPF) device at −196 °C and 2100 bar in a Leica EM Pact device (Leica Microsystems, Vienna, Austria). Afterward, the freeze substitution was carried out in an automatic freeze substitution system (Leica Microsystems, Vienna, Austria). Samples were cryosubstituted at −90 °C for 48 h in 100% methanol, containing 1% OsO_4 (w/v), 0.5% (w/v) uranyl acetate and 3.0% (w/v) glutaraldehyde. After 48 h substitution period, the temperature was raised to −50 °C, and the samples were washed three times in 100% methanol. Subsequently, the methanol solution was replaced gradually by the embedding medium Epon® (100%). This resin was replaced after 24 and 48 h with freshly made embedding medium. Finally, samples were transferred into a mould containing Epon® resin and incubated for 48 h at −50 °C under UVA radiation to facilitate polymerization. Ultra-thin sections were cut by using a Leica Ultracut UCT microtome, transferred to Formvar-coated grids, and examined in a JEOL 100 transmission electron microscope (Jeol, Tokyo, Japan).

2.8. HPLC analysis

All HPLC assays were performed isocratically at room temperature. The HPLC system consisted of a Waters 515 pump (Waters, Milford, MA, USA) with UV–vis 2487 detector (Waters, Milford, MA, USA) set at 305 nm. A reverse-phase column (Kromasil 100, 5 μm, 15 × 0.46 cm) with a flow rate of 0.8 mL/min was used. The mobile phase consisted of a mixture of 1% acetic glacial acid, acetonitrile:water (40:60 v/v). The injection volume was 50 μL and total run time 8 min.

2.9. Statistical analysis

Tests for significant differences between means were performed by Student's *t*-test or one way ANOVA by using the Prism®, V. 3.00 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at $P < 0.05$ level.

3. Results and discussion

3.1. Microspheres elaboration for buccal delivery

A buccal dosage form may be designed to deliver a drug to the systemic circulation, or merely indicated for local therapy of the oral mucosa. For this reason the selection of dosage forms is affected by the intended application. In order to avoid a potential systemic absorption the utilization of mucoadhesive patches or bioadhesive films was soon abandoned. The major drawback of buccal bioadhesive tablets was their lack of physical flexibility, leading to poor patient compliance (Salamat-Miller, Chittchang, & Johnston, 2005). Microspheres have additional advantages like biocompatibility, sustained release and much more intimate contact with the mucus layer due to a high surface to volume ratio. Alginate and chitosan were selected as ingredient polymers for their well-known low toxicity, biodegradability and bioadhesive properties; even this latter has been recognized as antimicrobial agent (Muzzarelli, 2009). After microspheres were prepared, the coating process with chitosan was carried out with 0.50% (w/v) chitosan solution concentration in view of its posterior best behaviour and in accordance with that found in other studies carried out previously (Martín-Villena et al., 2013). Furthermore, the methodology applied in AM2 elaboration let us to obtain a final gel containing microspheres. This was justified based on the need of effective mucoadhesion properties to prolong drug action by increased contact time and residence time of the dosage form at the mucus membrane (Friedl, Dünnhaupt, Waldner, & Bernkop-Schnürch, 2013).

3.2. Physicochemical characterization

Results obtained from the particle size of the microspheres investigated by LD are indicated in Table 1. The mean particle size in terms of $LD_{4,3}$ for unloaded vehicles ranged from 60.39 μm (AM2) to 89.03 μm (AM1) and 85.35 to 135.21 μm for loaded microspheres (AM2 and AM1, respectively). The coating procedure resulted in light decrease of particle size, it could be due to the electrostatic interaction between the positively charged —$NH3^+$ of chitosan and negatively charged —COO^- of alginate microspheres. The incorporation of Nys in the microspheres led to a significantly increase of the mean sizes ($P < 0.05$). The highest mean size was observed in AM1, 135.21 μm. An increase in particle size after drug incorporation is a general phenomenon observed with many drugs (Souto, Alsemi, Centini, & Müller, 2005). Span factor as a measure of polydispersity showed homogeneous distribution among particles being AM2 the widest with a span factor of 2.44.

Fig. 1 shows images of the morphological evaluation of the unloaded and Nys loaded AM1 (Fig. 1A and B) and CCM (Fig. 1C and D) by SEM. It can be observed a homogeneous aspect with a relative sphericity and a slightly rough surface. Moreover the drug loading did not affect the external morphology. A detail of the surface of the CCM (Fig. 1D) shows that

Table 1
Particle size distribution indicating mean diameter ($LD_{4,3}$) and diameters at the 90% ($LD_{0.9}$), 50% ($LD_{0.5}$), 10% ($LD_{0.1}$) over the volume distribution, zeta potential (ZP; $n = 9$), encapsulation efficiency (EE), loading content (LC) and percentage yield (PY) of unloaded and drug loaded microspheres ($n = 3$). Nystatin (Nys), alginate microspheres (AM1), chitosan coated microspheres (CCM), hydrogel microspheres (AM2).

Parameters		AM1	Nys-AM1	CCM	Nys-CCM	AM2	Nys-AM2
Volume distribution	$LD_{4,3}$ (μm)	89.03	135.21	81.20	103.98	60.39	85.35
	$LD_{0.1}$ (μm)	41.98	50.79	30.43	44.15	6.19	24.41
	$LD_{0.5}$ (μm)	82.55	118.85	74.82	98.14	59.43	61.47
	$LD_{0.9}$ (μm)	148.66	239.23	143.27	178.02	110.37	174.41
Span factor		1.29	1.58	1.51	1.36	1.75	2.44
Z P (mV ± SD)	pH 7.5	−35.30 ± 1.69	−39.46 ± 2.01	−5.78 ± 0.27	−08.51 ± 0.16	−34.19 ± 1.71	−37.42 ± 1.07
	pH 5.5	−32.31 ± 1.52	−35.04 ± 1.13	−3.84 ± 0.39	−11.07 ± 0.14	−32.53 ± 1.28	−35.22 ± 1.40
EE (%)	Surface	–	18.31 ± 0.18	–	06.63 ± 0.88	–	23.67 ± 2.72
	Inside	–	89.11 ± 4.28	–	70.50 ± 0.03	–	53.85 ± 3.41
LC (%)	Surface	–	02.18 ± 0.18	–	00.40 ± 0.42	–	07.63 ± 1.81
	Inside	–	10.58 ± 0.01	–	03.35 ± 0.07	–	17.45 ± 2.34
PY (%)		–	92.52 ± 5.70	–	70.96 ± 1.72	–	80.35 ± 3.29

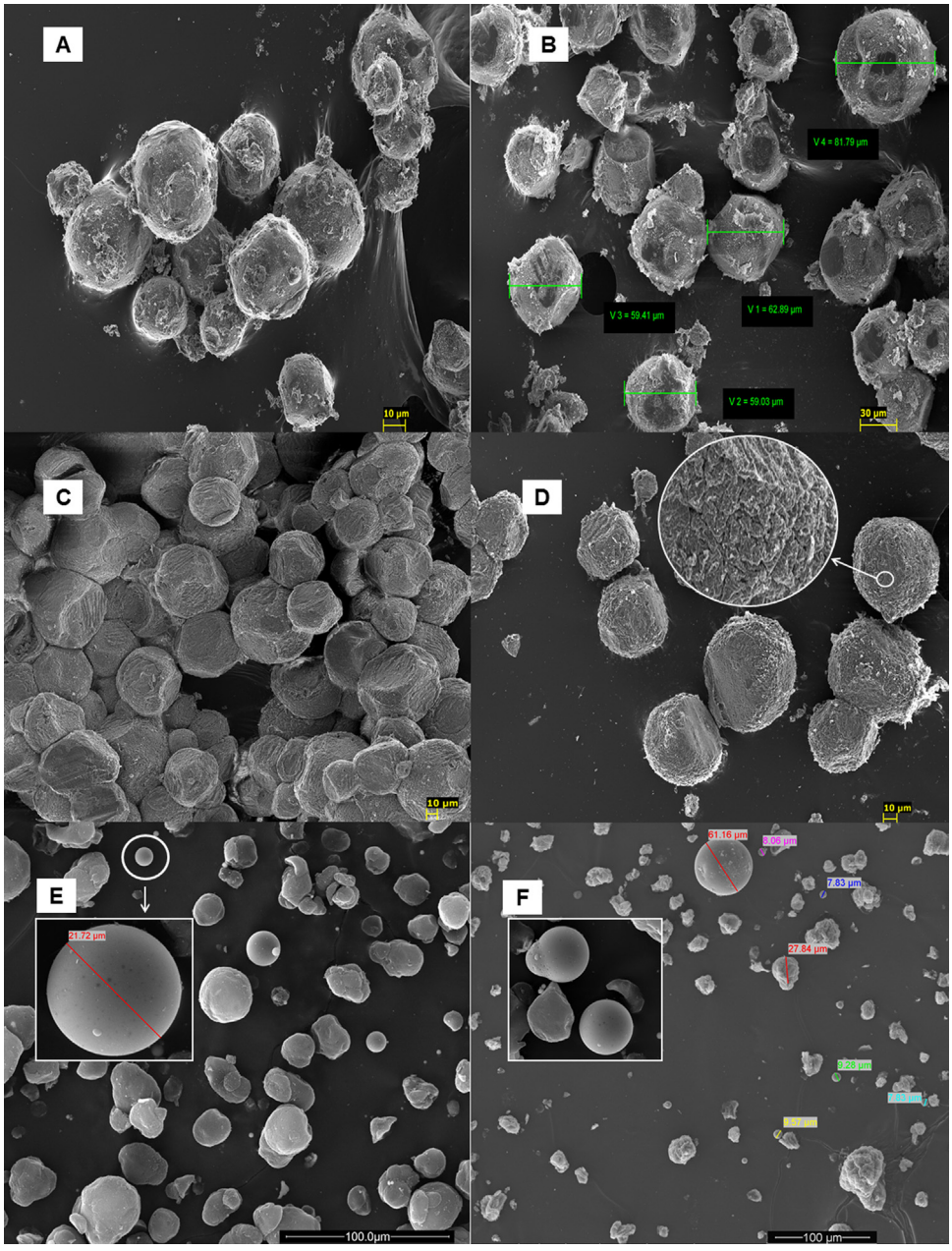

Fig. 1. Scanning electron microscope micrographs of the freeze-dried unloaded alginate microspheres (A), unloaded chitosan coated microspheres (C), unloaded hydrogel microspheres (E), drug loaded alginate microspheres (B), drug loaded chitosan coated microspheres (D) and drug loaded hydrogel microspheres (F).

pores are present on the rough surface of the microspheres. To obtain such images, the particles had to be sufficiently hydrated. Upon high pressures in the SEM apparatus fast dehydration occurred, followed by the microsphere subsidence due to the collapse of the hydrogel structure. Unloaded and drug loaded CCM exhibited a moderate tendency for aggregation. On the contrary, unloaded and drug loaded AM2 displayed more spherical shape and regular surfaces (Fig. 1E and F). Thus this elaboration method provided more regular shapes but according to the Span factor the widest particle size distribution. Good correlations were observed in particle size results between SEM and LD.

Measurements after 1–3 months did not show statistically significant differences.

The stability of many colloidal systems is directly related to the magnitude of their zeta potential. In general, zeta potential less than −30 mV or higher than +30 mV can be an indicator to assure the stability of particulate systems. Conversely, if the particle zeta potential is relatively small, the colloidal system will agglomerate. The charge properties were also important to the function of drug carrier, so it was necessary to examine the surface charge of microspheres for stability properties or adhesion onto biological surfaces. Equally, it is of substantial importance in all the production steps of these particles because the efficiency of the different steps is directly related to the establishment of electrostatic interactions (Gazori et al., 2009). Zeta potential measurements were performed at pH 5.5 and 7.5 because saliva is a weak buffer with a pH around 5.5–7.5 depending on the flow rate of saliva which, in turn depends upon three factors: the time of day, the type of stimulus and the degree of stimulation (Patel, Liu, & Brown, 2011). Table 1 also shows as the zeta potential measurements showed a negative surface charge in all cases. As expected, drug loaded AM1 and AM2 showed the most negative values at both pH conditions because of the carboxyl groups on the surface. After coating of chitosan on alginate microspheres core, the negative charges were partially neutralized by the interaction of amino groups of chitosan which made the surface exhibit lesser negative zeta potential values. As reported in literature, the introduction of chitosan could improve the bioadhesive of microspheres to specific regions of the buccal mucosa (Remunan-Lopez, Portero, Vila-Jato, & Alonso, 1998). No statistically significant differences were observed after three months storage period.

The values of PY, LC and EE of Nys loaded microspheres are also listed in Table 1. Being Nys an amphoteric molecule it is able to be electrostatically attached to such microspheres composed of alginate and chitosan with a high avidity, allowing its association with the stable colloidal drug carrier. The Nys molecule contains ionisable groups capable of attaining positive or negative charged polyelectrolytes like chitosan or alginate, respectively.

PY values varied between 92.52% ± 5.70 and 70.96% ± 1.72. The highest value of EE inside microspheres was for AM1 (89.11% ± 4.28) and the lowest for AM2 (53.85% ± 3.41), contrary AM2 surface adsorption was the best among microspheres (23.67% ± 2.72). The loss of Nys from CCM may be explained by the washing phase during the recovery and the agitation destabilizing effect during coating (Ribeiro, Silva, Ferreira, & Veiga, 2005). Equally AM2 exhibited the best total LC. A decrease of EE over time was observed in all microspheres after 3 month of storage period being more marked in AM2.

AM1 and CCM suspensions showed statistically smaller viscosity values than AM2 (1.05 ± 0.09, 0.95 ± 0.03 and 146.93 ± 3.7 mPa s, respectively) ($P < 0.001$). The flow and viscosity curves of formulations (shear stress versus shear rate, and viscosity versus shear rate) are shown in Fig. 2. The AM1 and CCM curves (Fig. 2A and B) presented constant viscosity values with an increasing shear rate from 0 to 100 s^{-1}, and thus Newtonian behaviour. A

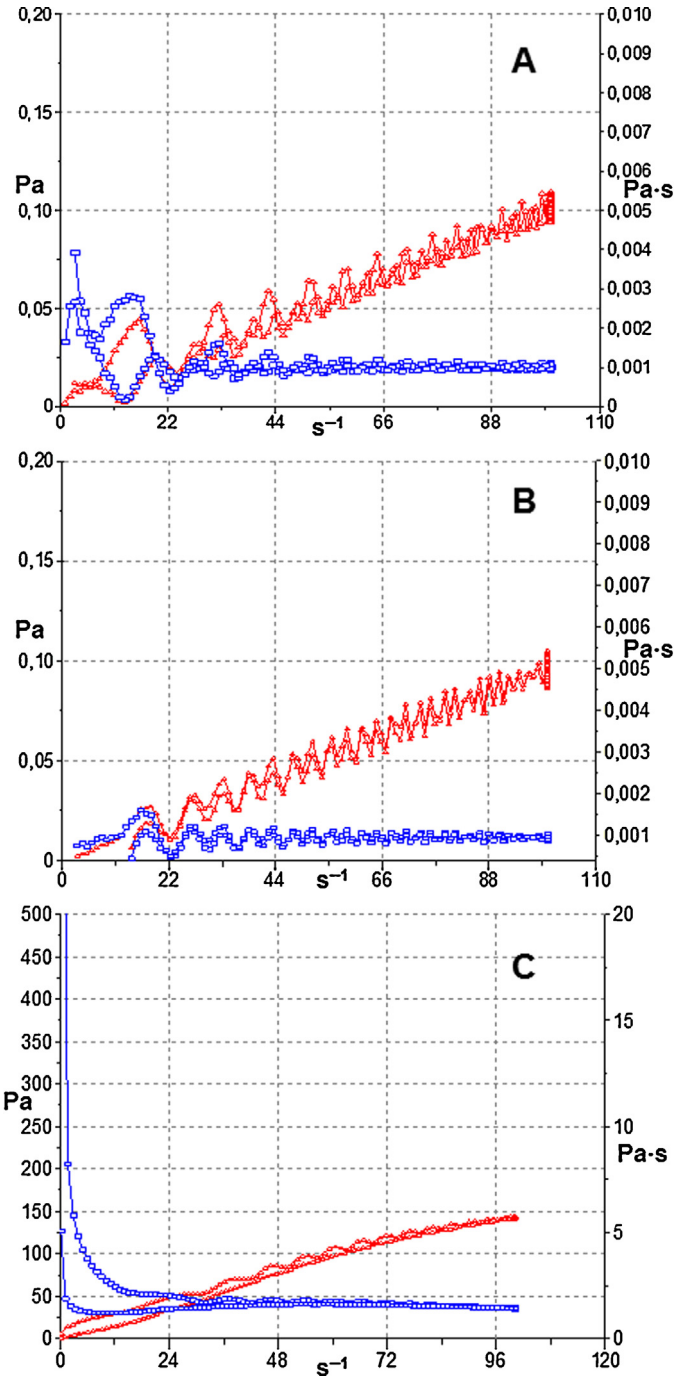

Fig. 2. Microsphere rheograms. It shows the shear stress (Pa) (in red) and the viscosity (Pa s) (in blue) of drug loaded alginate microspheres (Panel A), drug loaded chitosan coated alginate microspheres (Panel B), and drug loaded hydrogel microspheres (Panel C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

small decrease in the viscosity of AM1 was observed probably due to the swelling characteristic alginate. In both cases the hysteresis loop is inappreciable, which indicates that AM1 and CCM did not show thixotropy. In contrast, the AM2 curve (Fig. 2C) is dependent on shear rate, showing a consistent decrease in viscosity with increasing shear rate from 0 to 100 s^{-1}. This rheological behaviour could be described as typical for a non-Newtonian, pseudoplastic fluid. The hysteresis area, a pseudoplastic natural characteristic, was observed in the graph. AM2 thixotropy was 1695 Pa/s, $A(1) = 8487$ Pa/s, $A(2) = 836$ Pa/s, $A(3) = 7628$ Pa/s. Nonetheless,

concerning oral topical administration, the thixotropy property is a desired property because it contributes to increase the retention time of the formulation in the local of application (Silva, Amaral, González-Mira, Santos, & Ferreira, 2012). In this case, after fitting to mathematical models to identify the model that provided the best overall match of the experimentally observed rheological data, it was observed that Herschel–Bulkley equation was the best model that statistically described rheological behaviour in ascending stretches, with an r^2 of 0.9963. In descending stretches, both Cross and Herschel–Bulkley models exhibited the maximum r^2. After analyzing chi-square values of fit (1288 and 666.5 for Herschel–Bulkley and Cross, respectively), it was concluded that Herschel–Bulkley was also the most suitable. This model has been also found to be the most suitable for fitting experimental data from colloidal gels to quantify the dependence of the shear rate on the applied shear stress (Bonn & Denn, 2009). It is a generalized model of a non-Newtonian fluid, in which the strain experienced by the fluid is related to the stress in a complicated, non-linear way. Three parameters characterize this relationship, consistency, flow index (n), and the yield shear stress (τ_0). The consistency is a simple constant of proportionality, while n measures the degree to which the fluid is shear-thinning or shear-thickening. For n values of 0.8364 a pseudoplastic behaviour is evidenced. Finally, τ_0 quantifies the amount of stress that the fluid may experience before it yields and begins to flow (5.66 Pa). These results are important in terms of the *in vitro* performance of antifungal microspheres particularly with regards to release properties and consequent efficacy. This rheological behaviour of Nys-AM2 makes possible the administration in hard-to-reach anatomical areas as soft palate, posterior portion of the mouth, pharynx, etc. in a more controlled application and thereby making the application more efficient and effective.

The swelling behaviour of microspheres has a great impact on their stability, release of loaded drugs, adhesive properties and cohesiveness (Duchêne & Ponchel, 1992). After attachment with the underlying mucosal tissue, comprising polymers swell up *via* capillary action and diffusion process. In this regard, a moderate swelling is necessary to avoid over hydration and loss of adhesive properties (Roldo, Hornof, Caliceti, & Bernkop-Schnürch, 2004). As shown in Fig. 3A CCM displayed the best water uptake followed by AM1 and finally AM2. It could be related to the overall quantity of alginate and more entangled chains, which may lead to a greater expansion of the network. In this way CCM and AM1 are made up of equal alginate amount and larger than AM2. The different swelling behaviour exhibited by AM1 and CCM, which had similar alginate content could be interpreted according to the diffusion rate an extent of chitosan molecule to the three dimensional network of Ca-alginate, the large spatial size of chains for chitosan molecules results in high diffusion resistance leading to low diffusion rate and less diffusion extent. The reaction occurs mainly at the surface of Ca-alginate beads to form thin membrane (Liu et al., 2004). Finally it was observed that the swelling behaviour or microspheres was dependent on the exposure time. It could be due to the rupture of the microsphere structure.

The buccal region of oral cavity is useful for mucosal (local effect) drug administration. The mucoadhesive properties of a buccal delivery system are important for its influence on the prolonged permanence time. This facilitates the achievement of the site-specific release of the drug on the mucosa. The cationic polymer chitosan has a well-known bioadhesive nature, by the establishment of electrostatic interactions with sialic groups of mucins in the mucus layer. It consists of glucosamine and *N*-acetylglucosamine units with reported mucoadhesion properties.

In general, good adherent properties were observed in all microspheres. Although AM2 exhibited the highest bioadhesive strength

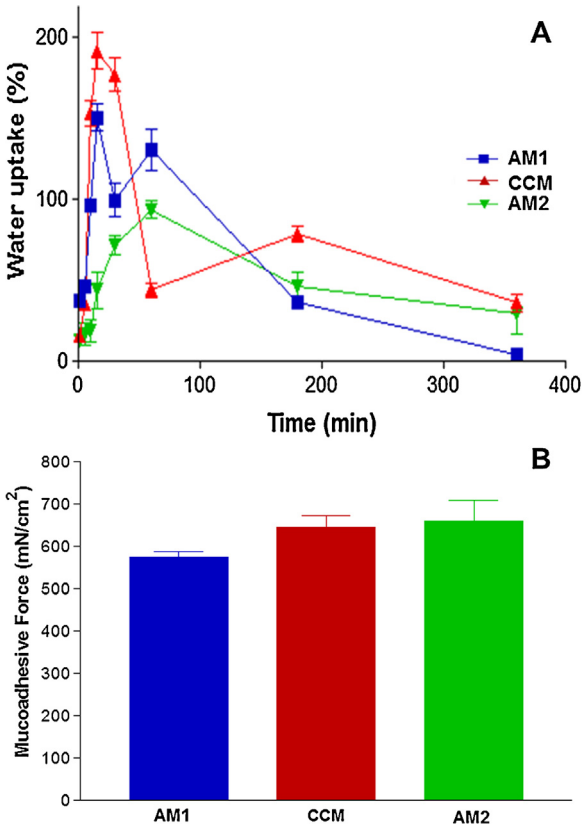

Fig. 3. Swelling behaviour (Panel A), and mucoadhesive force of microspheres (Panel B) for drug loaded alginate microspheres (AM1), drug loaded chitosan coated microspheres (CCM) and drug loaded hydrogel microspheres (AM2). Each value represents the mean ± SD ($n = 3$).

value possibly due to the adhesive effect of the gel (Fig. 3B). No significant differences were observed when compared with other two types of microspheres. CCM exhibited slightly higher bioadhesion properties than AM1, in spite of their different zeta potential. However, other mechanisms may be involved in this process (Woodley, 2001).

3.3. Drug release

Fig. 4A shows the different release profiles of Nys from AM1, CCM, AM2 and free Nys in DFM solution. In the first two cases, release profiles indicate a two-step process; an initial step with burst release which can be attributed to the surface associated drug, followed by a slower sustained release phase. This behaviour is lightly different in AM2 from which Nys release is released in a more constant manner. Statistically differences were observed between AM2 and the other two types ($P < 0.05$). Approximately 60% drug was released from AM1 and CCM for first 2 h, but only 25% from AM2. Contrary, at the end of the study Nys released from AM2 reached almost the same release rate that the other two microsphere types. This sustained released from AM2 could be due to the gel environment; it is well known that the microstructure of the materials plays an important role on the release behaviour of the encapsulated drug. On the other hand, the process of drug release from the alginate-drug matrix involves solvent penetration into the matrix, gelation of the polymer, dissolution of the drug, and diffusion of drug through resultant layer. In the film embedded with drug loaded microspheres (AM2), drug is stored inside microspheres and it has to dissolve in the film first before it can be released. All these factors could prevent or delay the release of drug. In addition the incomplete Nys

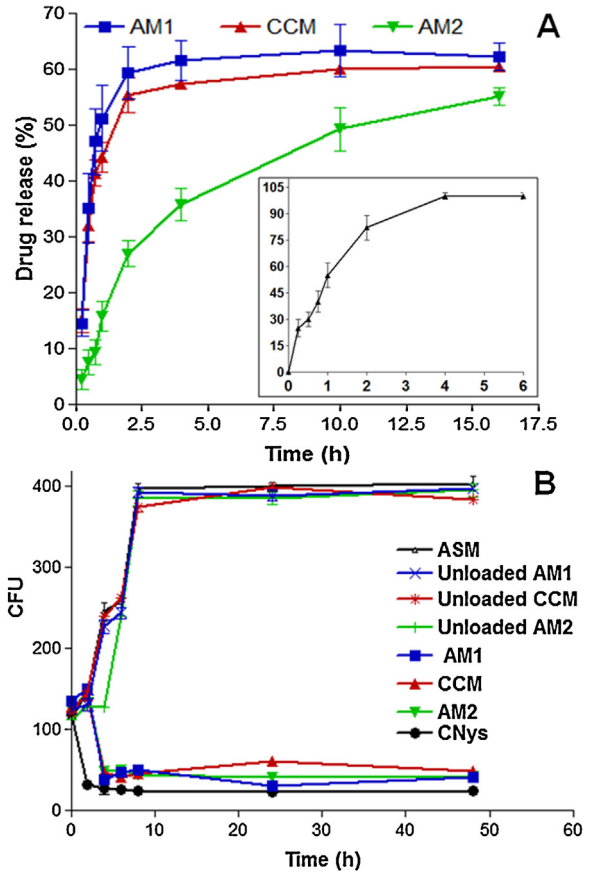

Fig. 4. *In vitro* release profiles of nystatin from microspheres in methanol:DMF:water (55:15:30, v/v/v) at 37 ± 0.5 °C, the inset shows the curve of pure nystatin in DMF solution (Panel A), and colony forming units (CFU) of different formulations in Sabouraud plates at 37 °C (Panel B). Nystatin (Nys), alginate microspheres (AM1), chitosan coated microspheres (CCM), hydrogel microspheres (AM2), nystatin commercial suspension (CNys), artificial saliva medium (ASM).

release from CCM suggests an electrostatic interaction between the active and the matrix (Ribeiro et al., 2005). Model fitting, according to the least AIC values, showed that AM1 and CCM followed the first order kinetic model. Pharmacokinetics parameters are also reported in Table 2. This means that Nys release from microspheres followed a concentration gradient pattern, based on the first Fick's law, where the released amounts are directly proportional to the amounts remaining into the dosage form offering sustained release of drug. No statistically significant differences were observed between release parameters of AM1 and CCM. Furthermore, Nys from AM2 followed the Weibull model, on the use of this function for the discernment of drug release mechanisms, estimates for $ß \leq 0.75$ indicate Fickian diffusion in either fractal or euclidian spaces while a combined mechanism (Fickian diffusion and swelling controlled release) is associated with $ß$ values in the range $0.75 < ß < 1$. For values of $ß$ higher than 1, the drug transport follows a complex release mechanism (Papadopoulou, Kosmidis, Vlachou, & Macheras, 2006). According to our results with a $ß$ value of 1.29 ± 1.33, a sigmoid curve indicative of complex release mechanism has been described. There are too many causes for diffusion to be non-Fickian since the mathematical description of the entire drug release process is somewhat speculative. There are too many factors involved, such as physicochemical properties, structural characteristics of the material system (mechanical and swelling properties), release environment, and the possible interactions between these factors. However, the final purpose of mathematical modelling is to simplify the complex release process and to gain insight into the release mechanisms (Domínguez-Villegas et al., 2014).

3.4. Antimicrobiological efficacy

Without Nys the increasing yeast concentration was observed in all unloaded microspheres after 24 and 48 h. This confirmed the capability of *C. albicans* to grow in ASM. As shown Fig. 4B, AM1, CCM and AM2 did not produce any growth inhibition effect at 0 h and 2 h, obtaining a yeast concentration of 2×10^6 CFU/mL approximately in all cases. On the other hand, commercial Nys suspension exhibited a much quicker effect, probably due to the direct contact with the yeast on the culture medium. After 4 h of inoculum contact, a growth inhibition effect was observed for all assayed samples, corresponding to the action of Nys concentrations from microspheres. These results are in concordance with time required for Nys release from microsphere and reach the yeast membrane (results of drug release studies). Fungicidal activity was demonstrated up to 48 h in all Nys loaded microsphere. No statistically significant differences were observed among microspheres or commercial product (after 4 h); in all cases Nys included in microspheres exhibited a marked fungicidal activity, since there was a drastic reduction on the *C. albicans* initial loading.

3.5. In vivo studies

After Nys loaded microspheres treatment on buccal mucosa of animals, no Nys was detected in plasma samples of animals from any kind of microspheres or commercial product. Therefore, flux of permeation values or other permeation parameters could not be estimated. This fact corroborated the safety of this delivery system, due to the reported systemic toxicity of Nys inducing haemolysis (Knopik-Skrocka, Bielawski, Glab, Klafaczynska, & Wulkiewicz, 2003). Subsequently, buccal mucosa tissue of the experimental animals were surgically removed and analysed for Nys content. Resulting amounts of Nys retained in the porcine mucosae were 3.79 ± 1.13, 4.73 ± 0.18 and 3.38 ± 0.25 μg/g tissue/cm^2 for AM1, CCM and AM2, respectively, without significant differences. No amounts of Nys were detected from the commercial suspension. Nevertheless according to the minimum inhibitory concentration (MIC) of Nys (0.78 μg/mL, equivalent to 0.79 μg/g) (Marín-Quintero et al., 2013), the retained amounts of Nys in the mucosa was between 4 and 6 times higher than the MIC value.

Table 2
Mean parameters obtained after fitting the release data of nystatin from alginate microspheres (AM1), chitosan coated microspheres (CCM) and hydrogel microspheres (AM2) to different release models.

Formulation	AIC	*K*	*ß*	*Td* (h)	Equation	Q_{max}
AM1	32.85	1.53(7.42%)	–	–	$\%R_t/\%R_\infty = 1 - e^{-1.53 \times t}$	62.26 (2.81%)
CCM	26.19	1.38(6.73%)	–	–	$\%R_t/\%R_\infty = 1 - e^{-1.38 \times t}$	60.55 (2.36%)
AM2	16.94	–	1.29 (4.7%)	0.68 (0.05%)	$\%R/\%R_\infty = 1 - e^{-(t/0.68)1.29}$	55.11 (2.57%)

$\%R_t/\%R_\infty$ is the rate of release, *K* is the release rate constant (h^{-1}) of the first order equation, *ß* is the shape parameter in the Weibull equation, *Td* (h) is the time in which the 63.2% of the drug is released in the Weibull equation, and Q_{max} (%) is the total percentage drug released at the end of the experiment, *AIC* is the Akaike's Information Criterion. The precision of the parameters estimation is also expressed as coefficient of variation (%).

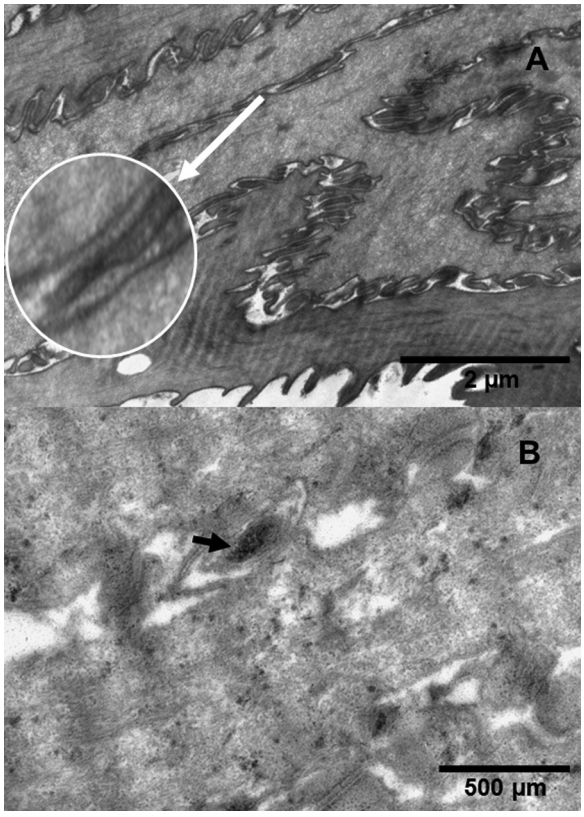

Fig. 5. Freeze-substitution transmission electron microscopy (FSTEM) micrographs of oral mucosa untreated (A), and treated with nystatin loaded hydrogel microspheres (B).

According to these results, retained amounts of Nys are more than enough to ensure its therapeutic effect. Nys exhibits concentration-dependent activity against a variety of *Candida* species. For values ranging from 0.5 to two times the MIC, Nys behaves as a fungistatic agent, whereas from two to four times the MIC a fungicidal effect is observed (Gunderson, Hoffman, Ernst, Pfaller, & Klepser, 2000). Thus with developed microspheres treatment both mechanism would be achieved.

The permeability barrier in nonkeratinized oral mucosa consists of lipids located in the intercellular spaces of the superficial epithelia layer. These can limit the penetration of nonpolar compounds, such as Nys. Thus, Nys may become trapped in a nonlipid or in a fluid-lipid intercellular compartment of the barrier layer. In order to evaluate the Nys permeation effect on the surface ultrastructure of the oral mucosa, electron microscopy analysis after Nys loaded microspheres treatment were taken. Fig. 5A and B shows histological microphotographs of control mucosa (untreated) and treated mucosa, respectively. The same intermediate layer region was evaluated for a better comparison of the treatment effect. Intercellular spaces of control mucosa were not widened (Fig. 5A magnification) and cells exhibited no cytoplasmic vesicles indicating intact healthy mucosa (Marín-Quintero et al., 2013). In treated mucosa, desmosomes remained intact (Fig. 5B arrow), indicative of the mucosal barrier structure conservation. As in control samples, epithelium maintains its structure with several cellular layers without any discontinuity or intercellular spaces and tight cell junction.

Commercial products for treatment of these diseases could contain irritating substances as propylene glycol or ethanol, which may cause further damage to the mucosa (atrophy, ulceration, and an inflammatory condition). According to our results Nys microspheres preserve the mucosa microstructure. Furthermore, chitosan possesses wound-healing properties particularly in the treatment of oral mucositis (Yang et al., 2010), and provided a sustained release of Nys decreasing the amount of drug and potentially reducing the frequency of application of the formulation.

Reported problems in other delivery systems such as liposomes are the susceptibility to enzymatic degradation and poor mucus penetrating ability due to the hydrophobic properties (Li et al., 2011). Nys nanoemulsions have been a reasonable alternative with optimal results but a short residence time in the oral cavity is the major shortcoming (Fernandez-Campos et al., 2013).

4. Conclusions

Microspheres have been designed as a new strategy that tries to resolve in part these problems with physical properties that enable them to make intimate contact with larger mucosal surface area than buccal tablets or lozenges. These microspheres loading Nys were successfully prepared by an emulsification/internal gelation method showing a clear inhibition effect on the *C. albicans* growth, suggesting their clinical potential use with no systemic absorption or tissue damage. Although three formulations were suitable and exhibited similar physicochemical properties, it seems that Nys-AM2 based on particle size, pseudoplastic behaviour with thixotropy, higher bioadhesive strength and the release of the drug in a more constant manner could be proposed as the best vehicle for the buccal action of Nys for the treatment of oral candidiasis.

Acknowledgements

Financial support from project MAT2011-26994 (MCNN-Ministerio de Ciencia e Innovacion) is acknowledged. Authors are also thankful to Mr. Alvaro Gimeno from the Animal Facility, for his support in animal studies and Dr. Lyda Halbaut Bellowa for excellent expertise.

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