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Novel microparticulate systems for the vaginal delivery of nystatin: Development and characterization

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ABSTRACT

To develop more effective antifungal microparticulate therapeutic systems for the treatment of *Candida* vaginitis, microparticles containing nystatin were elaborated by emulsification/internal gelation method. Three types of microparticles were successfully prepared, alginate microparticles, chitosan and poloxamer 407 coated alginate microparticles. DSC and FT-IR studies were performed to test the efficacy of the method. After physicochemical characterization, mean particle sizes ranged from 36.088 μm to 56.146 μm. The encapsulation efficiency was found to be similar for alginate and chitosan coated microparticles and lower for poloxamer 407 coated. Optimal mucoadhesive properties in all kind of microparticles where exhibited. Release studies showed the best kinetic parameters for poloxamer 407 coated microparticles. After ex vivo permeation studies through porcine vaginal mucosa, and determination of the amount of nystatin retained as well as microbiologic studies performed, it could be inferred that the developed microparticulate systems offered an antifungal effect against *Candida albicans* without toxic systemic absorption.

1. Introduction

Nystatin (Nys) is a polyene antifungal antibiotic, one of the oldest antifungal drug, produced by *Streptomyces noursei* strains (Kaur & Kakkar, 2010) commonly used for prophylaxis and treatment of candidosis by interfering with the fungal cell membrane of the antibiotic-sensitive organism by binding to sterols, chiefly ergosterol, and the formation of barrel-like membrane-spanning channels (Coutinho & Prieto, 2003) playing an essential role in antifungal chemotherapy. Nys is an active substance for treatment of susceptible cutaneous and mucocutaneous fungal infections caused by the *Candida* species, which have been reported to cause infection, and many of these are classified as emergent (Agarwal, Thakur, Kanga, Singh, & Gupta, 2008). But Nys also exhibits a broad spectrum of activity against fungi, including *Aspergillus*, *Coccidioides*, *Cryptococcus*, and *Histoplasma* ssp. and it has been shown that the antifungal activity of Nys is broader in comparison to amphotericin B other polyene antibiotic (Offner et al., 2004).

Vulvovaginal candidosis is estimated to be the second most common cause of vaginitis after bacterial vaginosis and up to 75% of all women suffer at least one episode of this infection during their lifetime, and 5–8% of adult women have recurrent vulvovaginal candidosis, defined as four or more episodes every year, in fact it is diagnosed in up to 40% of women with vaginal complaints in the primary care setting (das Neves et al., 2008). *Candida albicans* accounts for 85–90% of cases (Sobel, 2007).

Most patients with *Candida* vaginitis respond to topical treatment with nystatin or imidazoles (Richardson & Warnock, 2003). However excessive use of azole antifungal drugs as fluconazole, has increased the fungi resistances by multiple mechanisms (Bauters, Dhont, Temmerman, & Nelis, 2002), and non-*C. albicans*-related disease is less likely to respond to azole therapy, and particularly *Candida glabrata* does not respond to azole treatment (Phillips, 2005). Moreover azoles have a fungistatic effect, whereas Nys has both an antifungal and fungistatic activity (Recamier, Hernandez-Gomez, Gonzalez-Damian, & Ortega-Blake, 2010). But its clinical use is currently limited almost exclusively to the topical treatment of superficial *Candida* infections, since it is not effective when given orally and is severely toxic as an intravenous application (Schaffner, 2002).

Over the years much more attention has been paid to azoles versus Nys, but Nys has been found to possess broader spectrum of activity of the former drugs towards fungi in *Candida* vaginitis. On the other hand, vaginal administration has been reported to

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be more suitable than oral (das Neves et al., 2008) and in contrast to azoles, there is hardly Nys formulations for vaginal administration in the market; currently in some countries do not exist. In this sense, traditional vaginal drug delivery systems include solutions, suspensions, gels, ointments, foams, tablets, tampons, suppositories, pessaries and vaginal inserts, among others. These vaginal formulations often require a frequent dosing regimen owing to the self-cleansing action of the vagina, namely the secretion of mucus and humid site of administration (Valenta, 2005) in addition the chemist structure of Nys reveals formulation challenges by being both amphiphilic and amphoteric what contributes to its poor solubility in aqueous media.

A special attention has been focused on the development of novel and controlled release drug delivery systems to provide a long term therapeutic concentration of the drug following the application of a single dose in vagina (Mandal, 2000). A vaginal drug delivery system should have a long retention time to maximize drug release, a proper spreading over the vaginal epithelium to obtain fast absorption or to maximize the effect in case of local treatment and be easy to administer (allow self-administration) and not cause discomfort to improve patient compliance (Poelvoorde et al., 2009).

The main advantages of microparticles over traditional vaginal dosage forms are: the generation of sustained release of drug over time because microparticles provide an effective release of antifungal drugs, and long lasting method for the release of antifungal drugs (Yuen et al., 2012), the core content may be released by friction, pressure, change of temperature, diffusion through the polymer wall, dissolution or biodegradation of the polymer wall coating after deliberately tailoring the particle interior or wall properties (Yuen et al., 2012), a reduced number of doses required to the treatment of diseases, the protection of the drugs from inactivation, and capacity to reduce the drug toxicity. Furthermore, microparticles have exhibited a better stability in the biological environment than liposomes, and their highly reproducible formulation methods provide support to encapsulate hydrophilic and hydrophobic drugs. Due to the mucoadhesive properties of polymers make them able to promote intimate contact between the pharmaceutical form and the vaginal tissue and to prolong the residence time at the site of administration. In this way, multiple-unit mucoadhesive carriers such as microparticles, combine the abilities of the mucoadhesive dosage forms with the advantageous features of the multiparticulate delivery systems (Albertini et al., 2009).

Naturally occurring polymers, such alginate, an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid, are biodegradable materials with low toxicity, low cost and compatibility with the encapsulation of a wide range of drugs, with minimal use of organic solvents. They have been widely used as excipients in several pharmaceutical formulations for several decades (Bajpai, Saxena, & Sharma, 2006). Equally chitosan has increasing importance due to its dose dependent bioactivity, it has been reported to inhibit the adhesion of *C. albicans* to human vaginal epithelial cells (Knapzyk, Macura, & Paulik, 1992) and has been utilized as a membrane coating material to reduce microcapsule swelling, improve encapsulation efficiency and enhance stability (Li et al., 2009).

Poloxamers, are a class of non-ionic surfactants with the triblock poly(oxyethylene)–poly(oxypropylene)–poly(oxyethylene) structure, among these poloxamer 407 consisting of 70% (w/w) polyoxyethylene units, is a low toxicity excipient approved by FDA for different types of preparations as bioavailability enhancer has been utilized to obtain mucoadhesive systems for mucosa application (Jones et al., 2009) and was also selected as the biocompatible and non toxic polymeric shell on the microcapsules responsible for the vehiculisation of Nys and controlled release.

Extensive survey of literature and patent databases did not reveal any microcapsules formulation developed of Nys for the vaginal treatment of *Candida* vaginitis. Thus based on these considerations, the major aims of the present study were: (i) to develop the preparation of Nys loaded microcapsules by complex coacervation coated with polymeric surfactants chitosan and poloxamer 407 for the vaginal administration of nystatin, (ii) to characterize the formulation in terms of size, morphology, drug loading and physicochemical properties using DSC and FT-IR, (iii) to determine the antifungal activity of the Nys loaded microcapsules against a strain of *C. albicans*, (iv) to test the in vitro release profiles of Nys from the developed microcapsules, and finally (v) to evaluate ex vivo permeation across porcine vagina.

2. Materials and methods

2.1. Materials

Nys, Pluronic® F-127 (poloxamer 407) and sodium alginate were provided by Fagron Iberica (Terrassa, Spain). Low molecular weight chitosan was from Sigma–Aldrich (Madrid, Spain). Calcium carbonate, acetic acid, HPLC-grade methanol, N-dimethylformamide (DMF), acetonitrile and dimethilsulfoxide (DMSO) were obtained from Panreac (Barcelona, Spain). Vegetable oil, corn starch and Span® 80 were purchased from Guinama S.L.U. (Alboraya, Spain). Phosphate buffered saline (PBS) was obtained from Invitrogen (Alcobendas, Spain). Albumin solution 4% was obtained from Laboratorios Grifols S.A. (Barcelona, Spain). Double distilled water was used after filtration in a Milli-Q® Gradinet A10 system apparatus (Millipore Iberica S.A.U.; Madrid, Spain). Polysuphone membranes were purchased from Iberlabo S.A. (Madrid, Spain). Synthetic medium RPMI-1640 from Invitrogen (Barcelona, Spain), and buffer 3-(*N*-morpholino)-propanesulphonic acid from Sigma–Aldrich (Madrid, Spain), were also used.

2.2. Synthesis of alginate microcapsules and coating procedure

The formulation of the Nys loaded microparticles was based on the well known emulsification/internal gelation methodology (Silva, Ribeiro, Ferreira, & Veiga, 2006) with modification. The W/O emulsion was performed with a sodium alginate aqueous solution, calcium carbonate and Nys as the internal phase and vegetable oil as the external phase.

Briefly, 0.2 g of $CaCO_3$ was added to 40 mL of 3% (w/v) sodium alginate containing 474.55 ± 1.06 mg of Nys and placed in ultrasonic bath for 5 min. 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 with a mechanical stirrer to form uniform water in oil emulsion. With continuous stirring, 20 mL of vegetable oil containing 0.850 mL of glacial acetic acid was added to the W/O emulsion, to permit calcium carbonate solubilization. After 10 min under stirring, pregelified microparticles were separated from the oil dispersion by mixing with calcium chloride solution 5% (w/v). The supernatant was discarded and the alginate microparticles (AM) were centrifuged, collected and washed using 100 mL distilled water by vacuum filtration and stored at 4 °C in Petri dishes.

Low-molecular-weight chitosan and poloxamer 407 polymers were selected as coating materials for the microcapsules at different concentrations. Coating procedures followed the method that had already been previously reported (Liu, Rauth, & Wu, 2007).

Then, 10 g uncoated microparticles (AM) were immersed in three baths containing 100 mL of 0.25%, 0.5% and 1% (w/v) chitosan acetic acid solutions, respectively, and stirred at 300 rpm for 30 min on an orbital shaker for coating. The resulting chitosan-coated microparticles (CCM) were washed with 100 mL distilled water by vacuum filtration and kept at 4 °C. Equally for poloxamer 407 coating, 10 g of AM were suspended in two baths containing

100 mL of 1% and 2% poloxamer 407 solutions, respectively, and stirred at 300 rpm for 30 min on an orbital shaker. Finally, resulting poloxamer 407 coated microparticles (PCM) were washed with 100 mL distilled water by vacuum filtration and kept at 4 °C.

2.3. Physicochemical characterization

2.3.1. Particle size and morphological analysis

The particle size was evaluated by optical microscopy using an Olympus BX40 microscope equipped with a calibrated eyepiece micrometre and camera Olympus SC35 (Tokyo, Japan) under a magnification of 100×. The particle diameters of about 100 microparticles were measured randomly and the average particle size was determined.

The shape, surface morphology and internal of the microparticles were also examined by scanning electron microscopy (SEM) using a microscope Zeiss DSM 950 (Carl Zeiss AG, Oberkochen, Germany) set at 3 kV accelerating voltage. Measurements were repeated after 1, 2, and 3 storage months at 4 °C.

2.3.2. Determination of percentage yield, loading capacity and encapsulation efficiency

Determination was performed as follows, 0.5 g of AM and PCM 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 were liquefied in 50 mL sodium citrate solution (0.1 M) by gently shaking at room temperature for 45 min. After that, all samples were filtered through a 0. 22 μm filter and appropriate dilutions were carried out. Then samples were analyzed in triplicate by a validated HPLC methodology described in Section 2.7. Equally, for determination of the amount of Nys adsorbed in the microparticle surface, 0.5 g of each type of microcapsules was added to a methanol:DMF:water (55:15:30, v/v/v) solution and maintained at 25.0 ± 0.5 °C under mechanical stirring (50 rpm) for 1 h. Nys remaining in the supernatant (after microparticle centrifugation: 1 h at 10,000 rpm) was measured in triplicate by HPLC methodology.

Drug incorporation to microparticles was expressed in terms of percentage yield (PY, %), loading capacity (LC, %), and encapsulation efficiency (EE, %). EE were determined for all samples after the manufacturing process, and repeated after 1, 2 and 3 months stored in amber glass vials at 4 °C.

2.3.3. Zeta potential

Zeta potential measurements of diluted samples were made with a ZetaSizer® 2000 (Malvern Instruments Ltd., Malvern, UK). Zeta potential values obtained from ZetaSizer were average values from nine measurements made on the same sample. Measurements were performed at pH 4.5 in phosphate buffer solution (0.05 M) after elaboration and storage period of 1, 2 and 3 months stored in amber glass vials at 4 °C.

2.3.4. Fourier transform infrared spectroscopy measurement

Studies of infrared spectra of pure drug, raw polymers, and microparticles were conducted with a Nicolet 20 SXB spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) using the KBr disc method. The sample were diluted with KBr and then compressed into a tablet, 10 mm in diameter and 2–3 mm in thickness, using a manual tablet presser (Perkin Elmer Inc., Waltham, MA, USA) at 10 tonnes for 5 min.

2.3.5. Thermal analysis

The thermal analysis of pure Nys, raw polymers, physical mixture and microparticles was performed by differential scanning calorimetry (DSC) equipped with a thermal analysis data system Perkin-Elmer DSC 6 (Perkin Elmer Inc., Waltham, MA, USA). Samples weighting 5–6 mg were heated in flat-bottomed sealed aluminium pans over a temperature range 3–300 °C at a constant rate of 5 °C/min under nitrogen purge (50 mL/min).

2.3.6. Evaluation of mucoadhesive force

To evaluate the mucoadhesive force according to that reported by Li et al. (2012) two pieces of porcine vaginal tissue membrane (2.0 cm × 2.0 cm) 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 porcine vaginal tissue, 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 was calculated accordingly. The results were then reported as mean values ± SD (mN/cm^2) and at least 3 replicate measurements for each sample were performed.

2.4. Antimicrobiological efficacy

The present assay was performed to evaluate the antifungal activity of Nys microparticles 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 (Invitrogen, Madrid, Spain) medium at 35 °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 and counting in a Neubauer Chamber ($1–5 \times 10^8$ Colony Forming Unit, CFU/mL).

Growth kinetic of *C. albicans* ATCC 10231 was compared with the growth curves of the yeast cultured in presence of Nys in the following forms: (i) Nys loaded AM, (ii) Nys loaded CCM, and (iii) Nys loaded PCM. An appropriate amount of microparticles were weighted and diluted in sterile SVM to obtain a final Nys concentration approximately 20 μg/mL. Then 10 μL of inoculum were added to 1 mL of the microparticles suspension in SVM, obtaining a yeast loading of 10^6 CFU/mL. All these samples were incubated for 0, 2, 4, 8 and 24 h at 37 °C and shaken every 2 min to simulate in vivo conditions. After each time 10 μL of each sample were placed in Sabouraud plaques and incubated at 37 °C for 48 h and finally the colonies were counted by the naked eye (at 24 and 48 h). Chloramphenicol was added to Sabouraud plates to inhibit bacterial growth (Murray, Baron, Jorgensen, Pfaller, & Yolken, 2003).

2.5. Drug release studies

The release studies were performed using vertical Franz diffusion cell (FDC 400, Crown Glass, Somerville, NJ) through polysulphone membranes (Pall Corporation, Ann Arbour, MI, USA). 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 condition in the whole experiment (Fernández, Clares, Lopez, Alonso, & Calpena, 2013). The system was kept at 37 ± 0.5 °C and stirred continuously (600 rpm). Amounts of microparticles equivalent to 830 μg of Nys were added to the donor compartment. 200 μL samples were withdrawn at selected time intervals for 24 h and then replaced with the same volume of fresh receptor medium.

The concentration of released drug was measured by HPLC methodology. Values are reported as the mean ± SD of the five replicates.

2.5.1. Release parameters

Five different kinetic models (zero order, first order, Peppas-Korsmeyer, Higuchi and Weibull function) were used to fit the experimental data obtained in the drug release experiments (Costa & Sousa Lobo, 2001). A nonlinear least-squares regression was performed using the WinNonLin® Professional edition software, version 3.3 (Pharsight Corporation, Sunnyvale, CA, USA), and the model parameters calculated.

2.6. Ex vivo permeation through porcine vagina

2.6.1. Histological evaluation

This study was approved by the Ethics Committee of Animal Experimentation at the University of Barcelona. 3–4-month-old female pigs were used ($n = 6$) because porcine vaginal mucosa seems a good in vitro permeability model for human vaginal mucosa (van Eyk & van der Bijl, 2005). The vaginal tissue was purchased from the animal facility at Bellvitge Campus (University of Barcelona). The animals were sacrificed using an overdose of sodium thiopental anaesthesia and vaginal mucosa tissues were frozen by placing them in containers with a PBS mixture containing 4% albumin (extracellular cryoprotective) and 10% DMSO (intracellular cryoprotective) and stored at −80 °C.

Before permeation studies, fresh (used as control) and frozen vaginal mucosa tissues were evaluated in order to verify if the low temperature could cause histological changes. The mucosae were fixed in 10% neutral-buffered formalin for 15 h and then dehydrated by ethanol solution in different concentrations, then, were embedded in paraffin wax (Sigma–Aldrich, Madrid, Spain). Tissues were cut into 5 μm thick slices, mounted on slides and dried at 50 °C. After staining with haematoxylin and eosin (Sigma–Aldrich, Madrid, Spain) samples were examined by light microscope.

2.6.2. Permeation studies

Prior to the experiment, frozen porcine vaginal tissue specimens were thawed in Hanks' solution (Krackeler Scientific Inc., Albany, NY, USA) for 50 min at 37 °C under gentle shaking until total DMSO elimination. Subsequently, the vaginal tissues were dermatomed at a thickness of 300 ± 50 μm and mounted with a permeation orifice diameter of 9 mm (diffusion area: 0.63 cm^2).

The loading dose was 1.14 mg of Nys. Samples (300 μL) were drawn via syringe from the centre of the receptor compartment containing SVM at selected time intervals. Removed sample volume was immediately replaced with the same volume of fresh receptor medium.

2.6.3. Drug retention in porcine vagina

Amounts of Nys remaining on the vagina membrane were quantified. The mucosa was removed from the Franz cells, cleaned with gauze soaked in a 0.05% solution of dodecyl sulphate and washed in distilled water. The permeation areas of the mucosa were cut and weighed. Nys contended in the mucosa was extracted with acetonitrile/water (80:20, v/v) mixture during 20 min under cold sonication in an ultrasound bath (Campos, Calpena Campmany, Delgado, Serrano, & Naveros, 2012); the resulting samples were measured by HPLC.

2.7. HPLC analysis

Nys was quantified in samples using a high-performance liquid chromatography (HPLC) previously validated (Campos et al., 2012) according international guidelines. 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 cm × 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.

Stock solution of Nys (6.4 mg/mL) was prepared in DMSO. Working standards solutions were prepared daily by suitable dilutions in SVM for the quantification in permeation studies, and in methanol:DMF:water (55:15:30, v/v/v) for the quantification in release studies.

2.8. 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. Microparticle preparation

After Nys loaded and unloaded microparticles were prepared, the coating process with chitosan was carried out with three different chitosan solution concentrations 0.25%, 0.50% and 1% (w/v). Finally, only CCM coated with 0.50% (w/v) chitosan solution were selected for further studies. CCM coated with 1% (w/v) chitosan solution were discarded after preliminary encapsulation efficiency and release studies because the observations obtained were clearly disappointing (data not shown), increasing the chitosan concentration resulted in a thicker membrane; and even polymeric lumps rather than dispersed particles and the handling process became very difficult. Equally in preliminary studies CCM elaborated with 0.25% (w/v) chitosan solution provided lower Nys loading values than CCM elaborated with 0.5% (w/v) chitosan solution, 72.1% versus 94.27% respectively.

On the other hand, only PCM elaborated with 1% (w/v) poloxamer 407 solution were selected, since preliminary release studies results and morphology characteristics (agglomeration and fusion phenomena) of PCM elaborated with 2% (w/v) poloxamer 407 forced to discard.

3.2. Physicochemical characterization

The particle size analysis shows a homogeneous particle sized distribution. Unloaded microparticles ranged from 36.088 μm for AM to 56.146 μm for CCM. Mean diameter of microparticles was increased after coating in 20.058 μm for chitosan and 14.681 μm for poloxamer 407, indicating a high adhered quantity of the former versus the latter, and thus a thicker layer. When microparticles were loaded with Nys the mean sizes increased significantly in AM and PCM ($p < 0.05$) being the highest mean size for PCM, 60.001 μm (Table 1). According to these results it can be hypothesized that chitosan forms a stronger membrane than poloxamer 407 around the alginate microparticle, resulting in chitosan coated alginate microparticles further strengthens the alginate gel structure of microparticles, in this way could aid avoiding the particle size increase due to the Nys loading. Measurements after 1, 2 and 3 months storage did not show statistically significant differences except for Nys loaded PCM, in which the particle size decreased over time probably due to the thermogelation characteristics of poloxamer 407 resulting from interactions between different segments of the copolymer.

Optical microscopy images of the wet microcapsules are shown in Fig. 1. It could be observed the encapsulation of the drug and the particle size homogeneity. The morphological evaluation of the microparticles by SEM (Fig. 2) showed that AM and CCM exhibited a homogeneous aspect with a relative sphericity and a slightly

Table 1
Average particle size and Zeta potential (pH 4.5) of microparticles. Nystatin (Nys), alginate microparticles (AM), chitosan coated microparticles (CCM), poloxamer 407 coated microparticles (PCM).

Microparticle	Mean particle size (μm) ± SD	Zeta potencial (mV) ± SD
Unloaded AM	36.088 ± 9.27	−36.84 ± 0.14
Nys loaded AM	51.213 ± 9.35[a]	−42.33 ± 0.13
Unloaded CCM	56.146 ± 15.45	−6.213 ± 0.05
Nys loaded CCM	57.193 ± 13.48	−10.31 ± 0.12
Unloaded PCM	50.769 ± 12.84	−31.38 ± 0.12
Nys loaded PCM	60.001 ± 12.43*	−30.66 ± 0.14

[a] Express statistical significant differences in the parameter estimated ($p < 0.05$).

rough surface, it can be seen that the drug loading did not affect the external morphology. On the contrary, PCM displayed some shape irregularities; it could be due to the sample preparation method for SEM study, because optical microscopy showed well defined spherical shapes. The particle size of the dry CCM is smaller than the wet CCM which was observed by optical microscopy. This could be due to the oil in the microparticles leaked out of the chitosan shell which led to the shrinking and deformation of the microparticles (Yuen et al., 2012).

The values of PY, LC and EE of Nys loaded microparticles are also listed in Table 2. PY values varied between 79.67 ± 2.27% and 87.38 ± 6.86%. The highest value of EE inside macroparticles was for CCM (85.08%), but decreased over time (3 months) in all microparticles, being AM most affected, from 81.12% to 60.17%. Regarding to LC, the highest value was observed with CCM, it is evident the effect of chitosan coating on the LC percentage and EE of AM, this enhancement could be attributed to the formation of a strong complex membrane that would stabilize and strengthen the ion gel network and limit the loss of drug by diffusion.

Zeta potential less than −30 mV or higher than +30 mV can be an indicator to assure the stability of nanoparticulate systems. Zeta potential values were measured at pH 4.5 to emulate vaginal conditions. Table 1 shows as the Zeta potential measurements showed a negative surface charge in all cases. Zeta potential of AM depends on the high charge density provided by the acidic groups of alginate, thus remained minor than −35 mV. After complete coating of chitosan on alginate microparticles core, the value was about −5 mV for unloaded and about −10 mV for Nys loaded CCM, what implied the presence of chitosan layer on the surface by the interaction of the acidic groups of alginate with the amino groups of chitosan polymer. This Zeta potential value explained the difficulties that were found in preventing aggregation phenomenon with CCM when 1% (w/v) chitosan solution was utilized in coating process. Contrary, Zeta potential of PCM also remained under −30 mV at both pH conditions. These results indicated that the surface of the AM and PCM would produce electrostatic repulsion force to inhibit aggregation and behaved as stable colloidal microparticles systems. The measurements remained negatively stable after 1, 2

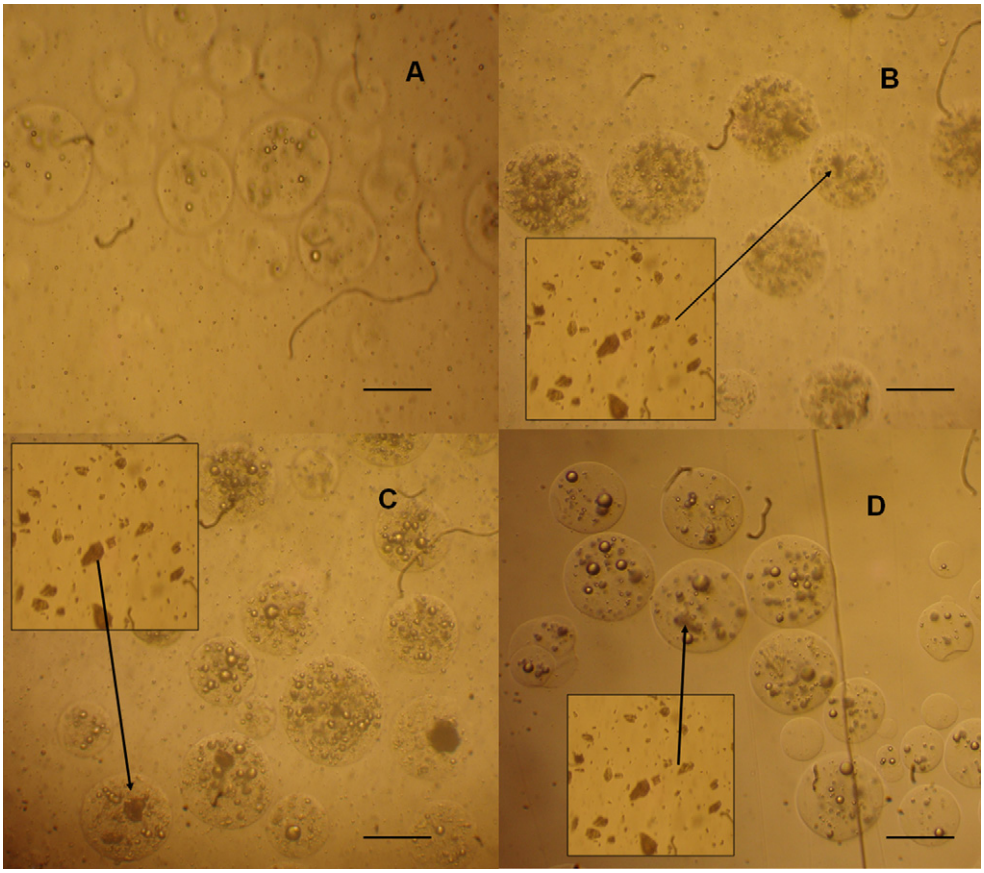

Fig. 1. Optical micrographs of plain nystatin (Nys) (A), Nys loaded alginate microparticles (B), Nys loaded chitosan coated microparticles (C) and Nys loaded poloxamer 407 coated microparticles (D), bar length 100 μm.

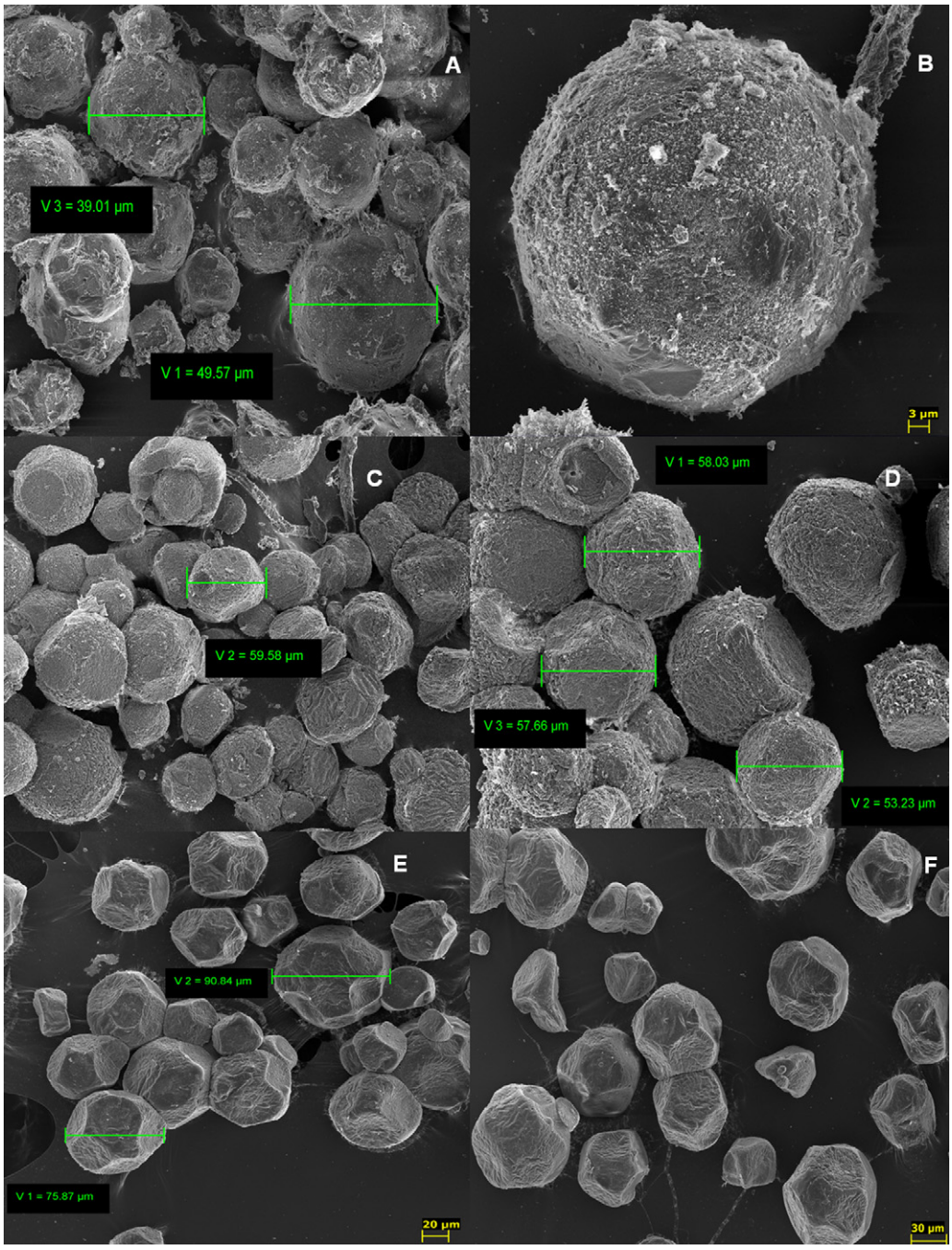

Fig. 2. Scanning electron microscope micrographs of the freeze-dried unloaded alginate microparticles (A), unloaded chitosan coated microparticles (C), unloaded poloxamer 407 coated microparticles (E), Nys loaded alginate microparticles (B), Nys loaded chitosan coated microparticles (D) and Nys poloxamer 407 coated microparticles (F).

and 3 months, no statistically significant differences were observed after storage period.

Fig. 3 shows the FT-IR spectra of raw materials and microparticles. Nys displays a broad intense absorption bands with the maximum at: 3410 cm^{-1} due to the stretching vibration of hydrogen bonded; 2924 due to asymmetric stretching vibration CH_2 group, 1708 cm^{-1} due to the stretching vibration of carbonyl from ester and carboxylic acids, 1576, 850 cm^{-1} due to the double bonds CH=CH, 1400 cm^{-1} due to the bending vibration ν(—CH), 1069 cm^{-1} due to hydroxyl groups (Silverstein, Webster, & Kiemle, 2005). The alginate spectrum exhibits characteristic absorption bands, 3439 cm^{-1} for primary amine, 1614.81 cm^{-1} and 1419.73 cm^{-1} due to the antisymmetric and symmetric stretching vibrations of the carboxylate group (Rao, 1963). On the other hand, the characteristic absorption of the chitosan is the band at 1650.98 cm^{-1}, assigned to the C=O stretching in amide group, 2924.36 cm^{-1} and 1384.37 cm^{-1} assigned to vibrations of C—H, another band at 3438.53 cm^{-1} is due to amine NH symmetric vibration. The peaks around 1105.44 cm^{-1} correspond to saccharide structure of chitosan, and absorption bands at 1153 cm^{-1} due to antisymmetric stretching of the C—O—C bridge (Krishna Rao, Vijaya Kumar Naidu, Subha, Sairam, & Aminabhavi, 2006). The poloxamer 407 spectrum showed a large band between 3650 and 2885 cm^{-1} for free O—H stretching vibration, and important peaks at 1112.40 cm^{-1} (C—O—C stretch), at 1343 cm^{-1} (in-plane O—H bend), and 2888.39 cm^{-1} (CH stretch aliphatic), and other bands at 1467.52 and 963.77 cm^{-1} as singlets and doublet respectively, due to the vibration of the ether bond (Albertini et al., 2010).

Table 2
Average percentage yield, loading capacity and encapsulation efficiency of microparticles. Nystatin (Nys), alginate microparticles (AM), chitosan coated microparticles (CCM), and poloxamer 407 coated microparticles (PCM).

		Nys loaded AM	Nys loaded CCM	Nys loaded PCM
Percentage yield (%)		83.26 ± 3.9	79.67 ± 2.27	87.38 ± 6.86
Loading capacity (%)	Surface	0.40 ± 0.08	0.91 ± 0.30	0.94 ± 0.43
	Inside	6.78 ± 0.8	4.87 ± 2.58	4.57 ± 0.45
Encapsulation efficiency (%)				
0 months	Surface	12.07 ± 2.16	9.19 ± 0.29	3.79 ± 0.34
	Inside	81.12 ± 3.9	85.08 ± 7.84	51.15 ± 6.1
1 months	Surface	6.1 ± 0.67	5.4 ± 4.55	3.76 ± 1.22
	Inside	72.95 ± 1.58	77.9 ± 6.68	42.19 ± 2.32
2 months	Surface	8.12 ± 1.89	6.7 ± 3.17	2.58 ± 0.20
	Inside	64.33 ± 3.43	78.23 ± 3.77	40.23 ± 1.34
3 months	Surface	8.61 ± 1.09	7.2 ± 2.56	3.41 ± 0.69
	Inside	60.17 ± 4.28	75.66 ± 7.66	41.52 ± 4.54

Alginate, chitosan and Nys spectra (Fig. 3A–C), exhibit a strong and broad band at about 3430 cm^{-1} that results from overlapping of the O—H and N—H stretching vibrations of functional groups engaged in hydrogen bonds (Rao, 1963).

A comparison of the spectra of pure Nys, alginate with the spectra obtained of microparticles suggested that Nys might interact with alginate as some of the peaks within the range of 1000–1300 cm^{-1} in microparticles were either not apparent or were shifted in comparison to the spectra of the separate materials. Such interaction between Nys and polymer is likely to be due to hydrogen bonding, which could involve the C=O, C—C—O and O—C—C chemical groups (Sakeer, Al-Zein, Hassan, Martin, & Nokhodchi, 2010) Nys loaded microparticles did not show significant differences between microparticles and alginate (Fig. 3E–G and A). Nys loaded microparticles showed several characteristic peaks, 1163 cm^{-1}, 1464 cm^{-1}, 1746 cm^{-1}, and a broad band with dual peak at 2854 cm^{-1} and 2925 cm^{-1} this latter is in line with the poloxamer 407.

For chitosan, the N—H stretching and bending vibrations in polyamide appear at 3438 cm^{-1}. The C—O stretching vibration band appears in the normal region at 1650 cm^{-1}.

Fig. 4 shows the thermograms of raw materials, physical mixtures and microparticles. Nys presented a single, well defined endothermic peak at 165 °C corresponding to the sharp phase transition, above this temperature rapid decomposition takes place.

On the one hand the DSC curves for isolated polymers: alginate and chitosan; and its physical mixtures showed a broad endothermic peak between 50 and 130 °C. All physical mixtures (alginate-chitosan, alginate-Nys, alginate-Nys-chitosan) showed an exothermic peak between 220 and 260 °C. Moreover, physical mixtures of alginate-Nys and alginate-chitosan-Nys also showed an exothermic peak at 165 °C due to the presence of Nys, which reveals the absence of interactions between the entities.

Poloxamer 407 showed an endothermic peak at 59 °C and a broad endothermic peak between 150 and 270 °C. Its physical

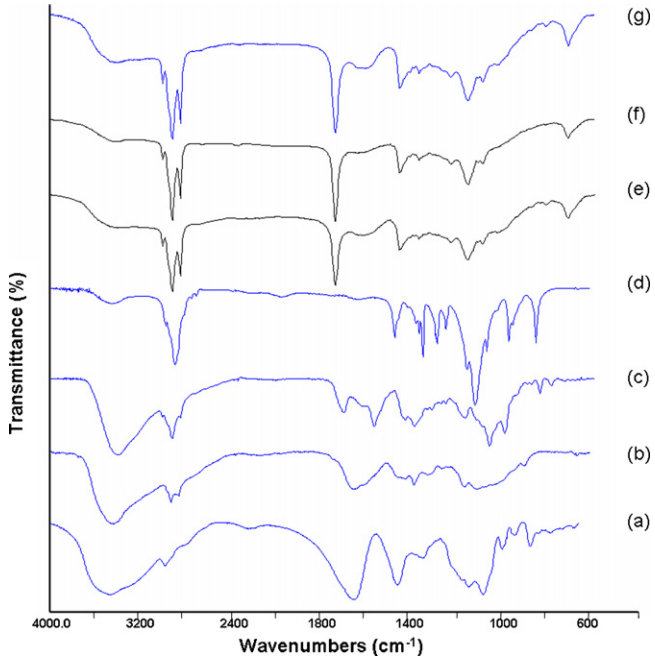

Fig. 3. FT-IR spectra of (a) alginate, (b) chitosan, (c) plain nystatin (Nys), (d) polaxamer 407, (e) nystatin loaded alginate microparticles, (f) Nys loaded chitosan coated alginate microparticles and (g) Nys loaded polaxamer 407 coated alginate microparticles.

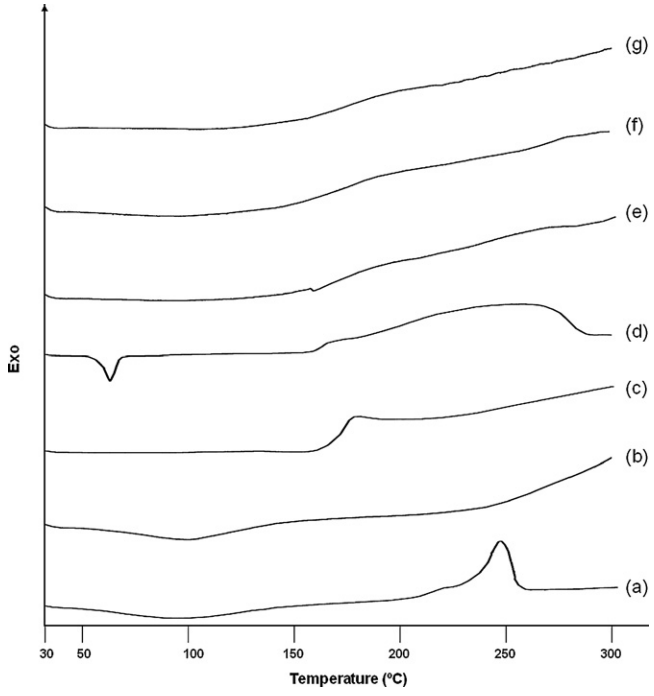

Fig. 4. Differential scanning calorimetry curves for (a) alginate, (b) chitosan, (c) plain nystatin (Nys), (d) polaxamer 407, (e) Nys loaded alginate microparticles, (f) Nys loaded chitosan coated alginate microparticles and (g) Nys loaded polaxamer 407 coated alginate microparticles.

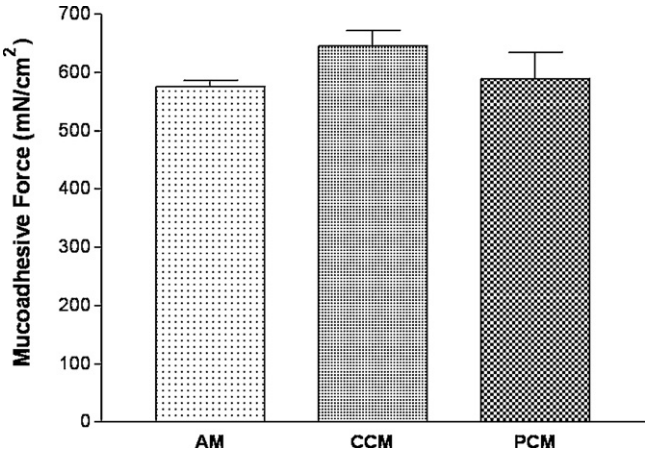

Fig. 5. Mucoadhesive force for alginate microparticles (AM), chitosan coated microparticles (CCM) and poloxamer 407 coated microparticles (PCM). Each bar represents the mean ± SD ($n = 3$).

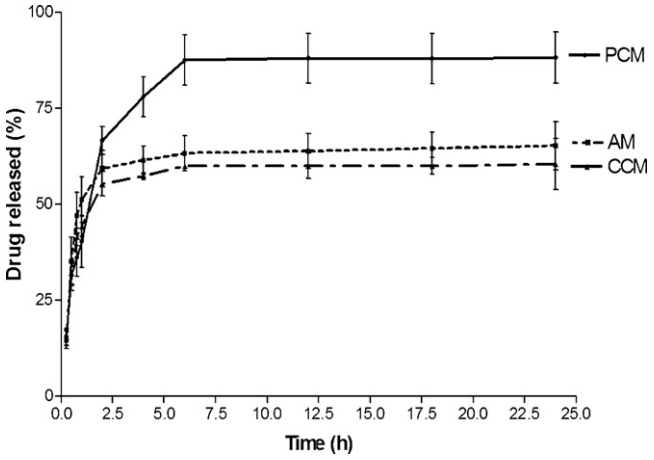

Fig. 6. In vitro release profiles of nystatin of alginate microparticles (AM), chitosan coated microparticles (CCM) and poloxamer 407 coated microparticles (PCM).

mixture with alginate showed a displacement of the endothermic peak of poloxamer at 55 °C and two exothermic peaks between 150–220 °C and 225–260 °C, respectively. However their physical mixture with alginate and Nys presented the melting endothermic of poloxamer shifted to 55 °C too, and three exothermic peaks between 150–175 °C, 190–215 °C and 240–260 °C, respectively.

Thermogram of uncoated and coated microparticles was similar. All of them showed a shoulder between 160 and 220 °C, and only in the case of uncoated microparticles, it can be observed a small exothermic peak moved to 155 °C corresponding with the presence of Nys. No differences in the DSC curves of the microparticles were detected after 3 months storage. These results, not shown for brevity, suggest the physical stability of the samples, at least for the examined time.

The use of alginate is limited due to its low physical stability in the presence of Ca^{2+}, chelating agents, monovalent ions and harsh environmental conditions (Smidsrod & Skjak-Braek, 1990). In order to improve the stability and effectiveness of encapsulation, coating has been widely reported to provide additional features. Two sorts of biocompatible mucoadhesive polymers with different chemical characteristics and mucoadhesion mechanism were selected for coating. Chitosan is a polycationic copolymer consisting of glucosamine and N-acetylglucosamine units that swells in contact with the acidic fluid and undergoes complete dissolution, its OH and NH_2 groups are considered essential for mucoadhesion (Valenta, 2005), moreover chitosan is also known to exhibit antimicrobial activity. Poloxamers are polyoxyethylene–polyoxypropylene–polyoxyethylene blocks polymers; they are thermosensitive polymers forming mucoadhesive gels at body temperature. The mucoadhesive properties of a vaginal delivery system are important for its influence on the permanence time of the drug on the vaginal epithelium. CCM exhibited the highest bioadhesive strength value (Fig. 5) slightly higher than PCM and AM, but no statistically significant differences were observed. It may be partly explained by the negative charge of mucin, a substance found in vaginal mucosa, as PCM and AM are also charged negatively (values of Zeta potential reported in Table 1) electrostatic repulsion forces would appear. But bioadhesion process is not yet well understood and other physicochemical process could be directly involved (Woodley, 2001).

3.3. Antimicrobiological efficacy

Without Nys the increasing yeast concentration was observed after 24 and 48 h confirming the capability of *C. albicans* to grow in SVF medium. Antimicrobial efficacy showed that AM and PCM did not produce any growth inhibition effect at 0 h, obtaining a yeast concentration of 2×10^6 CFU/mL approximately, in both cases. After 2 h of inoculum contact, a growth inhibition effect was observed for all assayed samples, corresponding to effectives Nys concentrations for AM and PCM. These results are in concordance with time required to Nys release from microparticles and reach the yeast membrane obtained in drug release studies (Fig. 6). Contrary, CCM were able to inhibit yeast growth from 0 h until the end of the study. In this case and take into account that at 0 h Nys had not reached the yeast it is possible to hypothesize that this yeast growth inhibition was due to an antifungal effect of chitosan as it has been reported by others authors (Cota-Arriola et al., 2011). In all cased Nys included in microparticles exhibited a marked fungicidal activity, since there was a drastic reduction on the *C. albicans* initial loading, thus a quick action onset would take place.

3.4. Drug release study

Fig. 6 shows the different release profiles of Nys from alginate, chitosan and poloxamer 407 microparticles. The release profiles indicate a two step process. The initial step shows a burst release which can be attributed to the surface associated drug, followed by a slower sustained release phase for all Nys loaded microparticles.

Statistically differences were observed between PCM and the other two types ($p < 0.05$). Whereas 80% drug was released from PCM within 4 h, only 60% drug was released from AM and CCM. In spite of lack of statistical differences ($p > 0.05$) between CCM and AM, there is a trend of enhancement in Nys release from AM, being the CCM which exhibited the slowest drug release. This delayed and sustained release of Nys from CCM compared with AM probably reflects the strengthening of the microparticles by ionic interaction between chitosan (NH^{3+}) and alginate (COO^-) ions. Contrary in PCM microparticles the hydrophilic portions of the poloxamer 407 probably contribute to the increased solubilization of Nys, in this sense it has been reported that the largest factors that appear to be contributing to solubilization of Nys by poloxamers are the number of micelles that these would form in the solution and the micellar core surface area accessible to Nys molecules (Croy & Kwon, 2004), due to the amphiphilic structure of poloxamers acting like a self-emulsifying system.

The kinetic model that best described the experimental data was selected based on the lowest Akaike's Information Criterion value as an indicator of the model's suitability for a given dataset. The release curve of Nys loaded into the three microparticles fitted to the first order kinetic model, and thus, statistically, described

Table 3
Mean parameters, obtained after fitting the release data from the formulations to different release models, where K is the release rate constant, R_∞ is the total percentage drug released at the end of the experiment, CV is the precision of the parameter estimation expressed as coefficient of variation, AIC is the Akaike's Information Criterion for first order model.

Microparticle	Parameter	Zero order	First order	Higuchi	Weibull	Korsmeyer-Peppas
AM	k	1.01 (% × h^{-1})	1.60 h^{-1}	20.55 (% × $h^{-1/2}$)	–	44.09 h^{-n}
	$\%R_\infty$	–	62.67%	–	68.77	–
	n	–	–	–	–	0.15
	t_d	–	–	–	1.01 h	–
	β	–	–	–	0.72	–
	CV	75.28%	8.65% (k) 2.49% ($\%R_\infty$)	21.42%	8.93% ($\%R_\infty$) 33.69% (t_d) 31.82% (β)	10.61%(k) 35.48% (n)
	AIC	77.65	35.57	70.48	55.25	57.58
CCM	k	1.08	1.45 h^{-1}	19.40 (% × $h^{-1/2}$)	–	40.38 h^{-n}
	$\%R_\infty$	–	59.26%	–	64.09	–
	n	–	–	–	–	0.17
	t_d	–	–	–	1.02 h	–
	β	–	–	–	0.73	–
	CV	63.00%	5.97% (k) 1.77% ($\%R_\infty$)	20.00%	6.20% ($\%R_\infty$) 23.34% (t_d) 22.15% (β)	9.68% (k) 29.03%(n)
	AIC	76.12	28.86	68.46	54.05	54.65
PCM	k	2.32	0.67[a] h^{-1}	25.95 (% × $h^{-1/2}$)	–	45.23 h^{-n}
	$\%R_\infty$	–	92.61[a]%	–	88.89	–
	n	–	–	–	–	0.25
	t_d	–	–	–	1.53 h	–
	β	–	–	–	0.87	–
	CV	43.23%	9.13% (k) 3.10% ($\%R_\infty$)	15.29%	2.97% ($\%R_\infty$) 9.85% (t_d) 8.59% (β)	12.53%(k) 22.53%(n)
	AIC	79.23	41.42	69.45	41.98	61.77

[a] Express statistical significant differences in the parameter estimated ($p < 0.05$).

the best release mechanism. Pharmacokinetics parameters are reported in Table 3. This means that Nys release from microcapsules 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. In order to compare the estimated first order parameters a one-way ANOVA was applied. The results showed statistically significant differences in the first order release constant (k) and in $\%R_\infty$ between PCM and the other ones ($p < 0.05$).

3.5. Ex vivo permeation experiments

Structural similarities between fresh and frozen-thawed vaginal mucosa tissues were shown (Fig. 7) confirming the tissue integrity prior to the permeation experiment. Both samples (fresh and frozen-thawed) show the standards mucosa sections: a non-keratinized stratified epithelium and underlying fibrous connective tissue or lamina propria with several structures (as blood vessels, sinus and lymphatic nodes). In both samples the epithelium

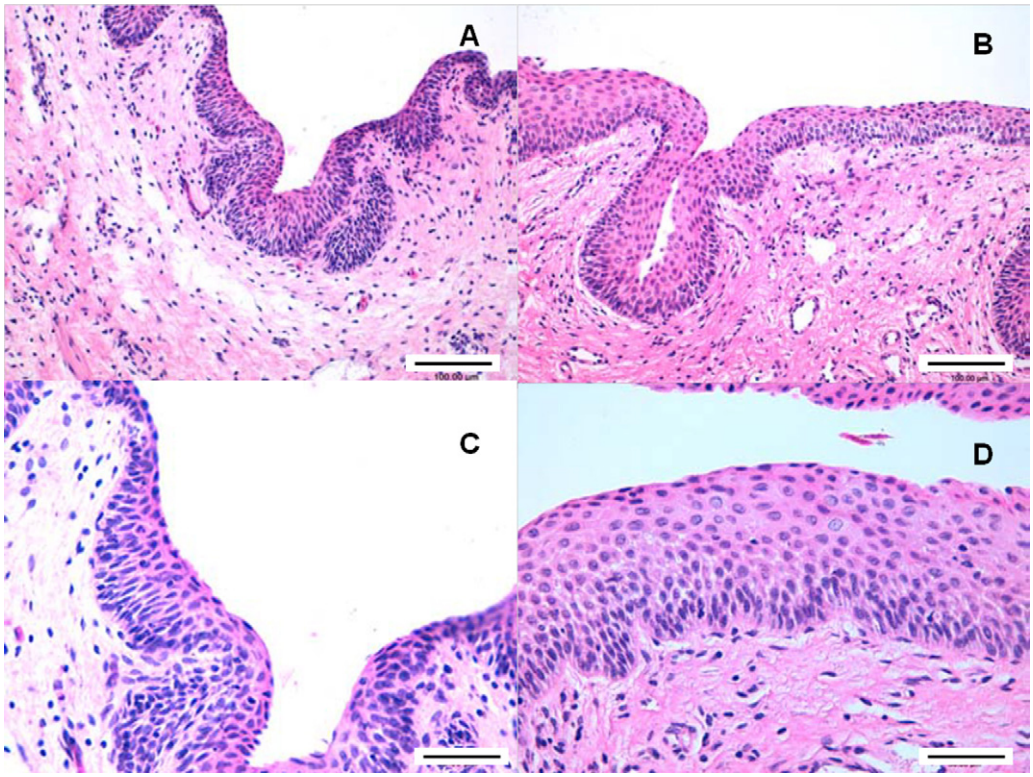

Fig. 7. Photomicrographs of porcine vaginal mucosa slices stained with haematoxylin–eosin technique (for histological studies) showing the morphological similarities before (A and C, respectively) and after (B and D, respectively) freezing process. Bar length of 100 μm for A, B, 50 μm for C, and 30 μm for D.

maintains its structure with several cellular layers without any discontinuity or intercellular spaces and tight cell junction.

The selectivity of the permeation assay was confirmed by the individual analysis of blank samples from porcine vagina and from the receptor side of the diffusion cells assuring that no interferences took place. No Nys amounts were found in the receptor compartment until the end of the experiment. Therefore, in any case the flux of permeation and any other permeation parameter could be estimated. The data showed that no significant Nys concentrations would be found in blood, based on the pharmacokinetics parameters reported (Groll et al., 2000) and the predictive equation which calculates the steady state concentrations after applying a transdermal formulation. The estimated flux required to achieve therapeutically concentrations would be higher than 1600 μg h/cm^2, far from the data presented in this work and thus assuring the safety of the potential treatment.

The amounts of Nys retained after 4 h with the Nys loaded microparticles were 170.01 ± 86.12, 147.48 ± 85.65 and 180.47 ± 39.49 μg/g tissue for AM, CCM and PCM respectively, corresponding to 14.92, 12.94 and 15.83% of the dose. Levene test showed the homoscedasticity of the retained amounts ($p = 0.333$), thus a one-way ANOVA was applied to check is there was differences among the different formulation assayed. Statistical test did not show statistical differences ($p = 0.815$). The type of polymeric coating did no exhibit any difference in the Nys retained amounts. Nevertheless according to the antimicrobial test and the retained amounts of Nys in the mucosa, the quantities of Nys were more than enough to ensure its efficacy.

4. Conclusions

In this paper three types of microparticles, including uncoated alginate, chitosan and poloxamer 407 coated microparticles were successfully prepared by an emulsification/internal gelation method. Size of the systems increased by incorporation of the drug, but not after storage periods except for Nys loaded PCM. These microparticulate systems showed spherical shape and slightly rough surface. Optimal values of percentage yield, loading capacity and encapsulation efficiency were obtained. Equally release studies gave a good fit to first order kinetic model indicating that Nys release from microcapsules 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.

The ability of these systems, to adhere to the vaginal mucosa has great appeal for the treatment of localized infection. Moreover Nys loaded microparticles exhibited a clear inhibition effect on the *C. albicans* growth, specially CCM suggesting their clinical potential use, once assured the security of the treatment by the permeation studies performed.

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