Mucoadhesive assessment of different antifungal nanoformulations

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1. Introduction

Oral candidiasis (OC) is an important opportunistic fungal infection caused either by bacteria, viruses or fungus, where *Candida albicans* is the most common cause [1]. This is a commensal fungus present in the mouth of 40%–65% of healthy adults, but it can cause an infection in immunosuppressed patients or those receiving anticancer radiotherapy. AIDS and other immunodeficient conditions that emerged in recent years, have paved the way for the reappearance of oral mucosal infections [2, 3]. In terms of treatment, polyenes and azoles are still the most used antifungal agents. However, most of these drugs present a poor oral absorption and, consequently, require a high frequency of administrations. One of the mostly used antifungal agent is nystatin (Nys) which is commercially available as a suspension [4]. This formulation is associated with a low or very limited systemic bioavailability. There are other alternative formulations present on the market, such as mouth solutions, rinses, tablets or gels, for the treatment of OC. However, even these formulations are unable to maintain the concentration of the drug in the saliva for a prolonged period of time [1].

Therefore, the major challenge is to improve the retention of the antifungal agents on oral mucosa, and the encapsulation into mucoadhesive drug deliv-
ery systems may be a possible strategy to achieve this objective. Mucoadhesive drug delivery systems, like oral gels (OGs) and films (Hydroxypropyl methyl cellulose, Polycarbophil, Ethyl cellulose, etc) and polymeric nanoparticles (NPs) based on Hyaluronic acid, alginate (Alg), polyactic acid (PLA) and poly lactic-co-glycolic acid (PLGA), have the capacity to adhere to the buccal mucosa and remain in place of action for a considerable period of time when compared with conventional delivery systems, like solutions or suspensions [5–7].

In this work, we have designed several types of mucoadhesive NPs, more specifically Alg, PLA and PLGA NPs, containing-Nys as model drug. The drug-loaded NPs were included in toothpaste (TP), OG and oral films (OF), for buccal administration. All the formulations have been fully characterized including their mucoadhesive capacity. The mucoadhesive capacity of the nanoformulations was tested in terms of their interaction with both mucin dispersions and mucus producing HT29-MTX cells. The effect of the formulations on the permeability of Nys through an HT29-MTX cell layer using the Transwell® setup was also assessed.

2. Materials and methods

For the development of NPs, sodium Alg from brown algae, acquired from Sigma-Aldrich (St. Louis, USA), PLGA (50:50) and PLA both obtained from Purac (Gorinchem, Netherlands) and Pluronic® F-127 obtained from Sigma (St. Louis, USA) were applied as encapsulation materials. For the preparation of the semi-solid formulations, the reagents used were sorbitol and sodium lauryl sulfate obtained from José M.Vaz Pereira S.A. (Lisbon, Portugal), glycerol and citric acid both acquired from Scharlau (Barcelona, Spain), calcium carbonate from Merck (Darmstadt, Germany), microcrystalline cellulose from Fagron (Barcelona, Spain), xanthan gum and sodium fluoride from Sigma-Aldrich (St. Louis, USA), hydroxypropyl methylcellulose (HPMC) from Norsk Medisinaldepot (Oslo, Norway) and glycerol from Apotek Produksjon AS (Oslo, Norway). Mucin from porcine stomach type II was obtained from Sigma-Aldrich (St. Louis, USA). Pure Nys was kindly donated by Angelini Pharmaceutical (Dafundo, Portugal). All other reagents were of analytical grade.

2.1. Development of the Nys-loaded NPs

PLGA and PLA NPs were prepared using the same method and according to previous work done by our group [8, 9]. Basically, an emulsion was prepared dissolving the PLGA or PLA with a concentration of 20 mg ml$^{-1}$ and Nys with a final concentration of 10 mg ml$^{-1}$ in a mixture aceton: ethanol (8:2, v/v). This solution was sonicated for a period of 15-20 min. After sonication, the solution was added dropwise to a 0.1% (w/v) aqueous solution of Pluronic® F-127, under a constant stirring speed of 800 rpm (figure 1). Subsequently, the organic solution was removed using a rotary evaporator at 60 °C for 20 min until NPs dispersed in the aqueous phase were formed [10].

The Alg NPs were prepared according to an emulsification/internal gelation method [11], also used by our group earlier [12]. In shorthand, the loaded-NPs are prepared from two different solutions. The aqueous solution is made by 50 ml of Alg solution (2%, w/v) and 3.5 ml of calcium carbonate (aqueous dispersion of insoluble and ultrafine calcium carbonate crystals at 5%, w/v). The resulting dispersion was emulsified within 50 ml of paraffin oil containing 1.5 ml of Span 80 by impeller-stirring homogenization (1600 rpm). After 15 min of emulsification, 20 ml of paraffin oil containing 300 µl of glacial acetic acid was added to the mixture and the stirring continued for 60 min to trigger the solubilization of the calcium from the carbonate complex, and concomitant gelation of the Alg NPs (figure 1). The oil-dispersed Alg NPs were recovered by centrifugation at 26230 × g with a Z36-HK high speed centrifuge (Hermle Labortecnik, Wéhingen, Germany) coupled with dehydrating solvents in acetate buffer to clean the NPs [12].

2.2. Characterization of the Nys-loaded NPs

2.2.1. Particle size analysis

The mean particle size and polydispersity index (PI) of the hydrated NPs (diluted aqueous suspensions, 1:20) were measured using dynamic light scattering with a Coulter Nanosizer Delsa NanoTM C (Coulter Beckman, Fullerton, CA, USA). All experiments were made at room temperature, with a detection angle of 165° and performed in triplicate [10].

2.2.2. Zeta potential (ZP) analysis

The ZP was measured in the same equipment and the same dilution factor described for size analysis but using the electrophoretic mobility technique. Each analysis was carried out at room temperature with a detection angle of 90° and in triplicate [10].

2.2.3. Determination of the encapsulation efficiency (EE)

After the preparation of NPs, a pellet was obtained by centrifugation at 23000 × g for 15 min in the case of PLGA and PLA NPs, and at 38000 × g for 10 min in the case of Alg NPs. Subsequently, the amount of free Nys was determined using direct quantification. The particles were disrupted and released Nys was analysed spectrophotometrically at a wavelength of 307 nm. To obtain a value of the amount of drug present inside the NPs, all measurements were within an established linearity range of 0.4–100 µg ml$^{-1}$ ($R^2 = 0.995$) for PLA and PLGA NPs and 0.4–50 µg ml$^{-1}$ ($R^2 = 0.996$) for Alg NPs. For the calculation of EE, equation (1) was used.
EE(%) = \frac{\text{Quantity of drug} - \text{Quantity of drug present in the supernatant}}{\text{Quantity of drug}} \times 100. \quad (1)

All the measurements were made in triplicate.

2.3. Development of the semi-solid formulations
The production of semi-solid formulations was achieved according to previous works done by our research group [12]. All formulations were optimized regarding some parameters like viscosity and pH values. In the following, preparation methods are reported very briefly.

2.3.1. Toothpaste preparation
The TP is constituted of two different phases: the first phase is a homogeneous mixture of 0.65 g of xanthan gum mixed with a small volume of sorbitol dispersed in 15 g of glycerol. The second phase is citric acid buffer (pH = 5) containing sodium fluoride (13 mg ml⁻¹). These two phases were mixed for 45 min and then 10 g of calcium carbonate with microcrystalline cellulose was added under agitation over a period of 30 min. Finally, the mint essence and sodium lauryl sulfate were added to the semi-solid dosage form [12]. After the preparation of the TP, the Nys-loaded hydrated Alg NPs were implemented in the ratio of TP:NPs = 1:2 (w/w). Optimization conditions were determined in a previous work done by our group [10].

2.3.2. Oral gel preparation
The OG preparation involves the addition of a few drops of an aqueous solution of sodium hydroxide (NaOH) with a concentration of 0.1 M to an aqueous solution of Carbopol 940 at 1% (w/v). After the preparation of the OG, the Nys-loaded-PLA NPs were implemented in the ratio of OG:NPs = 1:4 (w/w).
2.3.3. Oral films preparation

The OF were formulated using HPMC as the film-forming polymer and by using the solvent casting method [13]. Briefly, an aqueous solution containing 8% (w/v) of HPMC and 2% (w/v) glycerol was prepared and PLGA, PLA and Alg Nys-loaded-NPs were added to a final concentration of 7%, 4.45% and 0.72% (w/v), respectively. Then, the final volume was adjusted with water to 100 ml and left for an additional 30 min stirring to ensure a homogeneous distribution of the NPs in the final formulation [13–15]. After preparation, the homogeneous suspensions were allowed to rest for a period of 4 h to remove all the air bubbles and poured onto a clean levelled glass plate of the Coatmaster 510 (Erichsen GmbH, Essen, Germany), where a knife with a gap-opening of 1000 µm ensured an even spreading, and allowed to dry overnight at room temperature to obtain a larger film sheet. After complete drying, the film sheet was peeled off the glass plate and cut into pieces of single dose units of 2 × 2 cm and stored in desiccators to avoid moisture fluctuations. The prepared films presented a transparent and homogeneous appearance, even and flexible structure.

2.4. Characterization of the semi-solid formulations

2.4.1. Physical properties of the TP, OG and OF

The viscosity of the semi-solid formulations was assessed using a Rotating Viscometer Brookfield (Middleboro, MA, USA). The formulations were introduced into a 50 ml beaker and the spindle was positioned and rotated at a predetermined rpm value. The measurements were made with spindle 4 and rotation speed between 50–100 rpm in the case of the TP formulations and with the spindles 3, 5 and 6 with a rotation speed between 12–100 rpm in the case of the OG formulations. The viscosities and torques were recorded. Also, pH values (827 pH Lab, Metrohm, Herisau, Switzerland) and organoleptic aspects were assessed.

Also, the spreadability was assessed using Parallel-plate method [16]. The spreading capacity was measured 12 h after preparation by measuring the spreading diameter of 1 g of the OG between two glass plates, with a diameter of 15 cm. The mass of the upper plate was standardized at 125 g. To calculate the spreadability the following equation was used (equation (2)).

\[ S = m \times \frac{l}{t} \]

Where the \((S)\) represents spreadability capacity of the formulation, \((m)\) is the weight (g) tied on the upper plate, \((l)\) is the length (cm) of the glass plates, and \((t)\) is the time taken (s) for the plates to slide the entire length.

The OF was characterized with respect to thickness, weight, the water content and Nys content. Film thickness was assessed using a Micrometer ranging between 0–25 mm (Cocraft®, Manchester, UK). Water content was determined using the moisture Analyzer MA 30 (Sartorius AG, Goettingen, Germany). For the quantification of Nys present in each film, the films were dissolved in 10 ml of water during a period of 30 min. After this period, the samples were analysed using the UV-2550 UV–VIS spectrophotometer (Kyoto, Japan) at 307 nm wavelength.

Measurements were carried out on 10 pieces with a dimension of 2 × 2 cm for each film.

2.5. In vitro assays

2.5.1. In vitro mucoadhesion test

Mucoadhesion from the interaction of the samples with a 3% (w/v) dispersion of mucin in phosphate buffer (pH 7.4) was assessed [16]. Briefly, the mucin dispersion was evenly spread on the top of two different pieces of filter papers (25 µl on each) with an inert backing layer (Whatman® Benchkote, St. Louis, USA). In our previous work, this interaction with buccal tissue was already established using porcine oral mucosa [22]. The pieces of filter paper presented a dimension of 1.3 × 1.3 cm. Both pieces were attached with double sided adhesive tape; one of them was placed in the lower stationary part of a TA-XT2i Texture Analyzer (Stable Micro Systems, Surrey, England), and the other was attached to a specially designed larger, upper, movable part (11 cm × 11 cm).

To test the OF 1 × 1 cm squares were used whereas 0.01 g was the amount taken for OG and TP assessment. The samples were placed between the upper and the movable part, and the movable part was lowered until it touched the sample. Based on previous works, a preload force of 200 g was applied for a period of 100 s, after which the upper part was raised with a speed of 0.01 mm s⁻¹ [17]. The measurement was repeated ten times for each formulation and compared to the respective controls without NPs. In addition, the intermediate precision was tested by repeating the measurements the following day. The measurements were also repeated with pure buffer instead of mucin dispersion to allow distinction of the unspecific adhesion of the formulations from the mucin interaction. Also, this analysis was made only with filter paper in order to individual study the interaction of the formulation with the mucus layer.

Displacement and force of detachment were recorded. Based on the obtained force versus time curve, peak force \((F_{\text{max}}, \text{g})\) and the work of detachment (area under the peak, \(\text{AUC}, \text{g s}^{-1}\)) were obtained. The values obtained from testing the samples versus the mucin dispersions are designated the general mucoadhesion, and the values obtained from testing samples versus the phosphate buffer saline (PBS) are designated the unspecific adhesion. To estimate the mucin interaction for the semi-solid formulations, the unspecific adhesion was deducted from the general mucoadhesion [17].

After the in vitro mucoadhesion test, the mucoadhesive capacity of the most promising OF, OG and
TP formulations were also assessed using a retention model with HT29-MTX cells.

2.5.2. Cell culturing

The HT29-MTX cell-line was kindly provided by Dr Thécla Lesuffleur (INSERM UMR S 938, Paris, France). This cell line was selected because these cells produce mucus. These mucus-secreting cells have been adapted to and cultured for several passages in medium containing 10−6 M MTX and reversed for several passages in drug-free medium [18]. They do not need to be maintained in media containing MTX in order to differentiate after confluency.

Medium for cell growth: Dulbecco’s Modified Eagle’s Medium with high glucose (DMEM), L-glutamine, sodium pyruvate and phenol red, pH 6.8–7.2 (sodium bicarbonate buffer) (Sigma-Aldrich, St. Louis, USA), further supplemented with 10% inactivated Fetal Bovine Serum (Sigma–Aldrich, St. Louis, USA), Penicillin (100 units ml−1) with Streptomycin (100 µg ml−1) (Sigma-Aldrich, St. Louis, USA) and Gentamicin (50 µg ml−1) (Sigma-Aldrich, St. Louis, USA).

The cells were incubated at 37 °C under an atmosphere of 5% of CO2. For the preservation, the cells were passaged before reaching 80% of confluency with a solution of trypsin-EDTA (Sigma–Aldrich, St. Louis, USA). The medium was changed every two days.

Before the experiments, the cells were counted using a hemocytometer and seeded at an initial density 24000 cells cm−2. For the rising model experiments, cells were seeded on Nunclon® cell culture dishes (Sigma-Aldrich, St. Louis, USA) with a culturing area of 78.5 cm2 and cultured for 3 weeks. In these culture conditions, the cells are confluent after about seven days of culture and begin to differentiate, postconfluently, into a mixed population of mucus-secreting goblet cells and enterocytes [19]. Cells from passage number 26, 27, 28 and 29 were seeded, in triplicate, for the mucoadhesion test of the TP, the OG, the OF and TP formulations were also assessed using a retention model with HT29-MTX cells.

For the permeation and toxicity experiments HT29-MTX cells from passage number 27 were seeded on uncoated polycarbonate Transwell filter inserts (Costar, Corning) with a 0.4 µm pore size and a cell growth area of 1.12 cm2. The volume of the donor chamber and the acceptor chamber were 0.5 and 1.5 ml, respectively. The experiments were carried out after 3 weeks of cell culturing and in triplicate.

2.5.3. Rising model—mucoadhesive capacity

After 3 weeks of culturing, culture medium was removed from the dishes containing the cell monolayer and the cells were washed twice with Hank’s balanced salt solution (HBSS), modified, with 1.26 mM CaCl2 × 2 H2O, 0.81 mM MgSO4 and 4.17 mM NaHCO3, pH 7.2–7.6 (Sigma-Aldrich, St. Louis, USA).

In one side of the dish wall, a v-shaped indent was cut to allow HBSS to flow through. The dish was then mounted in the chamber and left there to acclimatise with the flow rinsing over (flow rate: 0.399 ml min−1) for a period of 10 min [20].

Afterwards, the flow was turned off and the samples were placed on top of the cell monolayer. In the case of the OF, film pieces samples of 2 × 2 cm were dipped in HBSS to wet and immediately attach onto cells. For the OG and the TP, 100 mg of samples were placed using a syringe. The samples were left on top of the cell monolayer for 2 min to let the samples attach onto the mucus present on the monolayer. The flow was turned on again at a flow rate 0.399 ml min−1. Four nozzles were used, yielding a total flow of 1.6 ml min−1. At pre-determined time points, the HBSS buffer that had rinsed over the formulation on top of the monolayer was collected and the amount of Nys determined, using the same UV method as described earlier (section 2.2.3).

2.5.4. Permeation and toxicity experiments

In this assay, we used the Transwell® setup. Before the experiments were carried out, the transepithelial electrical resistance (TEER) was measured with a Millicell ERS-2 Voltohmmeter, in order to verify the initial integrity of the cell monolayer.

Then, the cell monolayers were washed with HBSS and incubated with 0.5 ml samples in the case of OG, TP and pieces of 1 × 1 cm with 0.13 ml of HBSS buffer for the OF. Additionally, three control samples consisting of 0.5 ml of HBSS, 0.13 ml of HBSS buffer (presenting the same condition as the OF) and 0.5 ml solution of free Nys with a concentration of 1 mg ml−1 were placed in the donor chamber. Each sample was incubated in triplicate. 1.5 ml of HBSS buffer was placed in the acceptor chamber in all cases. After 2 h in a shaking incubator (60 rpm), the samples present in the donor chamber and the HBSS buffer present in the receptor chamber were recovered, and the two chambers, were washed with HBSS.

The samples from the receptor chambers were analysed using the same UV method as described earlier (section 2.2.3) to quantify the amount of Nys that had permeated the cell monolayer. After permeation of Nys, the permeability of carboxyfluorescein as well as TEER was measured in order to monitor any changes in the integrity of the cell monolayers caused by the formulations, i.e. toxicity.

For the carboxyfluorescein method, the samples present in the donor chamber and the HBSS buffer present in the receptor chamber were removed, and the two chambers including the filters containing the cell monolayers were washed with HBSS. 0.5 ml of a 15 µM solution of carboxyfluorescein in HBSS and 1.5 ml of HBSS buffer were introduced to the donor and the acceptor chambers, respectively. The plates were placed in a shaking incubator, 37 °C and 60 rpm. After 2 h, the amount of permeated carboxyfluorescein in the acceptors chambers were measured in triplicate using the fluorescence plate reader Victor® at λex 485 and λem 520 nm.
3.1. Characterization of Nys-loaded NPs

As demonstrated in our previous work, Nys is one of the most common antifungal drugs that present several problems in terms of solubility and permeation in the buccal mucosa [22]. So, the encapsulation into NPs systems and the implementation of these systems into semi-solid formulations will work as a vehicle of Nys [1]. The results obtained regarding the production of the Nys-loaded-NPs showed that they were successfully produced, presenting a mean size between 333–888 nm and with a surface charge between −15.65 and −43.62 mV (table 1), the polydispersivity index demonstrated that the PLA and PLGA NPs were more monodisperse than the Alg NPs. Also, the results regarding EE by direct determination showed that PLA, PLGA and Alg NPs entrapped as much as 71 to 95% of the added Nys, respectively as can be observed in table 1. This confirmed that the association established between the polymers and the drug is very high and it can be different when using diverse methods of production, since the hydrophobic polymer (PLA) showed a better EE to the purely water-soluble drug (Nys), increasing the possibility of achieving an efficient therapeutic effect. The chemical interaction established between PLA and Nys might be higher than for the other two polymers. Also, the influence of the chemical interaction of the polymers with the drug can be observed in the release profile, done in another work by our group, where the PLA and the PLGA NPs demonstrated a more controlled release profile in PBS buffer (pH 7.4) as compared to the Alg NPs [22].

3.2. Characterization of the semi-solid formulation with Nys-loaded NPs

After the characterization of NPs, they were implemented in the semi-solid formulation respecting certain NPs to formulation ratio, as described in section 2.3. The viscosity values of TP and OG with Nys-loaded NPs changed when compared with the same formulation without the NPs. These effects can be important since the viscosity can be directly correlated with the mucoadhesive capacity of the formulations. As observed in table 2, Alg NPs increased the viscosity of the TP formulation. It is described that formulations with high viscosity would present a better adhesive capacity and vice-versa [23]. Regarding the pH values, it can be observed that NPs will inflict a slight change in the pH value. Despite this, the values remained within the pH reference values based on commercial TP where the pH values normally recommended are 7.4 and it can be different when using diverse methods of production, since the hydrophobic polymer (PLA) showed a better EE to the purely water-soluble drug (Nys), increasing the possibility of achieving an efficient therapeutic effect. The chemical interaction established between PLA and Nys might be higher than for the other two polymers. Also, the influence of the chemical interaction of the polymers with the drug can be observed in the release profile, done in another work by our group, where the PLA and the PLGA NPs demonstrated a more controlled release profile in PBS buffer (pH 7.4) as compared to the Alg NPs [22]. The full characterization of all NPs was already published by our group, including release profiles and toxicity studies [22].
and re-mineralize when the mouth is alkaline [12]. In the case of the OG, the pH value must be between the interval of 4 and 5.5 so that the development of the teeth will not be hampered [24].

The different OF were also characterized in terms of chemical and physical properties (table 2). The films varied in weight, thickness, and in drug content where it can be observed that using NPs as vehicle for Nys increased the quantity of drug that was present in the films. This will increase the quantity of Nys present at the site of action. The residual water content in all films was less than 20% of the film weight.

3.3. **In vitro** assays

For the first test the mucoadhesive capacity of all formulations was measured using a mucin dispersion and a texture analyser. The maximum peak force and AUC were recorded. Results are presented in figure 2. The OF with PLGA NPs presented the highest value of general mucoadhesion. Regarding the unspecific adhesion, we observed that the incorporation of the NPs in the semi-solid formulations of the OF and the TP increased the peak force of the adhesion. The inclusion of NPs in the TP was beneficial for mucoadhesion, which can be influenced by many factors, one of them being the viscosity of the formulations and, as concluded before, the incorporation of NPs will increase the viscosity (see table 2). Also, when comparing the values of the different OF formulations we observed that different polymers constituting the NPs influenced the adhesion capacity in different ways similarly to other previous works [25]. In the case of the OG, the formulations containing NPs did not present any statistical significant differences when compared with the OG without the NPs.

The general mucoadhesion is a function that combines both the unspecific adhesion and the mucin interaction [26]. The estimated mucin interaction is generally a complex concept, influenced by degree of hydration of the formulations, the diffusion ability of the polymers and their capacity to engage in entanglements and intermolecular bonding with the mucin. The real value of the mucin interaction can be found by deducting the unspecific adhesion of the formulations (obtained from testing film versus buffer) from the general mucoadhesion (testing the formulations versus the mucin). As can be observed in figure 2(A), in all cases the general mucoadhesion was higher than the unspecific adhesion, indicating interactions between the polymer systems, semi-solid formulations and the mucin. The results obtained in terms of the maximum peak force correlated with the AUC values (figure 2(B)).

We observed that the TP with Alg NPs, the OG with PLA NPs and the OF with PLGA NPs were the best formulations in terms of achieving the highest interaction with mucin, when compared with the corresponding formulations without NPs, presenting values for the general mucoadhesion of maximum force of 290, 297 and 474 g, respectively. Moreover, the OF with PLGA NPs presented a significantly higher interaction with the mucin compared to all the other formulations ($p < 0.05$).

Before carrying out the rest of the *in vitro* assays, the HT29-MTX cells were grown for a period of three weeks on cell culture dishes, which is the time necessary to form a confluent monolayer of cells that will secrete mucus [27]. Considering this growth rate, several pictures from the moment of seeding, after a period of two days, one week, two weeks and in the moment of the experiments (three weeks) were taken (figure 3).

Regarding the rinsing model, three different formulations and a solution of free Nys to serve as a control were tested for mucoadhesive capacity. Comparing the results presented in figure 4, it can be observed that all the formulations increased the retention of Nys at the mucus-surface as compared to the solution. The amount of Nys attached to the cells after a period of 60 min under constant flow was $4 \pm 3\%$ for unformulated Nys (control samples). This value
Figure 3. Growth rate of the HT29-MTX cells after seeding (A) - after two days; (B) - after one week; (C) - after two weeks; (D) - in the moment of the experiments. The images were taken under the optical microscope with obj. 20. The scale bar equals 100 µm.

Figure 4. Retention of Nys to the HT29-MTX cell monolayer during continuous rinsing with saliva substitute (HBSS) at 1.6 ml min⁻¹ (n = 3).

Figure 5. TEER values of the HT29-MTX cell monolayers before (●) and after (○) the interaction with the formulations and % of variation (n = 3).
increased to 27 ± 7%, 19 ± 7% and 10 ± 1% for OF, TP and OG, respectively. The differences observed in figure 4 are statistically significant ($p < 0.001$) from control (5 min until 35 min) for the TP formulation, at 20 and 25 min for the OG formulation ($p < 0.05$) and at all time periods for the OF formulations ($p < 0.001$). The results showed that the mucoadhesive properties were improved when the NPs were present.

TEER measurement were made before and after incubation with the formulations. The results obtained are presented in figure 5. The values before the experiments were carried out are in the range of 200–300 Ω · cm², which are considered standard values [28]. This initial TEER can be compared to the values measured after the experiments had been carried out, to assess any toxic effects of the pre-incubation with formulations and free Nys compared to HBSS controls. The results are compiled in figure 5. It can be observed that the formulation of OF and the controls (solution of free Nys and HBSS buffer) did not influence the barrier integrity of the HT29-MTX cells monolayers. However, the TEER values decreases after pre-incubation with OG and TP. The pre-incubation with TP with Nys caused a TEER drop of 47% from the initial value, TP with Alg NPs caused a drop of 44%, OG with Nys caused a drop of 38% and OG with PLA NPs caused a drop of 27% from the initial value. In the case of the OF the results demonstrated an increment of the TEER value of 17% for the OF with Nys and 42% for the OF with PLGA NPs. These values demonstrate that the presence of the NPs will decrease the effects caused in the cells monolayer decreasing the side effects of the formulations. These results can be explained since the two formulations present a certain viscosity and the removal of the formulations to measure the TEER value can influence the integrity of the monolayer and remove some cells causing fragmentation in the monolayer. Also, some other explanations can be the interactions of some of the ingredients (like surfactants) that can compromise the monolayer. The paracellular permeability carboxyfluorescein confirmed these results. The amount of carboxyfluorescein that permeated the cell monolayers did not increase after pre-incubation with the OF formulations, compared to HBSS control, while pre-incubation with OG and TP formulations increased the permeability (figure 6).

In general, the formulations showed a high degree of biocompatibility, indicating low toxicity, since the amount of carboxyfluorescein that permeated was in all cases below 15% (figure 6). These results are in accordance with previous work where it was observed in a safety assessment study that the NPs did not interact with the natural growth of a fungus [22]. Although permeability enhancement is not the same as toxicity, a correlation between the two properties is common [28]. It can also be concluded that the use of NPs in all cases decreased the toxicity of the drug when compared with the respective formulation containing free Nys (figure 6). Therefore, we may advance with the assumption that the formulations should not have a potential toxicity effect for the cells, besides that caused by viscosity, but further studies must be conducted.

In the case of the permeation experiments, the results showed that the formulations increased the percentage of Nys that permeated the cell monolayer, achieving results of 26 ± 1%, 11 ± 1% and 17 ± 1% for the TP, OG and OF formulation, respectively, when compared to free Nys solution (2.63 ± 0.20%).
4. Conclusion

This study concludes that the bioadhesive NPs implemented in three different semi-solid formulations can be a promising approach to increase the efficacy of the buccal administration of Nys in the treatment of yeast infections of the oral mucosa. We observed that PLGA, PLA and Alg NPs were able to efficiently encapsulate Nys and also to improve the interaction of this antifungal drug with mucin and HT29-MTX cells. When focusing on the semi-solid formulations with the NPs, it can be observed that the OF with the PLGA NPs was the most promising formulation in terms of mucoadhesive capacity followed by the TP formulation with Alg NPs and the OG with PLA NPs. This study also suggested that these formulations may be non-toxic/non-irritating, but future studies also including natural products will be made in the near future.

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References

[1] Roque L, Molpeceres J, Reis C, Rijo P and Pinto Reis C 2017 Past, recent progress and future perspectives of nanotechnology applied to antifungal agents Curr. Drug Metab. 18 280–90
[19] Lesuffleur T, Barbat A, Dussaulx E and Zeebbaum A 1990 Growth adaptation to methtoxate of HT-29 human colon carcinoma cells is associated with their ability to differentiate into columnar absorptive and mucus-secreting cells Cancer Res. 50 6334–43