

Doctoral Program in Health Sciences D420

***Innovative nanotechnology formulations for
the treatment of yeast infections in oral
mucosa***

**Doctoral Thesis presented by:
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Innovative nanotechnology formulations for the treatment of yeast infections in oral mucosa

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CERTIFICAN:

Que la memoria para optar al Grado de Doctor, elaborada por Luís Miguel Vasques Roque, cuyo título es:

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ha sido realizada bajo su dirección y que reúne todos los requisitos necesarios para su juicio y calificación.

Y para que así conste, firman el presente certificado en Alcalá de Henares a dos de julio de dos mil dieciocho.

Catarina Pinto Reis

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To my parents, family, girlfriend and all my friends.

*“Without continual growth and progress, such words
as improvement, achievement, and success
have no meaning.”*
--Benjamin Franklin

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Resumen

La candidiasis oral es una infección provocada por hongos oportunistas que generalmente afecta a personas con inmunodeficiencia, bien por una infección viral o por un síndrome asociado a otras causas. Sin embargo, en algunos casos pueden verse afectadas personas sanas. Aunque *Candida albicans* es el principal agente patógeno causante de esta enfermedad, las infecciones por otras especies de *Candida*, como *C. krusei*, *C. glabrata* y *C. dubliniensis* también se han relacionado con la candidiasis oral. A pesar de los progresos en el tratamiento, polienos (como la nistatina) y azoles siguen siendo los agentes antifúngicos más utilizados para el tratamiento de estas infecciones. No obstante, la mayoría de estos agentes presentan algunas desventajas tales como la mala absorción oral del fármaco, el contenido en alcohol de las formulaciones y la necesidad de una administración muy frecuente.

Por lo tanto, el principal desafío en el tratamiento de las infecciones orales por *Candida* consiste en el aumento de la penetración y la permanencia de los antifúngicos en contacto con la mucosa oral o mucosa bucal. Para ello, la tecnología de la encapsulación constituye una posible estrategia. Así, el objetivo principal de esta Tesis ha sido el estudio de la encapsulación de agentes antifúngicos en varios tipos de partículas poliméricas con propiedades mucoadhesivas, incorporadas en formulaciones de aplicación bucal como pasta de dientes, gel oral y películas orales (sin alcohol) con la finalidad de aumentar su eficacia.

En este contexto, cabe destacar que tres de las formulaciones de nanopartículas evaluadas, nanopartículas de ácido poliláctico, de ácido poli(láctico-co-glicólico) y de alginato cargadas con nistatina, uno de los medicamentos antifúngicos más utilizados en el mercado, presentan ventajas por su tamaño de partícula, carga superficial y eficacia de encapsulación del fármaco. Cuando dichas nanopartículas se incorporan en una formulación semisólida se obtienen perfiles de liberación, permeabilidad, y mucoadhesión que aumentan la eficacia del tratamiento frente a una solución comercial de nistatina, sin detectarse toxicidad de los componentes de la formulación.

Asimismo, con la finalidad de convertir estas formulaciones en un tratamiento más natural, se determinó la actividad antifúngica de extractos de planta procedente de *Glycyrrhiza glabra* (regaliz). Una vez identificado el extracto con mayor actividad, y uno

de los principales compuestos responsables de la misma, se procedió a su encapsulación en los sistemas de nanopartículas anteriormente mencionados.

En conclusión, la formulación final desarrollada presenta un mejor perfil terapéutico en comparación con la solución comercial de nistatina, indicando que la encapsulación del fármaco antimicótico en asociación con un extracto natural podría constituir un futuro tratamiento con éxito.

Abstract

Oral candidiasis is an opportunistic fungal infection that generally affects people with immunodeficiency due to viral infection or acquired immunodeficiency associated to other causes but in some rare cases, it can also affect healthy people. Despite the advances in the treatment of the oral candidiasis, polyens (like nystatin) and azoles are still the most used antifungal agents. In addition, most of those treatments present some disadvantages such as poor oral absorption of the drug, alcohol content in dosage form and they generally require a very high frequency of administrations. Therefore, the major challenge in the treatment may be focused on the improvement of the oral absorption of the antifungal agents through oral or buccal mucosa using bioadhesive dosage forms.

This thesis aimed to develop a suitable buccal mucoadhesive nanoparticle (NP) formulation containing antifungal agents for the local treatment of oral candidiasis. Several types of polymeric particles with an encapsulated antifungal agent were prepared and further included into a toothpaste, an oral gel and oral films (all free of alcohol). Nystatin (Nys) was herein chosen as the reference drug because it is one of the main antifungal drug used. In this context, three different systems of nanoparticles (NPs), Polylactic acid, Poly(lactic-co-glycolic) acid and Alginate, were selected since they present a small particle size (300 to 800 nm), homogenous size distribution, appropriate surface charge, high encapsulation efficiency of Nys (above 70%), prolonged release profile, high adhesion capacity to oral mucosa, permeation capacity through the oral mucosa and safety profile. Also, the NPs successfully maintained the activity of Nys over time. Concerning the stability study the formulations were stable for 6 months.

In addition, instead of Nys, extracts from *Glycyrrhiza glabra* (Licorice) were prepared and their antifungal activity was studied. The results obtained showed that ethanolic extract of *Glycyrrhiza glabra* L. was the most active extract and that the loaded NPs were successfully produced, presenting a mean size between 100-900 nm and a negative surface charge with high encapsulation efficiency of the extract.

In conclusion, this thesis confirmed that all bioadhesive nanoformulations using Nys or extracts from plants are viable alternatives for improving drug bioavailability in the oral mucosa. Thus, all nanoformulations might potentially improve the patient compliance due to its extended action and reduced the overall required dosage.

BACKGROUND, HYPOTHESIS
AND OBJECTIVES

1

Background

Nowadays, oral candidiasis (OC) is an opportunistic fungal infection that generally affects people with immunodeficiency due to viral infection or acquired immunodeficiency associated to other causes ^{1,2}. However, it also can affect healthy people. This infection is predominantly originated by *Candida albicans* and where the main priorities of the treatment are rapid relief of the symptoms and the elimination of any factor that increases the recurrence of the disease ^{3,4}.

The effective treatment of the OC is based on four main grounds: the achievement of an early and accurate diagnosis of infection, correction of predisposing factors or underlying the disease caused by infection, the correct assessment of the type of *Candida* that is causing the infection and, finally, the use of antifungal agents suitable for the treatment of infection regarding the risk ratio/drug benefit in each case ⁵.

All fungal organisms are eukaryotes and exhibit certain characteristics like mammalian cells, and therefore there is a greater difficulty to obtain pharmacological selectivity. In general, pharmacological treatment includes the administration of antifungal agents. The most used therapeutic agents can be divided into two main classes: azoles and polyenes, which despite the recent developments in terms of other alternatives, they continued to be the most used antifungal agents. But those treatments present some disadvantages such as the poor oral absorption of the drug, high frequency of administration and rapid elimination on the target tissue ^{6,7}. Consequently, the major challenge in the treatment of OC may be focused on the improvement of the absorption of those antifungal agents and where the encapsulation technology may be considered as a possible strategy for achieving this objective ⁸.

In recent years, the encapsulation technologies have emerged with a great interest in in the administration of drug with enormous clinical relevance, such as the antifungal agents. Problems related to the uncontrolled release profiles, problems also related to the lack of specificity and irregular or absence of pharmacokinetic profile can be solved with the use of these techniques. Some new formulations involving the use of nanoparticles (NPs) as a carrier for the successful delivery of the drug have been developed, like micro suspension with amphotericin B and polymeric micro- and nanoparticles with nystatin

(Nys) have been developed to improve/solve some of the problems associated to antifungal therapy ⁹, since the polyenes can be toxic, causing serious side effects (like renal failure, hypokalemia, and thrombophlebitis) when they are intravenous administrated. In addition to this factor, they are poorly soluble in water, and can easily form aggregates when present in aqueous media. This feature can be related and responsible for the difficulties associated during the administration of polyene (where it can be included the poor absorption after oral administration and poor distribution of the antifungals in organs and tissues) ⁹. Despite these two antifungal drugs also clotrimazole have been studied using solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). In all cases, those studies showed an improvement in the efficacy of the treatment of OC by applying nanoparticles ^{10,11}.

Besides that, well-known antifungal agents, natural products like extracts from plants have been used, increasing the interest from pharma industry. In the present, they represent approximately one-third of the top-selling pharmaceuticals drugs, which are composed by natural products or their derivatives ⁹. Despite this fact, the association between extracts from plants and the use of nanoparticles as a drug delivery system can be beneficial, since it can improve the solubility of extracts and improve the probability to be absorbed in the target tissue, those reducing the number of administration and increasing the patient's compliance. At the same time the natural extract can decrease some of the adverse effects associated with the drugs used in the treatment ⁹.

Hypothesis

1. Considering the short-term residence of the antifungal agent in the target tissue, it is possible that mucoadhesive polymeric nanoparticles will allow a greater contact time in the site of action, improving the efficacy of the treatment of OC? If so, this contact by adhesive forces will be higher when those NPs are included in three semi-solid formulations (toothpaste, oral gel and oral films)? Can those nanoformulations be stable over the time? Can those nanoformulations be safe in the tested concentrations? Is there any nanoformulation that can be easily adapted in daily routine of the patient? Is there any nanoformulation that can be easily adapted to infants, free of alcohol? Is there any nanoformulation suitable in case of ulcerations?
2. On the other hand, is it possible to have a therapeutic strategy instead of classic antifungals agents? In this case, is it possible to apply extracts from plants? The optimized nanoformulations can be applied in those extracts and extended the time on the target tissue? If so, this contact by adhesive forces will be higher when those extract-loaded NPs are included in three semi-solid formulations (toothpaste, oral gel and oral films)? Can those nanoformulations be stable over the time? Can those nanoformulations be safe in the tested concentrations?

Objectives

Considering the previous points, we propose the development of different polymeric NPs implemented in the semi-solid formulations for the local treatment of the OC. To achieve these specific objectives, we have followed the subsequent steps:

1. Development of the polymeric nanoparticles and the development of the semi-solid formulation (toothpaste and oral gel)

In this part of the work, we have developed several polymeric nanoparticles (alginate, chitosan, dextran, xanthan gum, poly-arginine, polylactic acid and poly (lactic-co-glycolic) acid and accessed its characterization. At the same time, semi-solid formulations were developed, specifically a toothpaste and an oral gel. After the selection of the best systems of NPs in terms of small size, monodisperse size distribution, stability, cytotoxicity, encapsulation efficiency and controlled release profile of the encapsulated antifungal agent, they were implemented in the semi-solid formulations. Nystatin (Nys) was used as a model drug.

The main results regarding this milestone were reported in Article II, Article III:

“Innovative formulation of nystatin particulate systems in toothpaste for candidiasis treatment.”

“Bioadhesive nanoparticles as a strategy to improve the treatment of yeast infections in oral mucosa: *in-vitro* and *ex-vivo* studies.”

2. Development of the oral films and the *in vitro* adhesion studies

In this chapter, the optimized nanoformulations of the previous point were tested regarding the *in vitro* interaction with the pig's mucin and the *in vitro* attachment with the HT-29MTX cells. The tested nanoformulations were the toothpaste with alginate NPs, the oral gel with poly(lactic) acid and the oral film with poly(lactic-co-glycolic) acid nanoparticles.

The main results regarding this milestone were reported in Article IV:

“Mucoadhesive assessment of different antifungal Nanoformulations”

3. Preparation of natural extracts from *Glycyrrhiza glabra* (Licorice) study their anti-fungal activity

In this step of the work, the natural extracts were obtained from the *Glycyrrhiza glabra* (Licorice). In all case, extracts were tested in terms of their anti-fungal activity against several species of *Candida* (*C.tropicalis*, *C. glabrata*, *C.dubliniensis*, *C. krusei*, *C. guilliermondii* and *C. albicans*). Herein, the best extract was selected according its minimal inhibitory concentration, the minimal fungicidal concentration and the time-killing.

The main results regarding this milestone were reported in Article V:

“Development of a biodhesive nanoformulation with *Glycyrrhiza glabra* L. extract against *Candida albicans*”

4. Encapsulation of the ethanolic extract of *Glycyrrhiza glabra* into the three systems of the selected polymeric nanoparticles

In this final step of the work, the ethanolic extract of *Glycyrrhiza glabra* from the previous step was characterized with LC-MS and then it was encapsulated into the alginate, the poly(lactic) acid and poly(lactic-co-glycolic) acid. Again, these extract-loaded NPs were implemented in the respective semi-solid formulations and tested with the same tested done in the previous sections.

The main results regarding this milestone were reported in Article V:

“Development of a biodhesive nanoformulation with *Glycyrrhiza glabra* L. extract against *Candida albicans*”

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INTRODUCTION

2

Article I

Past, Recent Progresses and Future Perspectives of the Nanotechnology Applied to Antifungals Agents

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Past, Recent, Progresses and Future Perspectives of Nanotechnology Applied to Antifungals Agents

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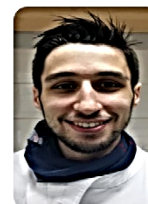
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Abstract:

Candida species remain a significant cause of nosocomial bloodstream infections, associated with prolonged hospital stay in the ICU and high healthcare cost. The incidence of *Candida* is very high in certain risk groups of patients (AIDS, diabetes, cancer, etc.). Recent developments of nanotechnology have strongly contributed to the design of new multifunctional drug carriers that improve drug bioavailability through a controlled and prolonged release profile or even through a more specific targeted delivery of the antifungal agent. Those types of systems have strongly increased with a progressive generation of new structures, permitting the conjunction of new materials, biomolecules, physical and chemical techniques, for better outcomes. Nanotechnology shows expanded possibilities within the medical field and in the case of the yeast infections it may overcome several issues related with the fungal proliferation or higher inhibition of the pathogen causing the infection. This review covers a period of the most representative research of Candidiasis since 1993 to the present.

Keywords: Fungal infections, Candidiasis, Encapsulation, Antifungal agents, Nanoparticles, Mucoadhesion

1. INTRODUCTION

The growth of opportunist infections result in the upsurge of the number of individuals sensitive to invasive fungal infections, causing the increasing of both mold and yeast pathogens.[1,2] Regarding this over the past 20 years, the number of invasive fungal infections remained the same, but in some cases the number of infected individuals has increased mainly related with the large number of individuals with severe immunosuppression.

It is demonstrated that the leading cause of the opportunist infections and the leading pathogen is the *Candida* species.[3]

Despite systemic infection with *Candida* being extremely rare in healthy individuals, it remains in the top five, being the fourth pathogen causing nosocomial bloodstream infections (BSI) acquired in the hospital, both in the USA and in European Union (EU). The based surveillance studies report that the yearly incidence of *Candida* infections is eight cases per 100.000 habitants.[4,5]

But in some places, this percentage can reach ten cases per 100.000 habitants. It also appears that there is a higher incidence observed in the African and African-American population, in newborns and in children under one year of age and in the elderly population over 65 years. Beside that in the case of the children, the newborns and the ones who present a low birth weight, the development of oral candidiasis (OC) is estimated to be between 5 and 20%.[6,7]

In addition, it is estimated that in certain risk groups, such as AIDS patients, the percentage that develops OC and BSI is between 5 and 31%, and is around 20% for patients with oncological problems. More specifically, the percentage and the incidence rate of OC and BSI infections in the population with cancer will depend on the type of cancer. It is calculated to be around 29.2% in the case of neck-cancer, 20.5% in the case of hematologic cancer and, finally, 17% for other solid tumors. In addition to these primary risk factors, the age and the use of dental prostheses are other risk factors that are normally associated with greater probability of incidence of OC and BSI.[8,9]

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Table 1 - Characteristics of main fungal infections worldwide [17]

Location	Pathogen type	Organ	Most frequent genus	Estimated incidence of infection
Superficial	Primary	Skin and hair	<i>Malassezia</i>	~140,000,000 cases/year
Cutaneous	Primary	Skin and nails	<i>Trichophyton</i> <i>Epidermophyton</i> <i>Microsporum</i>	~1,500,000,000 cases/year
Mucosal	Opportunistic	Gastrointestinal tract, urinary system and eye	<i>Candida</i> ;	~75,000,000 cases/year
			<i>Aspergillus</i> ;	~9,500,000 cases/year
			<i>Fusarium</i>	~1,000,000 cases/year
			<i>Candida</i> ;	~300,000 cases/year
Systemic	Opportunistic	Any organ (lungs, brain, etc.) bloodstream	<i>Aspergillus</i> ;	~350,000 cases/year
			<i>Cryptococcus</i> ;	~1,000,000 cases/year
			<i>Histoplasma</i> ;	~500,000 cases/year
			<i>Pneumocystis</i> ;	>200,000 cases/year up to
			<i>Coccidioidomyces</i> and so on	limits 300,000 cases/year

Currently, more than 150 known and studied species of *Candida* are described but only 15 of them have been isolated from individuals as an infectious agent. These 15 species of *Candida* are the following: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. lusitanae*, *C. dubliniensis*, *C. pelliculosa*, *C. kefyr*, *C. lipolytica*, *C. famata*, *C. inconspicua*, *C. rugosa* and *C. norvegensis*. [10] Despite these isolated species, their frequency of appearance may vary with their geographic location. In the last 20 to 30 years, many studies demonstrated that in 95% of *Candida* infections the main pathogens involved are *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. [11] Between these species, *C. albicans* still remains the most common pathogen in spite of its decreasing share. In humans, it generally colonizes some regions including skin, oropharynx, lower respiratory tract, gastrointestinal tract, and genitourinary system. The isolation rates of species other than *C. albicans* vary according to the features (age, underlying diseases, hospitalization ward, etc.) of patient population. To illustrate, *C. parapsilosis* causes 30% of candidiasis infections (CI) cases among newborns whereas the rate is 10–15% amongst adults. *C. glabrata* is a more common infectious agent among older and neoplastic patients. *C. tropicalis*, on the other hand, is more commonly seen among leukemia patients and neutropenic patients. Meanwhile *C. parapsilosis* colonizes the skin and it is also a common pathogen in catheter-related infections. *C. krusei*, on the other hand, is more common among hematopoietic stem cell recipients or neutropenic leukemia patients receiving fluconazole prophylaxis. [12]

In terms of treatment, there are several drugs called antifungals, but the main treatment problems relates to the possible specific resistance to drugs used for treating OC, such as fluconazole, which has a resistance of 4.5% cases, itraconazole with 11.7%, with 11.3% ketoconazole and caspofungin 44.1%. This fact has not been verified with amphotericin B and Nystatin (Nys). [13]

Recent developments in the area of Nanotechnology, have contributed widely in terms of drug delivery though the

achievement of new multifunctional drug carriers that lead to an improvement of the drug bioavailability, controlling drug release or achieving a more specific targeted drug delivery. [14] These types of systems have increased through the recent century and with a progressive generation of new structures, permitting the conjunction of new materials, biomolecules, physical and chemical techniques, for better outcomes. The main objective of these new types of systems is to improve the individual and general public health and compliance for a safer and efficient diagnosis and therapy. [15] These technologies present expanded possibilities within the medical field and in the case of the yeast infections they may overcome issues related with fungal proliferation or related with the higher inhibition of the pathogen causing the infection. [16]

I would also mention the hydrophobicity of antifungals and the improvement in biopharmaceutical properties after association with nanoparticles.

Assuming all information, there is still room for improvement in antifungal therapy and one of the alternatives is the nanotechnology application. This review describes an overview of the fungal infections, the developments made along the years in the antifungal treatment and the safety of current treatments, including the use of Nanotechnology as a way to improve the efficacy and safety of the treatment.

2. FUNGAL INFECTIONS

In the early beginning of the 20th century, bacterial infections were one of the global and most important causes of mortality. In contrast, fungal infections were practically not taken in account in terms of the mortality impact. But since the late 1960s when the antibiotic therapies where developed and started to be administered, a drastic increment in the number of fungal infections was observed until now, and they currently represent one of the global health threats. [17]

This drastic increase can be explained, not just by the fact of the developments in the medicinal area, but it is also influenced by the constant growing numbers of immunocompromised patients related to AIDS, cancer, the elderly population, diabetes and organ transplantation or other invasive surgical procedures.

As shown in table 1 fungal infections are normally caused by two different types of microorganism: the primary ones and the opportunist pathogens. The primary pathogens are naturally capable to establish by itself an infection in the healthy population. In disparity, the opportunistic pathogens, among them commensal microorganisms of the healthy population, are commonly present in the human microbial flora and are able to develop an infectious colonization of the human organism when specific criteria are present, such as immunosuppression.

In terms of the fungal pathogens they can be divided into two major groups: the filamentous fungi and the yeasts. Most of the primary microorganisms are the filamentous fungi, while on the other hand the most of the opportunistic pathogens are yeasts although some species of the filamentous fungi are increasingly identified as opportunistic pathogens. In addition, it is also important to note that the fungal infections can be also classified as a function of the tissue infected (Table 1), so that if the infection is present in the oral cavity it will be caused by opportunistic pathogens. [17]

On a more specific way the superficial mycoses, like the *Tinea versicolor*, are only limited to the most superficial and external part of the skin and hair. These types of infections are more commonly provoked by the species *Malassezia globosa* and by the *M. furfur*, which have been estimated to be present in the microbial flora of 2 to 8% of the healthy population worldwide and could lead to *T. versicolor* in some conditions that are still uncertain. [18]

When the infections occur in deeper skin layers they are usually designated as cutaneous and subcutaneous mycoses. This infection is normally caused by dermatophytes fungi that will affect the keratinized structures present in the body, usually present in the skin. The most frequent species of fungi involved in the dermatophyte are the *Trichophyton*, the *Epidermophyton* and the *Microsporum*. In most part of the cases, cutaneous fungal infections involve the immune system, and their incidence varies depending on the site of infection. For example, onychomycoses are very frequent in the global population, with the incidence ranges from 5% to 25%. [19] Oromucosal infections are frequently caused by opportunistic yeasts, and those belonging to the *Candida* genus are by far the most frequent ones present in this body cavity. Vaginal, esophageal, oropharyngeal, and urinary tract candidiasis (infections cause by *Candida*) mainly affect immunocompromised patients. Fungal infections of the eye can also be classified as mucosal fungal infections, but they are caused more frequently by *Fusarium* or *Aspergillus* species rather than *Candida* species. [20]

Theoretically, systemic mycoses may involve any part of the body, and a lot of species formerly considered as

nonpathogenic are now recognized as opportunistic pathogens responsible for mycoses. These infections are very common in immunocompromised patients and are frequently associated with an elevated mortality rate. The most frequent pathogens involved in systemic fungal infections are *Candida*, *Pneumocystis*, *Histoplasma*, *Aspergillus*, *Cryptococcus*, *Mucor*, *Rhizopus*, and *Coccidioidomyces*. [21–23]

Despite the existence of these different types of infections, candidiasis continues to be one of the most frequent opportunistic fungal infections.

3. CANDIDIASIS

The term *Candida* comes from the Latin word candid, which means white. The spores that are present in the *Candida* are dimorphic and they are inoffensive form of the dimorphic pathogen, which only becomes an invasive and pathogenic infection when there is a disturbance in the flora balance or immune fragility of the host. [24] The cause of some type of medical condition or disease by these commensal endogenous, may be associated with other pathogenic factors, in addition to those that are assigned by the organism itself. Thus, *Candida* presents a very unique feature when compared with other organisms, where virulence is considered a key factor in their pathogenesis. [25]

Infection caused by *Candida spp.* is usually a very common infection in the vaginal denominated vaginal candidiasis. It occurs also in the oral cavity, which is commonly referred to as OC. In recent years, various types of *Candida* infections, like the OC, have become more common and more severe, due to the abusive use of antibiotics, the appearance of some pathologies like AIDS, organ transplants and the use of invasive devices (such as catheters and artificial implants), which will increase the susceptibility of the occurrence of the infection. [26] In addition to these factors, recent studies have shown that certain characteristics, present in some percentage of the population, can increase the prevalence of *Candida* infections (CI) such as the age, parenteral nutrition and bacterial infections. [5]

In addition to these major risk factors, there still exists the influence of the geographical area, because there are differences in the distribution of each type of *Candida* species. For example the *C. parapsilosis* is the first or second etiologic role in CI, that is diagnostic in Australia, Latin America, the Mediterranean countries, Africa, Asia and USA. In the case of *C. glabrata*, it presents an important etiologic role in the USA more specifically in the Central and North zone of the USA. Another example is *C. tropicalis* that is also one of the first species present in the infection with *Candida* in Asia (Figure 1). [27]

One concern related with *Candida* infection is unacceptably high mortality. The percentage of mortality due to infection caused by *Candida* is usually associated with CI and the mortality rate is estimated to be above 30% and a crude mortality rate close to 50%. These mortality values large

Table 2 - Distribution of various species of *Candida* responsible for the development of OC ^a [32–35]

Species	Number of cases reported (%) ^b					
	1997/1998	1999	2000	2001	2002	2003
<i>C. albicans</i>	73.3	69.8	68.1	65.4	61.4	62.3
<i>C. glabrata</i>	11.0	9.7	9.5	11.1	10.7	12.0
<i>C. tropicalis</i>	4.6	5.3	7.2	7.5	7.4	7.5
<i>C. parapsilosis</i>	4.2	4.9	5.6	6.9	6.6	7.3
<i>C. krusei</i>	1.7	2.2	3.2	2.5	2.6	2.7
<i>C. guilliermondii</i>	0.5	0.8	0.8	0.7	1.0	0.8
<i>C. lusitanae</i>	0.5	0.5	0.5	0.6	0.5	0.6
<i>C. kefry</i>	0.2	0.4	0.5	0.4	0.4	0.5
<i>C. rugosa</i>	0.03	0.03	0.2	0.7	0.6	0.4
<i>C. famata</i>	0.08	0.2	0.5	0.2	0.4	0.3
<i>Candida spp.</i> ^c	3.9	6.0	3.7	3.3	7.9	4.9
Total of cases	22533	20998	11698	21804	24680	33002

a - Data collected by the global monitoring program ARTEMIS DISK

b - Includes some species of a total of 127 institutions from 39 different countries, globally

c - *Candida* species unidentified



Figure 1 - Geographical distribution of the most frequent *Candida* non-albicans species [27]

exceed what is widely reported and described for the most of the bacterial infections, thus making *Candida* infection one of the microbial infections with an elevated risk. Despite high values until 1989, year where there was a steady increase in mortality reaching the 0.62 deaths per 100,000 habitants, in recent years the mortality rates associated with invasive candidiasis has been declining, representing a decrease of 50%. This decline may be associated with the increased awareness in relation to this type of infection, but also due to the improved diagnosis and therapy.[27]

With these risk factors in mind the treatment of candidiasis has changed over the past years, resulting in the appearance of new classes of antifungals. However, the continued reported cases of resistance to antibiotics and antifungals, makes OC one of the most common oral infections (OI) that can be treated.[28] This type of infection is usually present in immune deficient individuals (carriers of a virus or immune deficient syndrome), women who are in the neonatal period or taking the pill (due to hormonal imbalance), individuals with an oral pH extremely acid, individuals who present a prolonged neutropenia or individuals that are to be subjected to a treatment with parenteral nutrition, the use of certain drugs such as steroids in treating asthma, but this infection can also occur very rarely in healthy people.[29,30]

OC generally causes oral discomfort, pain, loss of flavor and lack of appetite. There are many *Candida* species that lead to OC.[31] However, the most prevalent species is *C. albicans*. Over 90% of the oral infections caused by *Candida* are assigned to five major species: *C. albicans* (with 62.3%), *C. glabrata* (with 12.0%), *C. parapsilosis* (with 7.3%), *C. tropicalis* (with 7.5%) and *C. krusei* (with 2.7%), as shown in Table 2.[32–35]

In addition to the problems cited above, OCs are also typically characterized by the appearance of white exudates with a creamy reddish base which cover the tongue mucosa, the internal part of the cheeks and throat. These signals can lead to the appearance of more severe problems and pathologies, such as anorexia, the sudden appearance of small bleeding after the daily oral hygiene and also may influence the effectiveness of other oral medications.[36–38]

In the case of CI, the most prevalent species continues to be *C. albicans*. Although in the last few years its prevalence has decreased, it still represent 44% in the intensive care unit. Overall the CI can be brought about by five major species, like the OC: *C. albicans* (with 48.4%), *C. glabrata* (with 18.2%), *C. parapsilosis* (with 17.1%), *C. tropicalis* (with 10.6%) and *C. krusei* (with 2.0%), as shown in Table 3.[2]

In this case the diagnosis is not as straightforward as in the case of OC where the clinical signs and symptoms are specific, and, due to a low diagnostic yield of traditional blood cultures (still the gold standard for diagnosis), a significant percentage of CI goes undetected. The use of antifungal prophylaxis further hampers diagnostic sensitivity, while predisposing patients to infections due to fungi with intrinsic or acquired resistance to some antifungals. Despite that the most common signals and symptoms are fever, chills, skin rash, generalized weakness or fatigue, low blood pressure, vision changes or signs of eye infection and headaches.[39]

Table 3 - *Candida* species distribution in global studies (%) [2]

Year	Description	<i>Candida albicans</i>	<i>Candida tropicalis</i>	<i>Candida parapsilosis</i>	<i>Candida glabrata</i>	<i>Candida krusei</i>
1997–2007	Laboratory-based ARTEMIS	65.3	7.2	6.0	11.3	2.4
2003	Laboratory-based SENTRY	48.7	10.9	17.3	17.2	1.9
2008–2009	Laboratory-based SENTRY	48.4	10.6	17.1	18.2	2.0
2007–2011	Hospital-based, pediatric	44		22	11	3

4. ANTIFUNGAL DRUGS AND PROBLEMS IN THE TREATMENT

In the case of the treatment of OC, the main priorities are rapid relief of symptoms and the removal of any factor which can increase the disease whereas in the case of CI the main priorities consists in the inhibition of the proliferation of the infections and the rapid inhibition of the pathogen. The effective CO treatment is based on four main steps: the achievement of an early and accurate diagnosis of infection, the correction of predisposing factors or underlying disease caused by infection, the correct evaluation of the type of *Candida* that is causing the infection and, finally, the use of antifungal agents suitable for the treatment of infection with regards to drug risk / benefit ratio in each case. [40]

The efficacy of the treatment consists or is based on four main steps: the achievement of an early and accurate diagnostic of the infection, the treatment and elimination of the predisposing factors or the underlying diseases, the correct evolution on the type of pathogen responsible of the infection and finally the correct use of the antifungal drug that is more suitable for the treatment of the infection regarding the efficacy of the treatment.

Thus, two different strategies can be followed: the pharmacological and the non-pharmacological treatment. [41,42]

Regarding the non-pharmacological treatment several recommendations are made to individuals suffering from a chronic or prolonged candidiasis. Specifically, these recommendations consist in daily oral hygiene (adequate cleaning of teeth and/or dental prosthesis when applicable). If individuals are suffering from an acute or periodic candidiasis, beside the oral hygiene the recommendations will also include periodic oral examination. The use of a proper cleaning agent, for example, a cleaning solution with anti-*Candida* properties (such as 1% sodium hypochlorite or 0.2% of chlorhexidine) is strongly recommended. [41,42]

However, some side effects associated with previous treatment have been reported such as tooth discoloration and also the discoloration of the surface mucosa. Anyway, certain predisposing factors, such as the presence of an underlying disease (like leukemia or AIDS), make its control more difficult. In these more complicated cases, the non-pharmacological treatment is not sufficient to achieve full recovery. For that, it will be necessary to turn to a specific

antifungal therapy that will play a key role in the treatment. [43]

The pharmacological treatment includes the use of antifungal agents from the two main classes: azoles and polyenes. This simple classification is based on the pharmacological activity and mechanism of action of the antifungal agents, as showed in Table 4. [44,45]

Table 4 - Antifungal agents mechanism of action [44,45]

Antifungal Agents	Mode of action	Administration route
<u>Polvene</u>		
Nystatin	Connection to the ergosterol and degradation of fungal cell membrane	Topic
Amphotericin B		Topic
<u>Azoles</u>		
Fluconazole	Inhibition of ergosterol biosynthesis	Systemic
Miconazole		Topic / Systemic
Ketoconazole		Topic
Itraconazole		Systemic
<u>Flucytosine (5-Flucytosine)</u>	DNA / protein synthesis inhibition	Systemic (usually in combination with Amphotericin B)
<u>Echinocandins</u>		
Caspofungin	Inhibition of the synthesis of β 1,3-D-glucan	Intravenous
Micafungin		Intravenous

The most commonly type of antifungal used in the treatment of fungal infections is the polyenes, including drugs such as amphotericin B and Nystatin. The mechanism of action of this type of antifungals consists in the direct linkage to the ergosterol present in the cell membrane of the fungus. This connection with the drug will induce a structural change in

the membrane causing an increase in the permeability of the fungus. Then, fungus will lose the cytoplasmic content and other intracellular components provoking total degradation of the fungal cell.[46]

Furthermore, the polyenes are a group of antifungal drugs widely used mainly due to its lack of affinity for sterol, which is the equivalent to the cholesterol present in mammals. This very low affinity will make healthy cells, present in the host, less susceptible to any toxic effects. This class is very often used in the treatment of chronic candidiasis, for example, an oral suspension of Amphotericin B is commonly used in these situations since it is a very potent antifungal agent with a large spectrum of action. Amphotericin generally does not induce any type of resistance, beside that it is often used in the treatment of OC in HIV patients and also in the treatment of CI.[47,48]

However, polyenes are poorly absorbed in the intestinal tract. In the market, we can find tablets, oral suspension and suspension for topical application. However, they show limited therapeutic efficacy because, among other factors, the intestinal permeability is very low.[49]

On the other hand, azoles present a fungistatic action rather than the fungicide action presented by polyenes. The mechanism of action is based on the inhibition of the enzyme lanosterol 14 α -demethylase or the CYP51A1, which is a cytochrome P450 enzyme involved in the synthesis of ergosterol.[50] This inhibition will lead to a reduction in ergosterol levels present in the fungal cell membrane, thus increasing membrane permeability. Subsequently, the fungus will lose its intracellular components and cell death will occur.

The two antifungal drugs more frequently administered in the treatment of OC or CI are fluconazole and itraconazole which are easily absorbed in the intestine. In the case of fluconazole, which is designated as a synthetic triazole, it presents a large spectrum of action and therefore is one of the most frequently prescribed antifungal for the primary treatment of most *Candida spp* infections.[51,52] This antifungal was approved by the Food and Drug Administration (FDA) and presents different pharmacokinetic properties when compared with other class related antifungals. This difference is mainly due to the presence of two triazole rings that makes the drug a less lipophilic compound resulting in a lack of affinity for proteins. Furthermore, it is rapidly absorbed orally due to its log P value of 5.[4,53,54]

In the case of itraconazole, it is very similar to fluconazole and presents a unique advantage such as the activity against *Aspergillus spp*. [55,56]

Unfortunately, several *Candida* species are resistant both to polyenes and to azoles. This resistance can be explained since *Candida* species present small changes over the years in the targeted enzyme structure, resulting in a diminished effect. Also, they present the capacity to incorporate some sterols in the fungus membrane, which will act as an alternative to ergosterol, thereby decreasing the effects of polyenes. For example 35% and 75% species of *C. glabrata*

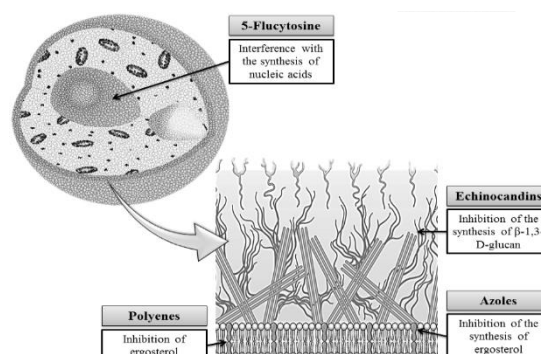


Figure 2 - Mechanism of action of the main antifungal drugs [43]

and *C. krusei*, respectively, showed resistance to azoles.[57,58]

Therefore, novel therapeutic options with antifungals are strongly required. As an alternative, echinocandins and flucytosine or 5-flucytosine, are considered (Figure 2).[43] Briefly, echinocandins are derived from the fermentation process of a variety of different fungi species and they act by inhibiting the synthesis of β -1,3-D-glucan (which is a necessary and essential enzyme in the synthesis of the fungal cell wall). Therefore, this drug will induce a change in the structural capacity of the fungal cell wall, causing the death of the fungal cell. Therefore, this drug is still being used since this enzyme is absent in mammalian cells, making the toxicity to the host cells very uncommon. The use of this type of agents such as the caspofungin and the micafungin are generally observed in invasive infections like the CI and not used in other types of infections such as CO.

In the case of flucytosine or 5-Flucytosine, it is a fluorinated pyrimidine analogue. It acts as an antimetabolite and despite the fungistatic activity against *Candida* it is usually not used alone due to development of resistance by the fungi. Usually,

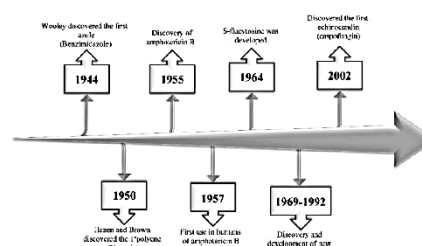


Figure 3 - Development in the antifungal treatment along the years [57]

this drug is used in combination with other classes of

antifungal agents such as amphotericin B. Unlike the other classes of antifungals, it will exert its action in the cell wall of the fungi, or will affect their structure. The mechanism of action interferes with the synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and essential cell fungal protein, thereby preventing the proliferation of the fungus.[58]

The major problem regarding these two new classes is that they have to be administrated by intravenous route, which will result in a decrease of therapeutic compliance.

Thus, despite the existence of several antifungals as shown in Figure 3, Nystatin still continues to be the principal and most widely used antifungal agent. Despite its low therapeutic efficacy, Nystatin remains the antifungal that presents or that has the lower number of side effects.[58]

5. NANOTECHNOLOGY AS A NEW WAY OF DELIVERY

In a general way, nanotechnology may be described by the manipulation of matter with one specific dimension size from 1 to 1000 nanometers. This technology is applied in many fields of science like organic chemistry, molecular biology, semiconductor physics, microfabrication, medicine, pharmacy, among others.[59]

One of the most interesting applications of nanotechnology consists in the use of encapsulation methods to improve the activity of some drugs or to design new drug delivery systems.[60–66]

However, there are still some problems associated with the release profile of drugs, problems associated with drug targeting to the specific site of action and also problems associated with the pharmacokinetic profile, which can be solved with these encapsulation methods.

5.1 Matrix Systems

Nanospheres as matrix systems are considered a dispersion of the drug in one or more substances, designated as a polymeric matrix, which are able to modify and control the release profile of the encapsulated drug over time as seen in Figure 4. Generally, the polymeric matrices are made of hydrophilic or inert polymers that can be further formulated and administrated in the form of capsules, granules or

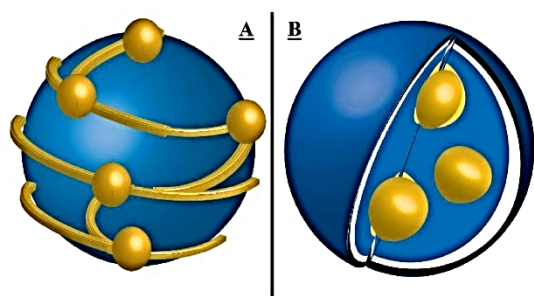


Figure 4 - Nanospheres (A) and Nanocapsules (B) [64] tablets. The release profile of the drug is generally controlled

by the characteristics of the polymer and can be conducted by different processes.[67]

5.2 Reservoir Systems

Nanocapsules are reservoir systems where there is an internal part which will serve as a reservoir and an outer portion that will serve to coat the core. In this case, the drug is generally released by a diffusion process.[68,69]

6. POLYMERS USED IN ENCAPSULATION METHODS

Polymers may be classified into three different types: natural, synthetic and semisynthetic ones. Some of the examples of each type of polymers are presented in Table 5.[70–80]

A polymer is considered a large molecule (macromolecules), which is composed by repeating structural units. These subunits are normally connected by covalent chemical bonds. Some authors suggest that synthetic polymers offer

Table 5 – Different types of polymer use to encapsulate antifungal drugs

Polymers		References
Natural Polymers		
Collagen		[72]
Hyaluronic Acid	✓	[72]
Dextran	✓	[72]
Alginate	✓	[72]
Chitosan	✓	[71,72]
Pectin		[72]
Agar		[72]
Synthetic Polymers		
γ-Polyglycolic Acid (γ-PGA)	✓	[77]
Poly(lactic Acid) (PLA)	✓	[73,77]
Poly(lactic-glycolic Acid) (PLGA)	✓	[73,77]
Polycaprolactone (PCL)	✓	[73,77]
Polydioxanone (PDS)		[77]
Polyethylene Oxide	✓	[69,77]
Polypropylene Fumarate		[77]
Semi-Synthetic Polymers		
Methyl cellulose		[70]
Hydroxyethyl cellulose		[70]
Carboxymethyl Cellulose		[70]
Hydroxypropyl Cellulose		[70]

more advantages when compared with natural polymers.[81,82] One advantage is the capacity of synthetic polymers that can be configured and adapted to obtain a wide range of structural and nanomaterial properties, becoming therefore more predictable as far as distribution and release profile of the drug are concerned, easier to work and greater reproducibility. Also, in the last case, their degradation is generally caused by a simple hydrolysis.

In the case of natural polymers they can be classified, by their origin, into two large groups: natural polymers obtained from plants (plant origin) like Cellulose, Hemicellulose, Agar, Starch, Pectin and Guar gum; or natural polymers obtained from animals (animal origin) like Chitin, Alginates and Xanthan gum.[74,83]

The major advantage over synthetic polymers, is they are structurally similar to natural tissues making it more likely the establishment of strong interactions between the polymer and human tissues or cells, increasing therefore the compatibility with the human organism and incising the bioavailability of the drug.[75,79]

Regarding to natural polymers, they show a highly organized structure that cannot be changed in a chemical way, both in at the molecular level and the macroscopic level. This capability can also be a huge advantage because this characteristic of natural polymers will allow them to present a favorable performance, since they will have high resistance properties, but will also present the ability to induce the growth of tissues.[71,84–86]

Both synthetic and natural polymers are available in the market but the use of natural ones for pharmaceutical applications is more attractive because this type of polymers are usually more economical, readily available and present non-toxic characteristics. They also present high biodegradability and with few exceptions, also biocompatibility. However, natural polymers are difficult in terms of isolation and purification.[72]

Finally, there are semi-synthetic polymers obtained by simple treatment of natural polymers, which will change its physical and structural properties. These types of polymers are less expensive when compared to the synthetic polymers.

7. NANOTECHNOLOGY APPLIED TO ANTIFUNGAL DRUGS

New formulations have been developed to improve/solve some of the problems associated to antifungal therapy (Table 6).

Amphotericin B was the first antifungal drug formulated as a delivery system in 1997 by Alexander Leenders. This system was developed in order to reduce the toxicity and increase the therapeutic index of amphotericin B. This liposomal formulation was designated as AmBisome®.[87]

In 2003, Karl V. Clemons and some collaborators, and also in 2004, V. B. Patravale developed a nanosuspension for the oral administration of amphotericin B through a high pressure homogenization method in order enable its use in

various dosage forms, including specialized delivery systems such as mucoadhesive hydrogel. Despite this new formulation exhibits a decrease of 30% of the pathogen present in the liver, this formulation presented smaller therapeutic effect when compared with free amphotericin B.[88–90]

Many formulations of clotrimazole have been studied over the last years. In this case, solid lipid nanoparticles based systems (SLN) and the nanostructured lipid carriers (NLC) are the most used in terms of nanotechnology and showed an improvement in the efficacy of the treatment.[91–95] Another recent formulation is a microemulsion formed by a thermodynamically stable isotropic liquid dispersion that presents a transparent appearance; these microemulsions have a low viscosity and are composed by an aqueous phase and an oil phase stabilized by a surfactant agent.

Those systems are very promising ways for delivering antifungal drugs because they increase drug protection against the enzymatic degradation in the gastrointestinal tract. Also they can be used in topical and transdermal formulations because drug solubility is increased and they may act as drug permeation enhancers through the skin.[96–98]

Table 6 – Encapsulation methods applied to antifungal drugs

Drug	Delivery System	References
Miconazole	Nanoparticles, Nanosuspension, Microcapsules, Niosomes, NLC and Nanoemulsions	[90-92]
	Microemulsions, SLN, Microparticles and Liposomes	
Econazole	SLN, Magnetic Nanoparticles, Niosome Gel and Microemulsions	[90-92]
Ketoconazole	Transfersomes, SLN, Microparticles, Niosomes, Nanosuspension, Microcapsules, Liposomes and Nanoparticles	[90-92]
Itraconazole	Nanosuspension, SLN and Microsuspension	[86]
Amphotericin B	Beads, Microparticles and Nanoparticles	[37]
Nystatin		

In this sense, a microemulsion with fluconazole has improved the drug safety profile showing a greater activity when compared with free fluconazole.[99,100]

With regard to miconazole, a microemulsion was developed and resulted in an increase of effectiveness when compared to free miconazole. It was also found that the type of formulation components strongly influences the amount of drug encapsulated within the formulation.

In addition to the microemulsion, there is still one more type of formulation commonly used in the encapsulation of

antifungal drug: niosomes (Figure 5). In the case of niosomes containing miconazole extensive studies by Firthouse in 2011 showed promising results.[101]

Regarding the case of Nystatin, some work has been done in the past years, one of the most recent one was made by Reis in 2015 where the NYS (check abbreviation) was encapsulated in three different systems of polymeric particles. In this case, alginate beads, microparticles and nanoparticles of alginate containing NYS showed that the microparticles improved the efficacy of the treatment over time when compared with free NYS.[37]

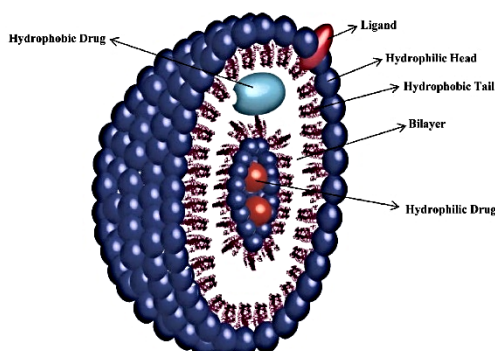


Figure 5 - Structure of a Niosome [101]

8. FUTURE PERSPECTIVES

With the development of new delivery systems, the effectivity of each treatment may be largely improved in many ways. Despite advantages, difficult production and scale up to industrial manufacturing are still one of the key factors. Future research should be focused on the improvement of antifungal treatment using an encapsulation method that will improve drug stability and permeability but with non or less toxic effects. Particle characteristics may strongly influence the therapeutic effect.

Despite that some questions are still waiting to be answered such as the influence of particle size on the efficacy of treatment or the type of polymer used to adhere to the oral mucosa, the most important question is how to combine nanotechnology with antifungals drugs in order to improve the treatment of OC and CI?

These questions, despite some recent studies, have to be in mind in future studies in order to improve antifungal therapy and to improve the life style of patients.

CONCLUSION

OC and CI are still one of the most common fungal infections in the world. Despite the lack of specific data, there are many reports that show that patients with some pathologies associated are more likely to present this type of infection. Literature demonstrates that it can be possible to achieve a reasonable therapeutic effect without toxic effect and without the concern of drug bioavailability and its degradation in the gastrointestinal tract. These factors could help in the discovery of a new way of treatment for OC and CI infection.

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Article II

Innovative formulation of nystatin particulate systems in toothpaste for candidiasis treatment

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RESEARCH ARTICLE

Innovative formulation of nystatin particulate systems in toothpaste for candidiasis treatment

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Abstract

Oral candidiasis is a mycosis on the mucous membranes of the mouth but not limited to the mouth. Nystatin is one of the most frequently employed antifungal agents to treat infections and may be safely given orally as well as applied topically but its absorption through mucocutaneous membranes such as the gut and the skin is minimal. The purpose of this study is to enhance the effectiveness of nystatin using particulate system such as beads, micro- and nanoparticles of alginate incorporated into toothpaste. Those particulate systems of nystatin were prepared by extrusion/external gelation for beads and emulsification/internal gelation for micro- and nanoparticles and characterized. Small, anionic charged and monodispersed particles were successfully produced. The type of particulate system influenced all previous parameters, being microparticles the most suitable particulate system of nystatin showing the slowest release, the highest inhibitory effect of *Candida albicans* over a period of one year. Those results allowed the conclusion that alginate exhibits properties that enable the *in vitro* functionality of encapsulated nystatin and thus may provide the basis for new successful approaches for the treatment of oral antifungal infections such as oral candidiasis.

Keywords

Candida albicans, encapsulation, nystatin, oral drug delivery

History

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Introduction

Oral candidiasis is a common opportunistic infection both in persons with immunodeficiency virus infections or with immunodeficiency acquired by other causes, but it can rarely appear in healthy people. Although *Candida albicans* is the major pathogen in this disease, infections with *Candida* species such as *Candida Krusei*, *Candida glabrata*, *Candida dubliniensis* have been also associated to oral candidiasis. Oral candidiasis is one of the most common, or the most common, treatable oral mucosal fungal infection, commonly observed, mainly due to impaired host defense mechanisms, in persons with an immunodeficiency virus or an acquired immune deficiency^{1,2} such as HIV patients, diabetics, women on neonatal period or taking the contraceptive pill (due to a hormonal imbalance), individuals who have an oral pH extremely acidic or poor oral hygiene, prolonged neutropenia or who are receiving parenteral nutrition³. The usual clinical manifestations of candidiasis is an oral thrush characterized by patches of creamy white exudate with a reddish base covering the mucous membrane of the tongue, cheeks, palate and pharynx. These signs and symptoms can lead to more severe problems such as anorexia and small hemorrhages after the oral hygiene^{4,5}. In the treatment of oral candidiasis, the main priorities are the relief of any identifiable factor and the management of the process. In this case, both physical and chemical reductions of *Candida* are essential to relieve the disease. Physically, this can be achieved by

good oral hygiene practices including tooth brushing and the use of antimicrobial mouthwashes. In terms of chemical methods, the two classes most used are the polyenes and the azoles. Polyenes include drugs like the amphotericin B and Nystatin (Nys) where their mode of action is through a direct binding to the ergosterol found within fungal cell membranes, causing changes in membrane permeability and, consequently, the output of the intracellular components⁶. Despite the existence of a wide range of antibacterial and antifungal agents on the market, an oral suspension of Nys remains one of the most prescribed for patients but it generally requires multiple administrations (four times a day or more) of 4–6 mL of suspension. Mouth irritation, diarrhea, nausea, vomiting or stomach upset may occur. Despite its wide use, oral formulations of Nys available on the market generally present additional problems such presence of alcohol, low solubility, a high frequency of daily applications due to the cleansing effect of the saliva, an unpleasant taste and a viscous consistency. Although certain marketed products have formulation correctors for flavor, like sucrose, this can lead to additional dental issues and might cause adverse reaction, for example, in diabetic patients^{7–9}. On the other hand, Nys has some toxicity when intravenously administered and it is not easily absorbed across intact skin or mucous membranes. Thus, the aim of this study was to use encapsulation techniques in order to obtain protective particulate drug systems such as beads (macroparticles), micro- (MPs) and nanoparticles (NPs), providing the following advantages: more achievable Nys therapeutic effect mainly due to the bioadhesive properties of alginate which may improve the adhesion of the particulate system to the mucus membrane of the mouth; a reduction of the frequency of

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administration and lower drug doses since we expected to retain the particles at the local target over a longer period of time^{10–12}. Besides, when applied to children (not infants) or adults we also expect that Nys particulate systems included in free of alcohol toothpaste will present a more pleasant texture, ultimately, leads to an increase of patient compliance. On the other hand, effective daily oral hygiene is an essential part of general health and well-being. Toothpaste is generally used to promote routinely oral hygiene and it is not intended to be swallowed. We expect that the patient will accept this novel dosage form of Nys as a simple routine action.

Materials and methods

Xanthan gum, Span 80, sodium fluoride, propylparaben and alginate were obtained from Sigma-Aldrich (St. Louis, MO). Sorbitol was obtained from the company José Vaz Pereira (Lisbon, Portugal). Glycerin, calcium chloride and citric acid were obtained from Scharlau (Barcelona, Spain). Calcium carbonate was obtained from Merck (Darmstadt, Germany). Microcrystalline cellulose was purchased from Fagron (Barcelona, Spain). Essence of mint was purchased from J. Santos Graça, Lda (Odivelas, Portugal). Sodium lauryl sulfate used was obtained from Himedia (Mumbai, India). Dimethylsulfoxide (DMSO) was obtained from Applichem (Darmstadt, Germany). Finally, a commercial suspension of Nys (Mycostantin®) was purchased from Bristol-Myers Squibb (Paço de Arcos, Portugal). Pure Nys was kindly donated by Angelini Farmacêutica (Dafundo, Portugal). All other reagents were of analytical grade.

Preparation of the toothpaste

A formulation of typical toothpaste normally contains a wide range of ingredients, each one with a purpose and with the potential to influence the performance and behavior of the toothpaste. The preparation of the semi-solid formulation (50 g) was performed according to previous works^{13–16}. All formulations and process parameters were optimized. Briefly, we separately prepared two phases: the first phase contains a homogeneous mixture of 0.65 g of xanthan gum in a minimum volume of sorbitol and 15 g of glycerol and the second phase contains 0.11 g of sodium fluoride dissolved in a citric acid buffer^{13,14}. These two phases were mixed over a period of 45 min and then 10 g of calcium carbonate and 0.1 g of microcrystalline cellulose were added under agitation over 30 min^{15,16}. Finally, the essence of mint (100 µL) and sodium lauryl sulfate (0.25 g) were added to the previous semi-solid dosage form.

Characterization of the toothpaste

Viscosity of the toothpaste was accessed using a Rotating Viscometer Brookfield (Middleboro, MA) with a spindle 7 with a rotation speed of 20 rpm.

The toothpaste was squeezed into a 100 mL beaker and the spindle was positioned and rotated at 20 rpm. The viscosity was recorded.

The pH value, the organoleptic and tactile aspects were also accessed. The reference values used (based on commercial toothpastes) for pH were between 5.66 and 7.35¹⁷.

Preparation of the alginate beads, MPs and NPs of Nys

Empty and Nys-loaded beads were prepared according to the technique of extrusion/external gelation^{18–20} meanwhile empty and Nys-loaded MPs and NPs were prepared according to emulsification/internal gelation^{18,21–24}. Briefly, for beads, Nys

were dissolved in 5 mL of a solution of alginate at 2% (w/v) and extruded into a aqueous solution of CaCl₂ 0.05 M.

For MPs and NPs, 10 mL of Nys were dissolved in alginate solution (2%, w/v) and calcium carbonate (aqueous dispersion of insoluble and ultrafine calcium carbonate crystals at 5%) was added. After homogenization, the resulting dispersion was emulsified within paraffin oil containing 1.5 mL of Span 80 by impeller-stirring homogenization (400 rpm for MPs and 1600 rpm for NPs). After 15 min emulsification, 20 mL of paraffin oil containing glacial acetic acid were added to the dispersion and stirring continued for 60 min to trigger instantaneous solubilization of calcium from carbonate complex, and concomitant gelation of the alginate particles. Nys-free particles (empty MPs and NPs) were also prepared using the same procedure.

Oil-dispersed alginate particles were recovered by using 12 500 g of centrifugation rate with a Z36-HK refrigerated high speed centrifuge (Hermle Labortechnik, Wehingen, Germany) coupled with dehydrating solvents in acetate buffer. After recovery, particles were resuspended in acetate buffer at pH 4.5 and stored at 4 °C. Alternatively, particles were frozen and lyophilized at 0 °C for at least 48 h (Freezone 2.5 L, Freeze-dryer Labconco, Kansas City, MO). After lyophilization, particles were stored at 4 °C.

Particle size analysis

Beads were macroscopically characterized and its image was digitally recorded.

The mean particle size, polydispersity index (PI) of the MPs and NPs were measured in hydrated particulate systems (diluted aqueous solutions, 1:1) with a Coulter Nanosizer Delsa Nano™C (Coulter Beckman, Fullerton, CA). A low value of PI factor indicates a more stable and less dispersed MPs and NPs distribution in size^{25,26}. All experiments were made at room temperature, with an angle detection of 165 °C and in duplicate.

Zeta potential analysis of the particles

Zeta potential was measured in Delsa Nano™C (Coulter Beckman, Fullerton, CA) by electrophoretic mobility technique. Each analysis was carried out at 25 °C with an angle of detection of 90 °C and in triplicate.

Particle morphology and surface

The morphology of the particles was assessed by scanning electron microscopy (SEM) before the centrifugation procedure. It is worthwhile noting that the sample with the empty particles was high and white dense suspension and the sample with the loaded particles was sparse and yellowish.

An aliquot of each sample was placed in a glass coverslip and left to dry in a desiccator for SEM analysis. The material was then coated with a thin layer of gold (with 500 nm of thickness) and observed on a JEOL 5200LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV. Images were digitally recorded.

Determination of Nys encapsulation efficiency

After the preparation of the beads, the supernatant was filtered and the amount of free Nys present in the supernatant was analyzed by a spectrophotometric method at 267 nm (UV-visible spectrophotometer, Evolution 300, Hertfordshire, England).

For the MPs and the NPs, supernatant was centrifuged 12 500 g during 15 min and the amount of free Nys present in the supernatant was spectrophotometrically analyzed at 267 nm. Measurements were made in triplicate and a standard calibration curve was made with a Nys standard solution. The established

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linearity range was 0.5–100 µg/mL ($R^2 = 0.987$) for the beads and 5–200 µg/mL for the MPs and the NPs ($R^2 = 0.998$).

In vitro drug release

The three systems (dried particles 50 mg) were placed in a recipient containing 50 mL of artificial saliva with a pH 6.5 and without enzymes and following sink conditions. Artificial saliva was prepared according to previous works²⁷. The system was maintained at 37 °C and in a constant stirring (130 rpm) in a multipoint laboratory magnetic stirrer (Thermo Scientific VARIOMAG® Telesystem Stirrers, Langenselbold, Germany). In appropriated time intervals, aliquots of the release medium were taken and replaced immediately with fresh saliva²⁷. Nys concentration was determined in triplicate using the UV-visible spectrophotometer and using a standard calibration curve in saliva. The established range was 0.5–32 µg/mL ($R^2 = 0.984$).

In vitro efficacy tests

The antifungal activity of the toothpaste with the different particle systems of Nys incorporated was tested against *C. albicans*. Briefly, an inoculum was obtained by suspending *C. albicans* colonies in sterile saline solution in order to reach a density equivalent to the 0.5 McFarland Standards. This inoculum was spread in Sabouraud agar media plates (wells of approximately 5 mm diameter) and filled with toothpaste containing each particle system of Nys. Two positive controls (pure Nys and toothpaste containing free Nys) and two negative controls (toothpaste with empty particles and toothpaste alone) were also poured into different wells in each agar media plate. Inoculated plates were incubated for 24 h at 37 °C and inhibition zones were measure in millimeters^{28–31}. Those studies were conducted over one year in order to evaluate the *in vitro* efficacy of the different particle systems and from the Nys along the time.

Determination of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to standard and legally accepted CLSI guidelines³². Briefly, after the *in vitro* drug release studies, those aliquots were also tested in order to determine the value of the MICs. Three microtiter plates with 96-wells were used, each for a different system of particles. Each well was filled with 100 µL of Sabouraud liquid medium and 100 µL of each aliquot was added with consequent two-fold serial dilutions. Finally, 10 µL of a suspension of *C. albicans* was added to each well. The plates were incubated in the dark at 37 °C for 24 h^{26,33–35}. The positive control was a solution of 1 mg/mL of Nys and the negative control was saliva.

Statistics

All results are expressed as mean ± standard deviation. One-way ANOVA analysis was applied to demonstrate statistical differences in all tested parameters. All analyses were performed using a software program (GraphPad Prism 5®, GraphPad Software, San Diego, CA) with a statistical significance level of 0.05.

Results and discussion

Oral candidiasis is a common opportunistic infection in medically compromised individuals. On the other hand, appropriate toothpaste is an essential key to daily oral hygiene routine. Toothpastes are pastes, gels or powders that help to remove plaque, a film of bacteria that forms on teeth and gums and to polish tooth surface.

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Table 1. Parameters of the tested and commercial toothpastes.

	Organoleptic parameters	Viscosity (mPa s)	pH
Tested toothpaste	Opaque (white) Paste can be easily cut off No trouble for punching The paste can keep its uniformity and homogeneity in the tube Very good foaming ability Great mint taste Great regular-flavor paste	19.4	6.8
Commercial toothpaste	Opaque with three colors Paste can be easily cut off Very good foaming ability Great taste	34.4	7

Toothpaste also improves the mechanical brushing and cleaning power of a toothbrush. In case of oral candidiasis treatment, effective antimicrobial agents are generally used in toothpaste to produce an inhibitory action on plaque formation and *Candida* colonization.

In this study, the pH values, the viscosity grade and the organoleptic and tactile aspects of the developed toothpaste and from commercial toothpaste were very similar (Table 1). The viscosity of the tested toothpaste was adequate since it was not hard to push out and extrude the paste from the tube³⁶. Commercial toothpaste was more viscous and disperses too slowly in the mouth and a sticky and thick paste formed in the oral cavity makes the washing of the mouth a hard task. These properties may not influence the function of the toothpaste but it could be an unpleasant situation especially for some age groups such as old people. In our toothpaste, fluoride was used to strengthen tooth enamel, remineralize tooth decay and based on a variety of mechanisms fluorides also may demonstrate some antibacterial and antifungal effects. Mild abrasive agent such as calcium carbonate was herein used to remove debris and residual surface stains. It may also reduce the numbers of acid living bacteria in the mouth as long as the mouth stays alkaline. In the last case, the value of pH of the mouth is very important. In our toothpaste, sorbitol was used as humectant agent, i.e. to prevent water loss in the toothpaste. Gum was applied as thickening agents or binders to stabilize the toothpaste formula. Sodium lauryl sulfate was used as detergent in order to create foaming action but also it has antimicrobial effects, which extend over several hours in the mouth and a moderate plaque-inhibitory action. However, our toothpaste by itself cannot eliminate the fungal as further we will demonstrate.

Particle characterization

Spherical alginate particles were obtained using standardized and reproducible conditions. Alginate beads were successfully produced by extrusion/external gelation method with a size range of 1–3 mm and depending on the dried or hydrated state (as seen in Figure 1). However, this extrusion method has at least three main drawbacks; the first being that size reduction is limited by nozzle diameter as well the viscosity of the solution. Particles less than 500 µm are difficult to produce. The second drawback is that the procedure is not suitable for industrial scale-up as producing particles on a large scale requires a large number of nozzles to be operated simultaneously. Finally, particles tend to be teardrop-shaped due to drag forces following impact with the gelation bath³⁷.

On the other hand, MPs and NPs were also easily produced through emulsification/internal gelation method. As demonstrated



Figure 1. Hydrated beads containing Nys (scale bar represents 5 mm).

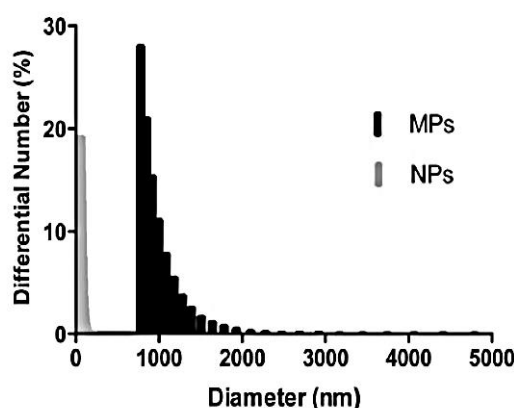


Figure 2. Size distribution of MPs and NPs in terms of number.

in Figure 2, MPs and NPs fulfilled the criterion that particle sizes should be less than 50 μm in diameter which was imposed in view of potential “mouthfeel” issues³⁸ where the size range was around 5.68 μm (PI 1.1) and 335 nm (PI 0.162) for MPs and NPs, respectively. By controlling the conditions under which the water-in-oil emulsion is produced, particle size can be easily controlled from a few nanometers potentially to millimeters in diameter. In internal gelation method, particles were very homogenous with rough surface as shown in Figure 3. In internal gelation method, calcium ions are homogeneously distributed in the alginate solution initially, thus diffusion of protons into the pre-gel droplets will induce gelation starting at the surface, giving rise to homogenous droplets. In all cases, freeze-drying did not preserve the spherical shape and the individual state of the particles.

Zeta potential was negative in all three systems ($-16.22 \pm 10.7 \text{ mV}$) and this value is according to previous studies³⁹. Moreover, zeta potential measurements suggested a weak stability to aggregation in solution due to the lower magnitude. This may result in a more favorable deposition from

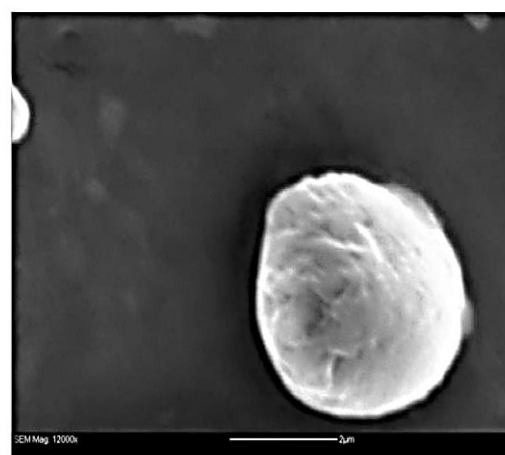


Figure 3. Morphology and surface characterization of particles produced by emulsification/internal gelation method (scale bar represents 2 μm , magnification 12000 $\times$).

the alginate onto the tissue like similarly described³⁷. Moreover, the high viscosity of the toothpaste could also be an important factor behind its greater binding to the oral epithelium and prolonged drug retention.

Alginate has been one of the most widely used polymers for the immobilization and microencapsulation of enzymes, cells and drugs. In this case, encapsulation efficiency of Nys significantly varied according the particle production method. For the Nys-loaded beads, encapsulation efficiency was around $88.87 \pm 2.56\%$; for the MPs was around $62.57 \pm 4.26\%$ and NPs was around $60.60 \pm 3.06\%$. Encapsulation efficiency was also determined by using a chelant agent of calcium (sodium citrate) which disrupts the alginate matrix and results were similar. In spite of the great capacity of the alginate to enhance encapsulation hydrophilic drugs^{40,41} and the logP of Nys is 0.5, which means that it exhibits poor hydrophilic characteristics. However, encapsulation efficiency values of the Nys in the MPs and in the NPs were lower than we expected⁴². In fact, emulsification methods are described as complexes processes since the recovery of the resultant particles generally produced a significant loss of the encapsulant agent during the centrifugation cycles. In addition, as previously described, internally gelled particles are more porous than externally gelled particles^{18,19}. The surface compactness of externally formed gels offers a higher resistance to diffusion in contrast to the more homogeneous gel formed by internal gelation^{18,19,43}.

In vitro drug release

The efficacy of conventional dosage forms for drug delivery to the oral cavity is often inhibited by the time residence (time contact) of the applied formulation. Salivation, the swallowing reflex, speech, mastication and food may affect displacement or dilution of the dosage form. Results showed significant differences: alginate beads have nearly an immediate and complete release within 3 h (Figure 4). This rapid drug release is also supported by previous studies⁴⁴. On the other hand, NPs prolonged Nys retention in saliva during 24 h and this effect was even more pronounced with MPs. Particle size might induce a more compact structure that limits the drug release.

In vitro efficacy tests

Results showed that the MPs was the system of particles which can provide and retain higher inhibition zone over a long period of

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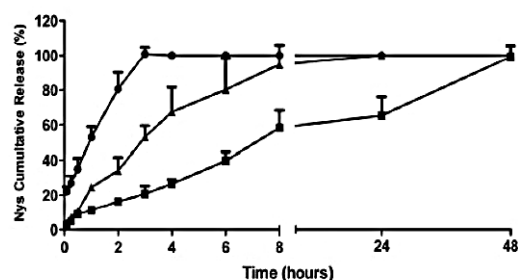


Figure 4. Cumulative release of Nys from the three particulate systems: beads (full circles), MPs (squares) and NPs (triangles).

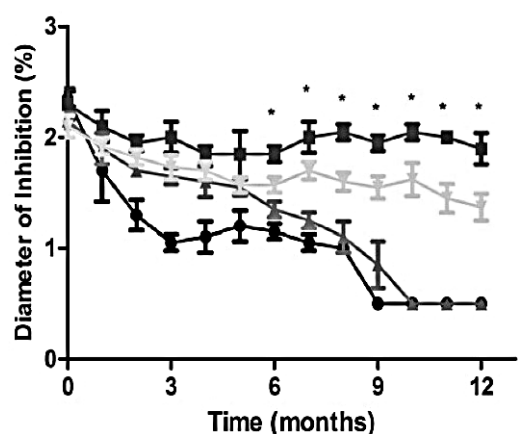


Figure 5. Profile of inhibition zone of *Candida albicans* over a period of one year: beads (full circles), MPs (full squares), NPs (full triangles) and Nys (grey and inverted triangles) with statistical differences between MPs and Nys (6–12 months). *Statistic significance of <0.05 when compared with the free NYS.

time, when compared to the beads, NPs or even Nys alone, as demonstrated in Figure 5. This high inhibition along the time may be explained through several points: first, beads leads to a rapid released of Nys as seen in *in vitro* drug release studies. Regarding to the NPs, NPs are very small in terms of size but their contact surface area is higher than MPs. In contrast to other studies⁴⁵, this last fact might induce some weakness when particles were incubated into saliva²⁷. Finally, free Nys was easily accessible to release medium and this fact explains the lower inhibition zone.

Determination of minimum inhibitory concentrations (MICs)

Results showed that the beads have a MICs value of $13.5 \mu\text{g/mL}$, MPs $5.21 \mu\text{g/mL}$, NPs $38 \mu\text{g/mL}$ ^{46,47} and negative control have adduced no inhibition. These results may be attributed to the prolonged and controlled release profile shown in the drug release assays and particle structure and size. When compared with the positive control, which presented a MIC value of $7.8 \mu\text{g/mL}$, the MPs are the most efficient system of particle since they present a lower MIC value to achieve a therapeutic effect. Like previously described, a nanoemulsion was also described as potential vehicle of Nys with very good *in vitro* results^{48,49}. We suggest that encapsulation technique using a bioadhesive polymer should potentially improve Nys therapeutic effect.

Conclusions

Candida is the most common fungal pathogen of the oral mucosa of immunocompromised patients. In general, gastrointestinal

absorption of antifungals like Nys is insignificant. Most orally administered Nys is passed unchanged in the stool and generally commercial Nys oral suspension requires several administrations along the day and at least 48 h after perioral symptoms have disappeared. This study described the preparation of three oral particulate systems of Nys and then included into a toothpaste instead of free Nys in suspension form. In this study, spherical polymeric particles loaded with Nys were successfully prepared. Microbiological studies revealed an enhanced antifungal effect of the Nys after encapsulation compared to free Nys. This study also showed that MPs allowed the lowest MICs and a high efficiency along the time (during one year) when compared with the beads or NPs or even free Nys. Thus, *in vitro* studies revealed that alginate MPs are promising vehicles for the delivery of actives in the oral cavity and it could be inferred that the developed formulation could be potentially applied for candidiasis infections during patient hygiene routine.

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Declaration of interest

The authors report no declarations of interest.

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Article III

Bioadhesive polymeric nanoparticles as strategy to improve the treatment of yeast infections in oral cavity: *in-vitro* and *ex-vivo* studies

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Macromolecular Nanotechnology

Bioadhesive polymeric nanoparticles as strategy to improve the treatment of yeast infections in oral cavity: *in-vitro* and *ex-vivo* studies



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ABSTRACT

Candidiasis is by far the most common fungal infection in the oral cavity and locally antifungal drugs are generally considered the first line therapy. However, some antifungal drugs like Nystatin (Nys) have very short time in the target tissue, which generally it requires several daily applications. One of the solutions to overcome those issues could be the incorporation of Nys into small, protective and polymeric nanoparticles (NPs). In this work, unloaded NPs were prepared and fully characterized and implemented in toothpaste and an oral gel. The three-best unloaded system (smallest, stable, safe and bioadhesive) was selected for further studies. After loading, NPs presented a size range from 300 to 800 nm, high encapsulation efficiency (above 70%), prolonged release and high adhesion capacity to oral mucosa when compared with free Nys. Free Nys lost its activity when it was not encapsulated. The presented results suggested that these new bioadhesive systems could be considered as good candidates to improve the time residence of Nys in buccal mucosa, reducing the number of applications during the day, using alcohol free-formulations and easily included in the daily lives of the patients.

1. Introduction

The mucosal and the transmucosal routes of drug delivery (i.e., the mucosal linings of the nasal, rectal, vaginal, ocular, and oral cavities) offer distinct advantages over per oral administration with pre-systemic effect. In the first case, the purpose is to achieve a site-specific release of the drug on the mucosa, whereas the second case includes drug absorption through the mucosal barrier to reach the systemic circulation. Between the various transmucosal routes, buccal mucosa has an excellent accessibility, an expanse of smooth muscle and relatively immobile mucosa, hence suitable for administration of controlled release dosage forms and new drug delivery systems like nanoparticles (NPs) [1].

Nanotechnology has demonstrated a high capability to target

specific areas of the body and to control the release of drugs. The most common types of NPs used for drug delivery are polymeric nanoparticles, solid lipid nanoparticles, liposomes, micelles and dendrimers. Each of these NPs has its own advantages and disadvantages as drug delivery vehicles [2–4].

Polymeric nanoparticles are one of the most tested and studies particulate systems. PLGA, Polyethylene glycol (PEG), Polyvinyl alcohol (PVA), PLA, Polycaprolactone (PCL), and Chitosan are the most common used polymers due to their biocompatibility, biodegradability, and the fact that they are easy to be functionalized. Besides these polymers others like Alginate (Alg) and Xanthan Gum are also widely used [5].

The use of Nanotechnology can be very useful to improve the efficacy of the treatment in some pathologies like in the case of

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opportunistic yeast infections. An opportunistic infection is an infection caused by pathogens (bacteria, viruses, fungi, or protozoa) that takes advantage of an opportunity not normally available, such as a host with a weakened immune system or a disrupted flora in a normal organism. Many of these pathogens do not cause any disease in a healthy host with normal immune system. However, in presence of a compromised immune system, a penetrating injury, or a lack of competition from normal commensals presents an opportunity for the pathogen to infect. In this type of infections, *Candida* spp. continues to be one of the most or the most common causes of opportunistic fungal infections [6,7].

The main objective of this work is the development and characterization of different types of polymeric NPs based on bioadhesive polymers. The secondary objective is to select the best system of NPs (smallest, stable, safe and adhesive), loaded with Nys and included both into toothpaste and an oral gel. Nys was herein chosen as the reference drug because it is one of the main antifungal drug used in the treatment of fungal infections. However, it generally requires a large volume of suspension in each administration (4–6 mL/6 h, 2–4 applications/day, 2 every 8 h). Also mouth irritation, nausea, vomiting or stomach upset may occur after Nys administration. Moreover, oral formulations of Nys available on the market generally present several problems such as high sucrose and ethanol proportions, their low solubility, the high volume of suspension to achieve the maximum efficacy, a high frequency of daily applications due to the cleansing effect of the saliva (minimum 3 per day) and some patients referred an unpleasant taste and a viscous consistency [6]. So, the encapsulation of Nys into bioadhesive NPs may have a great advantage to overcome those problems. This concept of bioadhesion is definitely very important in drug delivery. It describes the interaction established between synthetic or natural polymers with the mucus layer covering the mucosal epithelial surface. The improvement of the contact time and specific localization of the drug may improve the bioavailability of the encapsulated drug [8].

2. Materials and methods

Xanthan gum, Dextran from *Leuconostoc* spp., Pluronic® F-127, Miglyol® 812, Chitosan, Poly-(arginine), Alginate sodium salt (Alginate) from brown algae, Triphosphosphate (TPP) were supplied from Sigma-Aldrich (St. Louis, USA). PLGA and PLA used were obtained from Purac (Gorinchem, Netherlands). All other reagents utilized were also of analytical grade.

2.1. Empty NPs development

2.1.1. Method of preparation

PLGA and PLA NPs were prepared through emulsification/solvent diffusion method [9]. Briefly, an emulsion was prepared dissolving 50 mg of PLGA or PLA in a mixture acetone: ethanol (8:2). This solution was then placed in an ultrasonic bath for 15 min. After this period, the solution was then added drop by drop to 5 mL of an aqueous solution of Pluronic® F-127 at 0.1% (w/v) under a constant stirring speed of 800 rpm. After the last drop, the stirring continued for additional 15 min. Subsequently, the organic solution was removed using a rotary evaporator at 40 °C.

In the case of the poly-(arginine), the solvent displacement method was applied [10]. Briefly, an organic phase was prepared with 62.5 µL of Miglyol® 812 and 4.95 mL of acetone. This mixture was immediately poured on 10 mL of an aqueous solution which comprises poly-(arginine) (0.05% w/v) and Pluronic® F127 (0.25% w/v) under magnetic stirring. The suspension was then stirred for 10 min at 600 rpm.

Chitosan NPs were prepared according to the ionic gelation method [11]. Briefly, a chitosan solution with a concentration of 0.1% (w/v) was prepared by dissolving chitosan in a solution of 1% (v/v) of acetic acid. NPs were spontaneously formed upon addition of this first solution into a solution of TPP with a concentration of 0.3% (w/v). This addition occurred under gentle magnetic stirring (600 rpm) at room

temperature for a period of 2 h.

Chitosan/Dextran NPs were also prepared according to the ionic gelation method [11]. Briefly, a chitosan solution with a concentration of 0.1% (w/v) was prepared by dissolving low-molecular-weight chitosan in a solution of 1% (v/v) acetic acid (final pH 5). A second solution of dextran was also prepared in the same concentration 0.1% (w/v) (pH 3.2). To prepare the NPs the solution of Chitosan was added drop by drop into the Dextran solution, respecting the ratio of 6:4 (v/v), under magnetic stirring (800 rpm). After the last drop was added the stirring continued for a period of 2 h.

Xanthan gum NPs were prepared according to the *in-situ* polymer cross-linking method with some modifications [12]. In a short way, a combination of 500 mg Xanthan gum and 100 mg of Tween 20 were dissolved in 100 mL of water. At the same time, an organic solution was prepared containing 10 mL of acetone with 200 µL of Span 80. This organic solution was added drop by drop into the aqueous solution with a magnetic stirring (800 rpm) over a period of 2 h. After this period, a solution of glycerol was added and the agitation continued for a period of 30 min.

Alg NPs were prepared according to emulsification/internal gelation method [6]. Succinctly, 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%) were emulsified within 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 glacial acetic acid was added to the dispersion and the stirring continued for 60 min to trigger instantaneous solubilization of the calcium salt and concomitant gelation of the alginate particles. The oil-dispersed alginate particles were recovered by using centrifuge 26,230g (Z36-HK refrigerated high speed centrifuge (Hermle Labortechnik, Wehingen, Germany) coupled with dehydrating solvents in acetate buffer.

All NPs were produced in triplicate batches which were subsequently frozen at –80 °C without any cryoprotectant and lyophilized at –50 °C for at least 48 h (Freezone 2.5 L, Freeze-dryer Labconco, Kansas City, MO) to further determination of the NPs preparation yield. After lyophilization, NPs were stored at 25 °C. All the aqueous solutions used in the formulations of the NPs were prepared using ultrapure water 22 MΩ·cm at 25 °C.

2.1.2. Particle size analysis

The mean particle size and polydispersity index (PI) of NPs were measured using dynamic light scattering (DLS) method, in hydrated particulate systems (disperse in aqueous solutions), with a Coulter Nanosizer Delsa Nano™ C (Coulter Beckman, Fullerton, CA). A low value of PI factor indicates a monodisperse distribution. All experiments were made at room temperature, with a detection angle of 165 °C and in triplicate. This method was used for both the empty and the Nys-loaded NPs.

2.1.3. Zeta potential analysis

Zeta potential was measured in Delsa Nano™ C (Coulter Beckman, Fullerton, CA) by an electrophoretic mobility technique. Each analysis was carried out at room temperature with an angle of detection of 90 °C and in triplicate. This method was used for both the empty and the Nys-loaded NPs.

2.1.4. Particle morphology and surface

The morphology of the NPs was assessed by atomic force microscopy (AFM). To prepare the samples, a drop of each type of NPs was placed on freshly cleaved mica for a period of ten to fifteen minutes followed by rinsing off the excess liquid. After that, the samples were air-dried at room temperature and mounted on the microscope scanner of a Multimode Nanoscope III® (Digital Instruments, Bruker). Etched silicon tips (TESP, ca 300 kHz) were used for imaging in tapping mode, at a scan rate of ca. 1.6 Hz, under ambient conditions. The

morphological analysis of the NPs was performed in different zones of the mica. This method was used for both the empty and the loaded NPs.

2.1.5. Preparation yield

In order to predict the scale-up of this process, the yield of each preparation method was determined by weighting the pellets obtained after NPs centrifugation at 23,000g during 15 min or 38,000 × g during 10 min for Alg NPs and considering the weight of each component used in formulation. The yield of preparation (%) was determined following the Eq. (1).

$$\text{Yield} = \frac{(\text{Weight of the NPs after centrifugation})}{(\text{Weight of each compound present in the NPs formulation})} \times 100 \quad (1)$$

2.1.6. Preliminary in vitro safety assessment of empty NPs

Empty NPs were submitted to preliminary assessment of the biocompatibility based on a *Saccharomyces cerevisiae* model. This method, using a unicellular eukaryotic organism, was selected because it is a very fast method, economical and easy to execute. Regarding the study, it consisted on the incubation of NPs and yeast cells that were growing in YPD medium (1% yeast extract, 0.5% peptone, 2% glucose and 1.5% agar). Cell cultures of *S. cerevisiae* were prepared by transferring a cell colony growing on a Petri dish into a 50 mL sterile Erlenmeyer with 20 mL YPD medium and incubated at 30 °C with agitation (80 stirring cycles per min) for 24 h. This incubation leads to approximately 1.0×10^7 cells per mL. An equal volume of the yeast suspension was diluted with YPD medium in disposable cuvettes in order to obtain an initial absorbance for the tested NPs and control not higher than 0.10 at 525 nm. The control groups were prepared with medium without any exposing agent. The growth curve in the logarithmic phase for each tested group as well as for the control group was conducted by correlating the logarithm of the cell concentration (number of cells per mL) against the time of incubation. Each group was tested in four replicates. Cell concentrations were calculated by Eq. (2) previously described [13].

$$C = (\text{Sample}_{\text{Absorbance}} - 0.0327) / 6.8219 \times 10^{-8} \quad (2)$$

The growth inhibition (GI) of *S. cerevisiae*, in percent, was calculated from the linear slope of the logarithmic phase of each tested substance and concentration. The inhibition of the growth capacity caused by the cytotoxicity exerted on *S. cerevisiae* by the tested products gives an indication of their safety use.

2.1.7. Stability of empty NPs using harsh storage conditions

All formulations of empty NPs were studied over a period of 6 months in terms of their size and zeta potential. Samples of freshly prepared NPs were kept (in triplicate) as suspensions in closed 50 mL recipients at three different conditions: refrigeration conditions (Temperature = 4 ± 2 °C) and at residual humidity (RH) of 70%, at room temperature (Temperature = 25 ± 2 °C) and at RH of 50% and at higher temperature conditions (Temperature = 37 ± 2 °C) and at RH of 20%. Size, PI and the zeta potential were measured as described previously in the section “Particle size analyses” and “Zeta potential analyses”. This assay was also done for the Nys-loaded NPs.

2.2. Development of semi-solid formulations (SSF) with empty NPs: Toothpaste and oral gel

2.2.1. Method of preparation

The preparation of SSF (50 g) was performed according to previous works [14] and all formulations and process parameters were optimized. The toothpaste was composed by two phases. The first phase contains a homogeneous mixture of 0.65 g of xanthan gum in a minimum volume of sorbitol and 15 g of glycerol. The second phase

contains 0.11 g of sodium fluoride dissolved in a citric acid buffer. These two phases were mixed over a period of 45 min and then 10 g of calcium carbonate and 0.1 g of microcrystalline cellulose were added under agitation over 30 min. Finally, mint essence (100 µL) and sodium lauryl sulfate (0.25 g) were added to the previous semi-solid dosage form [6]. In the case of the oral gel, Carbopol 940 was the selected gelling agent. Basically, the preparation of the gel consists in the addition of a few drops of a solution of NaOH to an aqueous solution of Carbopol 940.

The final formulations were prepared considering 1:2 ratio in the case of the toothpaste (1g of NPs for 2 g of toothpaste) and 1:4 in the case of oral gel (1 g of NPs for 4 g of oral gel).

2.2.2. Characterization of the SSF without empty NPs: toothpaste and oral gel

Toothpaste and oral gel were tested in terms of their stability using the accelerated stability test. These two formulations (5 g) were submitted to heating (50 ± 1 °C) in a water bath and after they were centrifuged at 26,230g during 1 h. At the end, the samples were checked to see whether there was any change. All the formulations were tested in triplicate. Also, a heating/cooling cycle test was performed. Both formulations were stored in a fridge/oven and temperature was changed between 4 ± 2 °C (RH of 70%) and 40 ± 3 °C (RH 20%) every 24 h, respectively, during a period of 7 days. This thermal stress testing was performed one week after the preparation of the samples.

Parallel-plate method was used for determining the spreadability of both formulations [15]. The spreading capacity was determined 24 h after preparation by measuring the spreading diameter of 1 g of the gel 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 (Eq. (3)).

$$S = m \times l / t \quad (3)$$

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.

2.2.3. Characterization of the SSF with empty NPs: Toothpaste and oral gel

Viscosity of the final formulations was assessed using a Rotating Viscometer Brookfield (Middleboro, MA, USA). Formulations were squeezed into a 50 mL beaker and the spindle was positioned and rotated at a determined value of rpm. The viscosity and the torques were recorded. The pH values (pH electrode meter, 827 pH Lab, Metrohm, Switzerland) and the organoleptic and tactile aspects were also assessed. The reference values were based on commercial toothpastes [6].

2.2.4. Biodhesion testing of the toothpaste and oral gel with empty NPs

The adhesion capacity of the particles was determined using a TA-XTplus Texture Analyser (Stabel Micro Systems, Surrey, England) in the texture profile analysis mode and using the penetration application. The final formulations (30 g) were placed into recipients and were left to rest for a period of 24 h before testing, to remove the air of the formulations. Texture profile analysis involved an analytical probe (150 mm in height) being depressed into a standard sample (the two SSF without the NPs) to a defined depth (15 mm), at a defined rate (15 mm s^{-1}). At least 3 replicate analyses of each of the final formulation (SSF with the empty NPs) were performed at controlled ambient temperature of 25 ± 3 °C and from the resultant force-distance plot the hardness, compressibility and adhesiveness properties were estimated.

2.2.5. Stability tests of the toothpaste and oral gel with empty NPs

The stability was tested by monitoring pH and viscosity values of each formulation along 6 months.

2.3. Development of the Nys-loaded NPs

2.3.1. Method of preparation

After the characterization of empty NPs, the most promising formulations were selected to encapsulate the antifungal drug. NPs systems were selected taking into consideration the following parameters: small mean size of the particles, high adhesion capacity, safety and their stability along the time. Three of the seven NPs formulations were selected: NPs made of PLGA, PLA and Alg. To prepare the loaded-NPs, the model drug used was the antifungal agent Nys like we did in a previous work [6]. All the NPs were produced in triplicate. Before the initiation of the assays regarding the characterization of the loaded-NPs, the solubility of Nys was tested in Artificial Saliva (AS) pH = 6.5 and in PBS buffer pH = 7.4 to ensure sink conditions in further assays according previous references [16].

2.3.2. Method of quantification the Nys

The quantification of Nys was made using the spectrophotometer (UV Evolution 300, from Thermo Scientific, England) with a wavelength of 307 nm. All measurements were made in triplicate and standard calibration curves were made with Nys standard solutions. The range of each calibration curves were done according with the amount of Nys present in each system of NPs and used solvents used in NPs preparation. So, there was one for the Alg NPs and one for the PLA and PLGA NPs, with an established linearity range between 0.5 and 50 µg/mL ($R^2 = 0.9943$) and 0.5–100 µg/mL ($R^2 = 0.9928$), respectively. Also, different calibration curves were made for AS with an established range 5–60 µg/mL ($R^2 = 0.9903$) and for PBS buffer with range 3–150 µg/mL ($R^2 = 0.9943$).

2.3.3. Nys encapsulation efficiency (EE) in NPs

After the preparation of NPs, the supernatant was centrifuged at 23,000g for 15 min in the case of PLGA and PLA NPs, and at 38,000g for 10 min for Alg NPs. After the centrifugation, the amount of free Nys was determined by indirect and direct quantification methods: in the indirect method, the Nys present in the supernatant and in the direct method, the Nys presented inside the NPs was spectrophotometrically after particle disrupting [6,9,17]. All the samples were analyzed by the method describe in Section 2.3.2.

2.3.4. In vitro Nys release from NPs

Amounts of NPs corresponding to Nys 2.96 ± 1.29 mg were weighed and placed in 50 mL of AS (pH = 6.5) without enzymes under sink conditions (USP XXX). AS was prepared as described previously [6]. The system was maintained at 37 °C and in a constant stirring (130 rpm) in a multipoint laboratory magnetic stirrer (Thermo Scientific Variomag® Telesystem Stirrers, Langenselbold, Germany). At appropriate time intervals, aliquots of the release medium (2 mL) were taken and replaced immediately with fresh. Nys concentration was determined in triplicate using the UV–visible spectrophotometer. This assay was also performed with PBS buffer at pH = 7.4. In this case, the amounts of NPs corresponded to 1.63 ± 0.06 mg Nys and samples were withdrawn at appropriate time interval points. All the measurements were tested in triplicate [6] and quantified with the method described in Section 2.3.2. The solubility of Nys was determined before this assay according international guidelines to ensure that the assay was conducted under sink conditions. Before the measurements all of the aliquots were centrifuged at 7,250g for 5 min to obtain a clear sample.

2.3.5. Bioadhesion testing of Nys-loaded NPs

The bioadhesion capacity of the particles was determined using a TA-XTplus Texture Analyser (Stabel Micro Systems, Surrey, England) in the texture profile analysis mode and using the penetration application. Three samples of the final formulations dissolved in 3 mL of AS were prepared and measured to determine the final viscosity when the

formulations are applied in real life terms. These formulations with loaded NPs were also tested with the previous method used to determine the adhesion properties of the empty NPs, so that the formulations can be compared.

The mucoadhesion capacity of the selected systems of NPs was also accessed using the *in vitro* mucin binding testing of the NPs with the modified pig mucin method [18]. Briefly, NPs were mixed with 400 µg/mL pig mucin suspension in PBS buffer with a pH 7.4, respecting a ratio of 1:1 (v/v). After incubation at room temperature for 2 h, samples were centrifuged at 18,000g for 30 min (Centrifuge 5430R, Eppendorf, Hamburg, Germany). Subsequently, the absorbance of the remaining free mucin present in the supernatant was determined by UV spectrophotometry (UV-2550R, Shimadzu Corporation, Japan) with a wavelength of 251 nm with an established linearity range between 1.56 and 400 µg/mL ($R^2 = 0.9975$). The mucin-binding efficiency of the NPs was calculated using (Eq. (4)):

$$\text{Binding efficiency} = ((C_0 - C_s)/C_0) \times 100 \quad (4)$$

where C_0 represents the initial concentration of mucin and C_s is the concentration of free mucin present in the supernatant.

2.3.6. In vitro efficacy tests

After drug release assessment, the efficacy of Nys-loaded NPs was tested using the well diffusion method on *Sabouraud* dextrose agar medium in order to verify the activity of the NPs before and after the release of the Nys and if the encapsulation process changed Nys activity [19,20]. Briefly, the assays were performed on *Sabouraud* dextrose agar (SDA, Biokar Diagnostics, Beauvais, France) plates using as inoculum 0.1 mL of *C. albicans* (ATCC 10231) suspension (in sterile water) adjusted to 0.5 McFarland standard. Wells of 5 mm in diameter were made on the agar surface and filled with 50 µL of each compound: the systems of NPs containing Nys, before and after the release assays. Three positive controls (Nys at a concentration of 3.59 mg/mL, Nys after incubation in PBS pH = 7.4 for 7 h and Nys after incubation in AS pH = 6.5 for 4 h) and two negative controls (PBS buffer and AS) were also poured into different wells in each agar media plate. The plates were incubated at 37 °C for 24 h. The antifungal activity was assayed by measuring the diameter of the inhibition zone and reported in millimeter (mm). Each assay was performed in triplicate.

2.3.7. Ex-vivo permeation tests in porcine mucosa

According previous work [21], the mucosa excised from porcine mouth was obtained from the slaughter house. The buccal mucosa was separated from the full thickness of the tissue and the fatty layers were removed by scalpel, and the buccal mucosa was isolated from the underlying tissue. Finally, the mucosa was washed with isotonic phosphate buffer, pH = 7.4. These mucosae were mounted between the donor and receiver chambers of Franz diffusion cells with the same available diffusion area and the donor chambers were filled with the formulations containing the NPs and empty NPs as was done in the previous test. The amount of Nys present in the PBS buffer from receptor compartment of each sample was determined using the method described in Section 2.3.2.

2.3.8. Stability testing of the Nys-loaded NPs

After the physical characterization of the final formulations composed by the toothpaste and the oral gel with the NPs, these formulations were stored at temperature of 4 ± 2 °C and 70% RH and their stability was tested. To achieve this purpose, the pH and the viscosity values of each suspension were measured after 3 and 6 months.

2.4. Statistics

All results are expressed as mean $\pm$ standard deviation. One-way ANOVA and Two-way ANOVA analysis were applied to demonstrate statistical differences in all tested parameters. All analyses were

Table 1
Characterization of different polymeric NPs (n = 3).

NPs systems	Mean size $\pm$ SD (nm)	Polydispersity Index (PI)	Mean zeta potential $\pm$ SD (mV)	Yield (%)
Alg	574.5 $\pm$ 223.6	0.516 $\pm$ 0.010	−1.97 $\pm$ 1.67	59.85 $\pm$ 1.00
Chitosan	3419.8 $\pm$ 477.5	0.984 $\pm$ 0.034	+23.26 $\pm$ 1.00	50.00 $\pm$ 0.50
Chitosan/dextran	1452.4 $\pm$ 357.6	0.566 $\pm$ 0.117	−0.11 $\pm$ 0.03	41.33 $\pm$ 2.25
PLA	194.2 $\pm$ 2.8	0.103 $\pm$ 0.029	−0.14 $\pm$ 0.03	91.82 $\pm$ 1.10
PLGA	219.6 $\pm$ 3.3	0.096 $\pm$ 0.022	−2.57 $\pm$ 0.10	69.45 $\pm$ 2.50
Poly-(arginine)	231.0 $\pm$ 12.1	0.215 $\pm$ 0.134	+1.85 $\pm$ 0.01	9.71 $\pm$ 0.25
Xanthan gum	664.7 $\pm$ 151.4	0.314 $\pm$ 0.020	−4.60 $\pm$ 4.46	50.27 $\pm$ 1.50

performed using a software program (GraphPad Prism 5*, GraphPad Software, San Diego, CA) with a statistical significance level of 0.05.

3. Results and discussion

3.1. Empty NPs

In this work, NPs were successfully prepared by the respective production methods, with a size range between 200 and 700 nm for PLA, PLGA, Poly-(arginine), Alg and Xanthan gum NPs (Table 1). Despite the Chitosan and Chitosan/Dextran NPs being successfully produced, they produced agglomerates ranged 3–5 μ m and this issue may represent a drawback for buccal delivery. According to previous reports, to permeate through oral mucosa, NPs would have to present a size up to 500 nm. Non-mucoadhesive particles with a diameter of 500 nm can diffuse between mucin fibers in the microarchitecture and mesh spacing of the mucin network [22–25]. In addition, chitosan-based NPs may present another issue; chitosan can be used as a substitute of chitin in the synthesis of the fungal cell wall, and then it may indirectly interact with the fungal proliferation [26,27].

AFM analysis (Fig. 1) confirmed the presence of spherical shaped nanostructures for all the different NPs types. In spite of the variations in the average sizes of the NPs, they all exhibit a diameter lower than 500 nm. In the case of Alg, PLGA and PLA NPs (Fig. 1A, C and D, respectively) one can depict individual and well-defined NPs, where the size of the latter two is in agreement with the estimated by DLS (Table 1), confirming a low tendency for aggregation. The image of Xanthan Gum NPs also shows round particles; however, the image resolution is affected by the adhesion established between the sample and the tip. Concerning Poly-arginine, Chitosan and Chitosan/Dextran (Fig. 1F, G, and E, respectively) spherical NPs are co-adsorbed with compounds that did not form distinct NPs.

Table 1 also shows the results of the zeta potential. Most of the NPs presented a negative or very slightly positive charge (low values) except Chitosan/TPP NPs. The low values may suggest a weak stability leading to aggregation in solution. Somehow, this may originate a more favorable deposition from the NPs onto the tissue as previously described [28,29].

During 6 months, the NPs were tested in terms of their size and zeta potential, to verify the stability of the particles in different environments. The results showed that PLA, PLGA, Poly-(arginine) and Xanthan Gum NPs were very stable at different temperatures since the size and the surface charge of the particles remained unaltered. These results were also similar to previous works [30,31]. In the case of the Alg NPs, they were also very stable regarding the size of the particles, but the surface charge presented a significant statistical difference ($p < 0.001$). In terms of the most unstable one, it was demonstrated that NPs based on chitosan were not very stable over time since they demonstrated a significantly statistical difference when compared with the initial values ($p < 0.001$) as showed in Table 2. Depending on the method of production, chitosan NPs tend to be stable after a period of 12 months [32]. The storage temperature influenced the particle size; the majority of NPs increased the PI at higher temperature with values ranging between 0.169 and 2.344. In general terms surface charge, this

value was maintained, but in some cases the charge changed at all temperatures. This fact can be explained because NPs surface charge can also change depending on the pH of the storage solution [33,34].

Preparation Yield evaluation assay indicated a wide range of variation from 10% to 91%. The Poly-Arginine presented the lowest percentage (9.71%) and the PLA the highest percentage (91.82%), as can be observed in Table 1. These values are in accordance to previous reports where PLA NPs can lead to high values from 88.73% to 92.73% [10,35–38].

The preliminary safety *in vitro* studies performed with NPs demonstrated that their concentration does not affect the growth capacity of *S. cerevisiae*. Moreover, it was observed that the size of the particles does not influence the assay but the type of polymer will influence the final values. In some cases, the polymer will also contributed to the background absorption at 525 nm, showing absorptions higher than 0.9 at the later growth phase [39]. Despite that, and assuming the values obtained in the controls and standards tested, performed only with NPs in the medium, the NPs may be safe when used as an oral delivery system, since that the only systems of NPs with a percentage of inhibition higher than 20% were the Chitosan/Dextran NPs (Fig. 2).

3.2. Semi-solid formulation (SSF) characterization

Until this section, the results obtained in terms of small mean size, high mucoadhesion capacity, high stability and high preparation yield suggested that the most promising systems of NPs were the PLA, PLGA and Alg NPs. But in order to verify their bioadhesion capacity, these three systems were implemented into the semi-solid formulation (SSF). The choice of two different SSF (toothpaste and oral gel) was based in different factors: the toothpaste is an essential key to daily oral hygiene routine in adult's life and an oral gel is normally used in the new-born and young children. On the other hand, the toothpaste also improves the mechanical brushing and cleaning power of a toothbrush. In case of fungal infections treatment, effective antimicrobial agents may be generally used in toothpaste to produce an inhibitory action on plaque. Those properties can also be observed in the oral gel with foaming absence so it can be used for children and babies [6,40].

The developed toothpaste and gel were based on previous work [6] and commercial formulations present in the market. As shown in Table 3 (samples 1 for the toothpaste and sample 8 for the oral gel), the viscosities of the tested formulations were adequate for their future application since it was not hard to push out and extrude the paste or gel from the tube. Regarding the pH, the pH values ranged between the 3.67 and the 11.13 that are regular values based on commercial toothpastes. All the formulations presented similar pH values. The target pH value should be above the 7.5, where the teeth should become stronger and easily can re-mineralize [41,42]. The toothpaste was very stable along time since no dual phase formation was evident during stability testing. The viscosity and the pH values remained unchanged during cycles testing. In the case of the oral gel, cycles testing showed that the viscosity and the pH value were influenced along time, showing less stability than the toothpaste. Furthermore, before the inclusion of the NPs, the two formulations were tested in terms of their spreadability. Knowing that each person applies SSF with a slightly

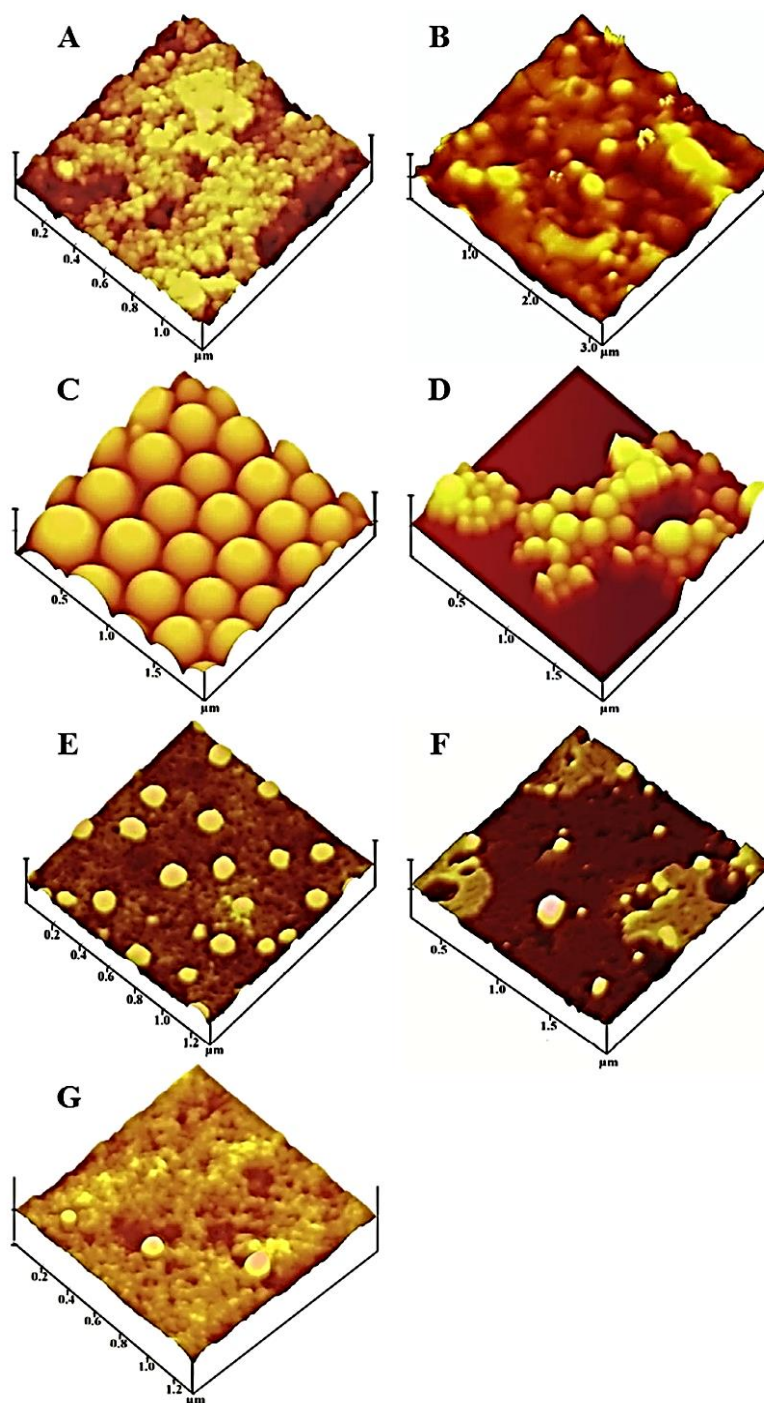


Fig. 1. AFM image in 3D from the seven different empty-NPs systems: Alg NPs (A), Xanthan Gum NPs (B), PLA NPs (C), PLGA NPs (D), Chitosan/Dextran NPs (E), Poly-arginine NPs (F) and Chitosan NPs (G).

Table 2
Stability of the empty NPs during 6 months (n = 3).

NPs systems	Tested conditions	Time		3 months				6 months			
		Mean size	± SD (nm)	PI	Mean zeta potential	± SD (mV)	PI	Mean size	± SD (nm)	PI	Mean zeta potential ± SD (mV)
Alg	4 ± 2 °C	825.8 ± 27.4		0.326 ± 0.020	-3.14 ± 2.17			1098.4 ± 222.2		0.397 ± 0.076	-1.34 ± 0.10
	25 ± 2 °C	1030.9 ± 37.7		0.373 ± 0.014	-2.85 ± 2.02			3566.7 ± 452.5		0.846 ± 0.083	-2.64 ± 1.44
	37 ± 2 °C	827.1 ± 50.1		0.334 ± 0.013	-7.68 ± 2.45			491.5 ± 96.3		0.215 ± 0.049	-2.10 ± 1.02
Chitosan	4 ± 2 °C	16283.0 ± 1918.8		0.336 ± 0.262	+0.16 ± 0.06			55594.6 ± 3631.8		0.395 ± 0.180	-0.13 ± 0.04
	25 ± 2 °C	-		-	-			-		-	-
	37 ± 2 °C	-		-	-			-		-	-
Chitosan /dextran	4 ± 2 °C	19562.8 ± 9873.6		1.830 ± 0.678	-0.15 ± 0.06			-		-	-
	25 ± 2 °C	6329.7 ± 3475.9		1.786 ± 0.827	-0.15 ± 0.10			-		-	-
	37 ± 2 °C	9280.4 ± 9003.3		2.344 ± 1.601	-0.26 ± 0.07			-		-	-
PLA	4 ± 2 °C	214.9 ± 1.7		0.036 ± 0.032	+0.05 ± 0.01			233.4 ± 2.8		0.084 ± 0.027	+0.32 ± 0.14
	25 ± 2 °C	230.6 ± 0.40		0.238 ± 0.008	-0.12 ± 0.05			211.5 ± 8.3		0.079 ± 0.016	+0.17 ± 0.07
	37 ± 2 °C	67.6 ± 7.3		0.254 ± 0.021	-0.13 ± 0.04			351.7 ± 80.5		0.294 ± 0.081	+0.19 ± 0.11
PLGA	4 ± 2 °C	242.7 ± 9.5		0.167 ± 0.032	-0.67 ± 0.01			223.0 ± 9.0		0.032 ± 0.026	+0.22 ± 0.14
	25 ± 2 °C	198.2 ± 4.4		0.086 ± 0.028	-0.30 ± 0.01			260.4 ± 9.3		0.162 ± 0.022	+0.10 ± 0.04
	37 ± 2 °C	252.4 ± 7.3		0.169 ± 0.041	+0.06 ± 0.04			438.8 ± 65.7		0.197 ± 0.027	-0.10 ± 0.00
Poly-(arginine)	4 ± 2 °C	180.7 ± 2.1		0.110 ± 0.028	-0.09 ± 0.01			183.1 ± 92.1		0.191 ± 0.001	-0.85 ± 0.29
	25 ± 2 °C	197.6 ± 2.6		0.095 ± 0.007	+4.73 ± 0.10			225.9 ± 1.2		0.046 ± 0.064	+0.12 ± 0.05
	37 ± 2 °C	216.4 ± 3.0		0.220 ± 0.014	+1.95 ± 0.63			255.3 ± 8.0		0.160 ± 0.003	+1.51 ± 0.12
Xanthan gum	4 ± 2 °C	316.2 ± 7.9		0.302 ± 0.019	-7.46 ± 1.50			366.2 ± 17.2		0.307 ± 0.017	-6.94 ± 1.00
	25 ± 2 °C	917.0 ± 98.8		0.373 ± 0.032	-3.70 ± 0.10			1083.5 ± 42.7		0.425 ± 0.009	+0.09 ± 0.01
	37 ± 2 °C	1359.2 ± 180.5		0.483 ± 0.052	+0.09 ± 0.01			1354.5 ± 54.0		0.439 ± 0.014	-8.25 ± 1.50

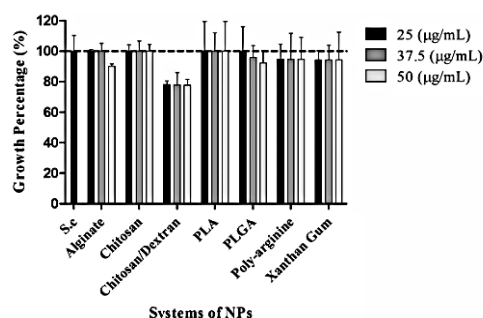


Fig. 2. Safety assessment of the empty-NPs in *S. cerevisiae* (S.C.) model, according to the preliminary *in vitro* safety test (n = 4).

Table 3

Characterization of the SSF with empty NPs (n = 3).

Samples	Adhesiveness (N/mm)	Viscosity (mPas)	pH
1	-0.168 ± 0.005	1706.00 ± 16.56	7.47 ± 0.20
2	-0.280 ± 0.007	3072.33 ± 61.33	5.72 ± 0.18
3	-0.166 ± 0.004	1346.45 ± 17.09	6.09 ± 0.06
4	-0.185 ± 0.003	1546.33 ± 15.14	7.35 ± 0.15
5	-0.187 ± 0.004	2932.33 ± 11.02	5.64 ± 0.27
6	-0.160 ± 0.005	1456.67 ± 33.55	7.39 ± 0.12
7	-0.176 ± 0.005	1576.67 ± 11.93	7.45 ± 0.16
8	-0.489 ± 0.019	83330.67 ± 8.50	6.06 ± 0.01
9	-0.004 ± 0.002	690.00 ± 65.57	4.71 ± 0.04
10	-0.055 ± 0.001	841.67 ± 28.02	4.51 ± 0.12
11	-0.140 ± 0.006	2028.00 ± 22.65	4.76 ± 0.14
12	-0.343 ± 0.008	2739.00 ± 13.53	5.33 ± 0.11
13	-0.483 ± 0.014	66774.67 ± 12.66	4.92 ± 0.33
14	-0.464 ± 0.013	60703.67 ± 20.11	5.07 ± 0.13

Sample definition: (1) Toothpaste control; (2) Toothpaste with Alg NPs; (3) Toothpaste with Chitosan/TPP NPs; (4) Toothpaste with Chitosan/Dextran NPs; (5) Toothpaste with Xanthan Gum NPs; (6) Toothpaste with PLA NPs; (7) Toothpaste with PLGA NPs; (8) Oral Gel control; (9) Oral Gel with Alg NPs; (10) Oral Gel with Chitosan/TPP NPs; (11) Oral Gel with Chitosan/Dextran NPs; (12) Oral Gel with Xanthan Gum NPs; (13) Oral Gel with PLA NPs; (14) Oral Gel with PLGA NPs.

different motion, stroke, and rate, any rheological estimation of this process involves some degree of error. Nevertheless, good approximations can still be made. Tests and estimations are important in the development of the formulations and can influence their final behavior [15,43]. In terms of the spreadability, both SSF presented comparable

values 49.98 ± 5.47 and 32.12 ± 11.89 g/cm/s for the toothpaste and the oral gel respectively, also those values were similar to previous works [44–46].

After the preparation and characterization of the SSF, the NPs were incorporated and the final formulation was fully characterized as seen in Table 3. In the case of the toothpaste, it improves the viscosity after NPs loading and Alg NPs was the best one. In the case of the oral gel, the best ones were the PLA and the PLGA NPs. This result can be explained by the fact that using polymers with mucoadhesive properties and gelling properties like Alg strongly influenced the viscosity of the SSF. The amount of polymer used may increase or decrease the viscosity depending on the type of SSF [47,48]. The pH values were also determined and all values were in accordance to commercial formulations [49]. The values of the toothpaste must be between 5.5 and 8.0 (the recommended value is above 7.5, as explained above) and the values of the oral gel must range from 4.5 to 6.5, according to the previous literature [49]. The adhesiveness of the final formulation was also analyzed and measured by calculating the force needed to detach the probe from the formulations. Alg NPs presented a higher adhesion when implemented in the toothpaste formulation and the PLGA and the PLA NPs in the oral gel formulations. On the other hand, the correlation established between the values of viscosity and the values of adhesion was according with the theoretical predictions. It means that when the formulation present a higher value of viscosity the adhesion capacity would be better and vice-versa, which was also verified in other works [50]. Regarding the stability of NPs incorporated in SSF, pH values and viscosity parameters are shown in Table 4. These changes can be explained by two facts comprising the degradation of polymers along time (i.e. PLA and PLGA degradation from several days to several months depending on the molecular weight and co-polymer composition) [51], and the interaction established between NPs and SSF components with time, thereby changing the viscosity and the pH values [52–55].

Considering the results obtained with empty NPs in terms of characteristics correlation, particle size and morphology and adhesion capacity, Alg NPs represents the best option to be incorporated in the toothpaste formulation. Previous works have shown that Alg can be a good polymer to encapsulate antifungal drugs and can be orally administered [6]. On the other hand, PLGA and PLA NPs showed to be a promising NPs system loading drugs to be further implemented in the oral gel.

3.3. Nys-Loaded NPs

Nys was herein selected as a model drug since it is one of the most common antifungal drugs used in the treatment of fungal infections, and also because it presents a broad spectrum of activity. However,

Table 4

Stability of the empty NPs incorporated in the SSF (n = 3).

Samples	3 months		6 months	
	Viscosity (mPas)	pH	Viscosity (mPas)	pH
1	1910.00 ± 10.58	8.85 ± 0.22	1460 ± 0.00	9.08 ± 0.01
2	3449.33 ± 2.31	6.42 ± 0.06	2808 ± 0.00	6.69 ± 0.02
3	1262.00 ± 6.93	7.69 ± 0.16	1461.3 ± 1.15	7.79 ± 0.06
4	1308.67 ± 5.03	8.48 ± 0.16	1089.3 ± 212.0	8.91 ± 0.08
5	3157.33 ± 54.60	8.26 ± 0.12	3656 ± 0.00	8.96 ± 0.06
6	1263.33 ± 7.57	8.82 ± 0.11	1084.7 ± 161.67	9.21 ± 0.02
7	1359.33 ± 74.22	8.46 ± 0.21	1248.7 ± 8.08	8.60 ± 0.02
8	92003.67 ± 5.51	5.19 ± 0.07	48356.7 ± 176.16	6.06 ± 0.05
9	638.67 ± 18.77	4.60 ± 0.15	595 ± 161.01	4.59 ± 0.02
10	741.67 ± 62.80	4.36 ± 0.12	779.3 ± 194.82	4.38 ± 0.02
11	1801.33 ± 24.11	5.11 ± 0.16	2008 ± 0.00	5.25 ± 0.01
12	6607.00 ± 11.27	5.09 ± 0.07	6272 ± 21.50	5.22 ± 0.02
13	61428.67 ± 62.29	5.64 ± 0.12	37023.3 ± 549.76	5.17 ± 0.03
14	49128.33 ± 25.66	5.09 ± 0.07	49138.8 ± 25.66	5.13 ± 0.01

The samples present the same definition of Table 3.

several problems related to its solubilization in water and low permeation through oral mucosa limits its use. It is described that Nys aqueous solubility is 0.1 mg/mL but no consistent data is available [56,57]. Thus, we decided to mimic the biological environment of Nys in oral mucosa. The solubility of saturated solutions of Nys was tested in PBS buffer (pH = 7.4) and in AS (pH = 6.5) after a period of 72 h. The results obtained showed that the solubility in PBS buffer was 0.04 ± 0.03 mg/mL (25 °C) and 0.16 ± 0.02 mg/mL (37 °C), whereas a greater solubility of 0.19 ± 0.04 mg/mL (25 °C) and 0.39 ± 0.11 mg/mL (37 °C) was obtained in the case of AS. This means that Nys should be soluble in AS and in PBS buffer after released from NPs. Nys assays demonstrated no degradation of the active pharmaceutical ingredient during the encapsulation process, which can potentially occur during this procedure. First, encapsulation efficiency assay was made by analyzing the quantity of Nys present in the supernatant after the preparation and centrifugation of the NPs (indirect method) but also Nys present inside the NPs after the disruption of the NPs (direct method). In the indirect method, the most efficient system was PLA NPs with an EE value of $94.74 \pm 0.41\%$. Lower values were obtained with PLGA and Alg NPs, $71.05 \pm 9.60\%$ and $71.78 \pm 15.65\%$, respectively. This fact shows that there is a higher affinity between PLA and Nys as compared to the other two polymers. The direct method led to similar values and conclusions.

The characterization studies showed that the encapsulation of Nys did not influence the PLA and PLGA NPs particles in terms of their mean size, showing a mean diameter of 332.8 ± 172.0 and 413.1 ± 186.8 nm and a surface charge of -0.10 ± 0.01 and -2.97 ± 0.10 mV, respectively. In terms of the Alg NPs, they presented a higher particle size (886.7 ± 13.7 nm) when compared with the empty particles observed in the DLS analysis and presented a surface charge of -0.34 ± 0.05 mV. Corroborating results were obtained by AFM analyses (Table 5). As far as bioadhesion capacity to the oral mucosa is concerned, Table 6 shows that Nys-loaded NPs increased the adhesion capacity of Nys when compared with its commercial formulation with and without the presence of AS. Also, the results present in Fig. 3 showed that the interaction established between the mucin and

the loaded-NPs is extremely higher when compared with the solution of free Nys, where the NPs presented a percentage of binding to the mucin between 97 and 99% and the free Nys a value of $19 \pm 0.9\%$. No significant difference was observed between the NPs of different polymers. Drug release profiles showed that all NPs display an immediate release in AS between 2 or 3 h (Fig. 4A). In case of PBS buffer, a more prolonged released profile of 6–7 h was observed (Fig. 4B). These results showed that despite two of the formulations achieving a 100% release profile after 3 h (PLA NPs and PLGA NPs) and the formulation containing Alg NPs achieved a 100% release profile after a period of 2 h, PLA NPs presented a better profile showing a good linearity over time. However, Alg NPs led to the most rapid drug release in AS achieving $71.25 \pm 5.60\%$ in the first 30 min. This Alg NPs behavior was confirmed in PBS. In contrast, the release of Nys was slightly different in the case of PLA and PLGA NPs, achieving 100% after a period of 6 and 7 h, respectively. The results obtained can be explained because of the properties of particles in the case of Alg NPs. Alg NPs may be more porous when compared with PLA and PLGA NPs. Thus, a burst release of Nys will be more predictable to happen. This fact was observed by Nazila Kamaly in 2016 [58]. On the other hand, any difference observed could be also related to the pH values of each tested medium, as concluded by Banu S. Zolnik in 2007 [59]. In general, acidic pH conditions can be utilized to accelerate drug release from PLGA microspheres [59,60].

The efficacy of Nys after encapsulation into NPs was tested and compared versus free Nys. Nys-loaded NPs showed a higher inhibition zone when compared to free Nys as shown in Table 7 showing therefore an advantage in the encapsulation of an antifungal drug [61]. The results obtained showed that the encapsulated Nys will present the same inhibition activity before and after the release assay, showing that during the period of release of the drug from the NPs the activity will not be influenced. Also, when compared with the free Nys the systems of NPs present a great advantage, since free Nys loses all of its inhibitory activity at the end of the release assay. In any case, the excipients showed no inhibitory effect on the yeast growth, proving that no interferences took place.

Table 5
Characterization of the SSF with loaded NPs.

Sample	AFM images (topography) of loaded NPs in 2D	AFM images (profile) of loaded NPs
PLA NPs		
PLGA NPs		
Alg NPs		

Table 6
Characterization of the SSF with Nys-loaded NPs based on Alg, PLGA and PLA (n = 3).

Samples	Adhesiveness with synthetic membrane (N/mm)		Viscosity (mPas)	
	Without saliva	With saliva	Without saliva	With saliva
1	-0.051 ± 0.004	-0.048 ± 0.005	1706 ± 16.56	71.80 ± 8.73
2	-0.542 ± 0.154	-0.052 ± 0.001	15110 ± 20.50	187.2 ± 1.50
3	-0.274 ± 0.017	-0.025 ± 0.010	3649 ± 54.31	63.4 ± 0.06
4	-0.253 ± 0.038	-0.046 ± 0.002	3072 ± 61.33	75.8 ± 2.31
5	-0.090 ± 0.010	-0.079 ± 0.005	83330 ± 8.50	169.4 ± 0.6
6	-0.677 ± 0.050	-0.256 ± 0.019	70830 ± 50.50	1511 ± 1.60
7	-0.642 ± 0.244	-0.156 ± 0.014	68910 ± 49.00	1418 ± 1.64
8	-0.520 ± 0.024	-0.059 ± 0.003	60703 ± 20.11	72 ± 0.00
9	-0.599 ± 0.065	-0.061 ± 0.018	66774 ± 12.66	106.1 ± 0.00
10	-0.535 ± 0.118	-0.051 ± 0.012	66690 ± 51.96	96.7 ± 0.58
11	–	-0.016 ± 0.001	–	42.56 ± 1.00

Sample definition: (1) Toothpaste control; (2) Toothpaste with loaded Alg NPs; (3) Toothpaste with free Nys; (4) Toothpaste with empty Alg NPs; (5) Oral Gel Control; (6) Oral Gel with loaded PLA NPs; (7) Oral Gel with loaded PLGA NPs; (8) Oral Gel with empty PLGA NPs; (9) Oral Gel with empty PLA NPs; (10) Oral Gel with Free Nys; (11) Commercial Nys.

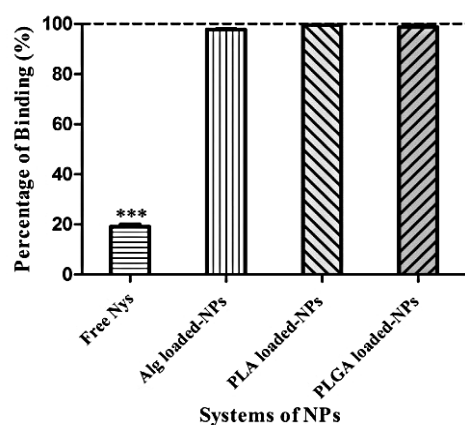


Fig. 3. *In vitro* mucin bioadhesion profiles of the Nys-loaded NPs (n = 3), ***p < 0.0001.

Additionally, it can be observed that the Nys-loaded NPs when implemented in SSF have the capacity to maintained the inhibition activity of Nys during a period of 6 months like we previously observed without SSF [6].

In terms of NPs stability in solution and when implemented into the SSF, they showed a great stability after a period of 3 months as observed in Table 8 and in Table 9, respectively. Despite the fact that the results also demonstrated that the particles presented a larger size and PI after 6 months, this may be explained because the analyses were made regarding the mean volume size. This factor will not distinguish different NPs size populations, resulting in a relatively small population of

Table 7
Inhibition zone (n = 2 or 3).

Samples	Inhibition zone (mm)
PLA NPs (before) - 3.59 mg/mL	16
PLGA NPs (before) - 3.59 mg/mL	16
Alg NPs (before) - 3.59 mg/mL	18
Alg NPs (after in PBS) - 0.054 mg/mL	11
Alg NPs (after in AS) - 0.059 mg/mL	11
PLGA NPs (after in PBS) - 0.054 mg/mL	10
PLGA NPs (after in AS) - 0.059 mg/mL	12
PLA NPs (after in PBS) - 0.054 mg/mL	12
PLA NPs (after in AS) - 0.059 mg/mL	13
Negative control (PBS)	0
Negative control (AS)	0
Positive control- Nys in PBS - 0.072 mg/mL	0
Positive control- Nys in AS - 0.072 mg/mL	0
Positive control- Nys (before) - 3.59 mg/mL	30

aggregates being responsible for an abrupt change in the mean value whereas a high percentage of particles still remain within the original size range. Also regarding the results corresponding to PLGA NPs at 37 °C after 6 months of storage, they presented a value of zero because the particles were totally dissolved in the medium and the equipment failed to obtain any value. This indicates that at high temperature the PLGA particles after 6 months were not very stable as they tend to dissolve in the medium.

Finally, *ex-vivo* permeation assays were also conducted. In this case, all loaded NPs when implemented in the SSF were tested after 3 min massage of the samples in excised porcine oral mucosa. A Nys solution was also tested in the same conditions to serve as control.

Table 10 and Fig. 5A, B and C (for PLA, PLGA and Alg NPs respectively) showed that Nys encapsulated in the NPs presented a better permeation profile when compared with the free Nys. Higher diffusion

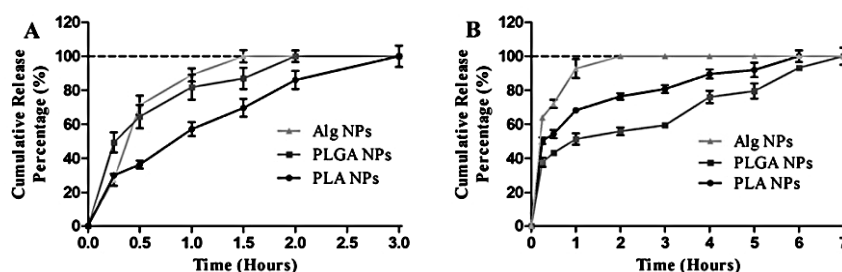


Fig. 4. Release profile of Nys-loaded NPs in AS with a pH = 6.5 (A) and in PBS buffer with a pH = 7.4 (B) (n = 3).

Table 8
Stability of the Nys-loaded NPs made of Alg, PLGA and PLA (n = 3).

NPs	Time (months)	Temperature (°C)	Mean size $\pm$ SD (nm)	PI	Mean zeta potential $\pm$ SD (mV)
Alg	3	4 $\pm$ 2 °C	1019.3 $\pm$ 109.6	0.385 $\pm$ 0.035	−6.39 $\pm$ 0.10
		25 $\pm$ 2 °C	1944.2 $\pm$ 291.0	0.510 $\pm$ 0.043	+0.17 $\pm$ 0.06
		37 $\pm$ 2 °C	2596.9 $\pm$ 546.5	0.638 $\pm$ 0.123	−3.96 $\pm$ 3.86
	6	4 $\pm$ 2 °C	1613.5 $\pm$ 504.6	0.565 $\pm$ 0.149	−2.22 $\pm$ 0.10
		25 $\pm$ 2 °C	3113.5 $\pm$ 1331.7	0.594 $\pm$ 0.268	−11.94 $\pm$ 0.19
		37 $\pm$ 2 °C	1880.7 $\pm$ 1540.8	0.691 $\pm$ 0.504	−0.95 $\pm$ 0.01
PLA	3	4 $\pm$ 2 °C	1088.63 $\pm$ 119.2	0.396 $\pm$ 0.054	−0.21 $\pm$ 0.15
		25 $\pm$ 2 °C	792.1 $\pm$ 66.6	0.320 $\pm$ 0.020	+0.12 $\pm$ 0.01
		37 $\pm$ 2 °C	2943.6 $\pm$ 290.7	0.916 $\pm$ 0.108	+0.09 $\pm$ 0.01
	6	4 $\pm$ 2 °C	2798.5 $\pm$ 235.2	0.732 $\pm$ 0.030	−0.02 $\pm$ 0.01
		25 $\pm$ 2 °C	2711.4 $\pm$ 540.5	0.860 $\pm$ 0.136	−0.07 $\pm$ 0.02
		37 $\pm$ 2 °C	2435.7 $\pm$ 162.3	0.787 $\pm$ 0.115	−0.13 $\pm$ 0.01
PLGA	3	4 $\pm$ 2 °C	888.77 $\pm$ 111.9	0.367 $\pm$ 0.026	−0.08 $\pm$ 0.03
		25 $\pm$ 2 °C	1053.0 $\pm$ 57.9	0.401 $\pm$ 0.012	−11.19 $\pm$ 1.50
		37 $\pm$ 2 °C	655.9 $\pm$ 116.3	0.274 $\pm$ 0.047	+0.23 $\pm$ 0.08
	6	4 $\pm$ 2 °C	2074.0 $\pm$ 240.8	0.701 $\pm$ 0.056	−0.75 $\pm$ 0.01
		25 $\pm$ 2 °C	1516.1 $\pm$ 20.0	0.533 $\pm$ 0.021	+0.08 $\pm$ 0.03
		37 $\pm$ 2 °C	–	–	–

Table 9
Stability of the Nys-loaded NPs (Alg, PLGA and PLA) in the SSF (n = 3).

Polymeric NPs	Conditions Tested	3 Months	6 Months
Alg	Viscosity (mPas)	17573 $\pm$ 324	8283 $\pm$ 362
	pH	8.58 $\pm$ 0.01	7.38 $\pm$ 0.08
PLA	Viscosity (mPas)	54033 $\pm$ 1701	96600 $\pm$ 917
	pH	4.25 $\pm$ 0.01	4.10 $\pm$ 0.02
PLGA	Viscosity (mPas)	69533 $\pm$ 2673	88533 $\pm$ 2250
	pH	4.25 $\pm$ 0.01	4.21 $\pm$ 0.01

Table 10
Diffusion rate of Nys in the SSF (n = 3).

Samples	Nys diffused in the porcine mucosa (%) (Mean $\pm$ SD)
Solution of free Nys	74.76 $\pm$ 2.71
Oral gel with free Nys	50.46 $\pm$ 7.20
Oral gel with Nys-loaded PLGA NPs	75.27 $\pm$ 1.21
Oral gel with Nys-loaded PLA NPs	87.61 $\pm$ 5.88
Toothpaste with free Nys	44.71 $\pm$ 5.70
Toothpaste with Nys-loaded Alg NPs	59.29 $\pm$ 6.32

rates of Nys were observed with the PLGA NPs and the lowest was observed in the PLA NPs. It should be noted that all experimental variables including the diffusion area (0.95 cm²), the cell volume (4.00 $\pm$ 0.13 mL), the thickness (75 μ m) and porcine mucosa as membrane were constant for all the formulations. The fluxes in the different formulations of Nys were also calculated using the slope obtained by the diffused amount of drug over time. Alg NPs led to a flux of 26.59 $\pm$ 1.75 μ g/cm²h^{−1} whereas the PLA and PLGA NPs showed similar values 21.65 $\pm$ 0.44 μ g/cm²h^{−1} and 31.13 $\pm$ 5.64 μ g/cm²h^{−1}, respectively. It can be verified that the values are according with different characteristics. First, the size of the particles influenced the percentage of permeation. Smaller NPs led to higher percentage. In a second point of view, the physical chemical characteristics of the SSF also influenced the percentage of Nys that diffused through the mucosa. In this case, toothpaste has a lower value when compared with the oral gel because the nature of formulations is different, i.e., the oral gel is 99% water and the toothpaste is constituted by to phases (oil and water) and may be more occlusive. We also observed that hydroalcoholic

solution of Nys with SSF displayed a high percentage of Nys diffused. As previous described, Nys is not soluble in water and it should be dissolved in a minimum quantity of ethanol. The presence of ethanol may result in a destruction of the lipid composition that the protective layer of oral mucosa and disrupt the normal order of epithelial lipid molecules, resulting in a gap between epithelial cells and increasing oral mucosal permeability [62]. Regarding the results of the *ex-vivo* permeation studies, the overall systems of NPs have shown an advantage in terms of permeation. It seems that SSF may provide a longer contact time between the NPs and the oral mucosa, allowing a better permeation of Nys through in the mucosa by a factor of 1.5 or 1.7 with PLGA or with PLA both in the oral gel or by a factor of 1.3 with Alg NPs included in the toothpaste.

4. Conclusion

The success of fungal infections normally depends on the bioavailability of the drug used in their treatment. Bioadhesive NPs really provides a promising approach to increase the efficacy of topically administered Nys in the treatment of oral candidiasis. With this purpose, PLGA, PLA and Alg NPs were able to encapsulate one of the most used antifungal drug present in the market, Nys. All chosen polymers were bioadhesive and stable along 3 and 6 months. The resultant Alg, PLA and PLGA NPs also showed to be efficient encapsulation systems for Nys. No toxic effects of the empty NPs were observed in *S. cerevisiae* model. On the other hand, a high adhesivity to oral mucosal was achieved. This novel application of Nys-loaded NPs could lead to the development of improved formulations of other antimicrobial agents. Further studies will include the encapsulation of natural extracts using these NPs systems for its application in the treatment of oral candidiasis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.eurpolymj.2018.04.032>.

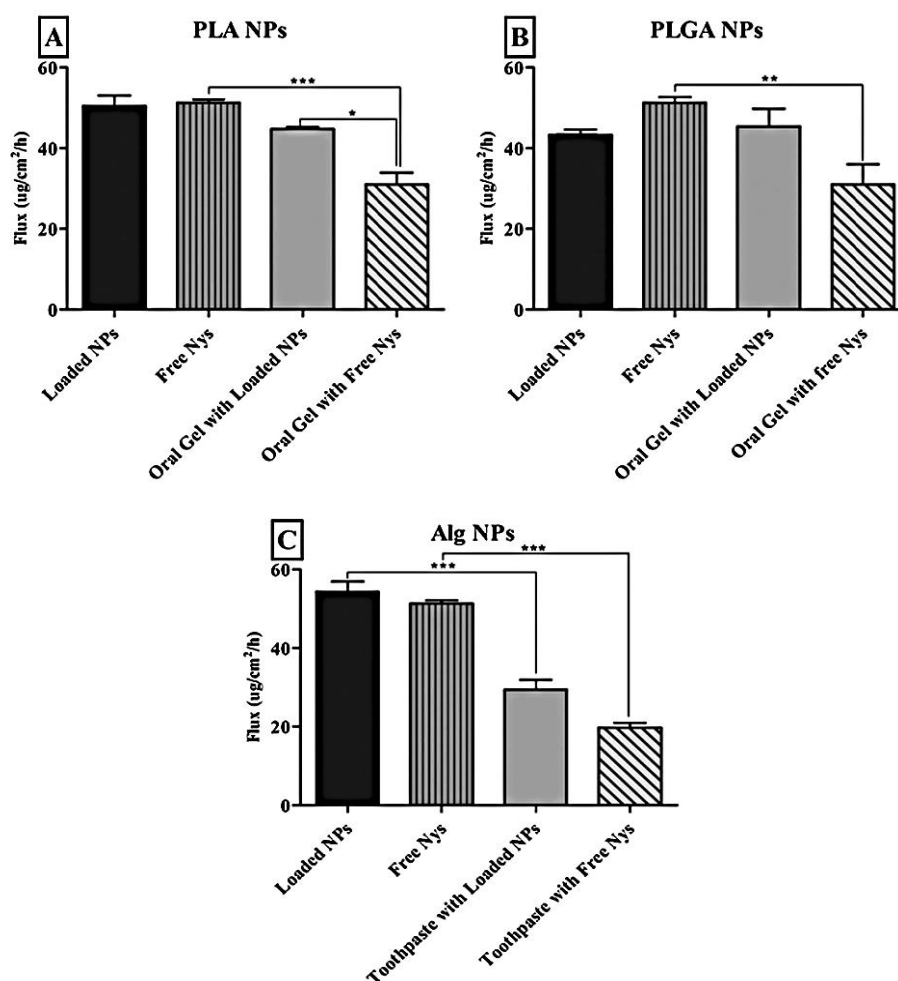


Fig. 5. Fluxes over the time of the NPs in the permeation assay for (A) PLA NPs where * $p < 0.05$ and *** $p < 0.0001$; (B) PLGA NPs, ** $p < 0.001$; (C) Alg NPs, *** $p < 0.0001$ ($n = 3$).

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Article IV
**Mucoadhesive assessment of different antifungal
nanoformulations**

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Abstract

Oral candidiasis is an important opportunistic fungal infection and polyenes and azoles are still the most used antifungal agents. However, the oral absorption resulting from most available treatments is poor and, consequently, a very high frequency of administrations is required. Therefore, the major challenge is to improve the retention of the antifungal agents in buccal mucosa, and the encapsulation into mucoadhesive systems may be considered as a possible strategy to achieve this objective. Three types of mucoadhesive polymeric nanoparticles (PLA, PLGA and alginate) were prepared using nystatin as model drug. The drug-loaded nanoparticles were included in toothpaste, oral gel and oral films respectively. The results obtained demonstrated that the loaded nanoparticles were successfully produced, presenting a mean size between 300-900 nm and with a surface charge between -15.65 and -43.52 mV. Also, the determination of the encapsulation efficiency of all nanoparticles showed values above 70%. In terms of the *in vitro* mucoadhesion, the best formulation was the oral film loaded with the PLGA nanoparticles followed by the oral gel with PLA nanoparticles and thirdly the toothpaste with alginate nanoparticles. This was confirmed in an *in vitro* rinsing model with mucus producing HT29-MTX cells, where the percentage of nystatin retained to the cells after 40 min of simulated saliva flow was between 10-27% when formulations were used and only 4% for free nystatin. Further studies will include *in vivo* testing using animal models.

Keywords: Nanoparticles, HT29-MTX cells, Mucoadhesion, Antifungal, Nystatin

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% to 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 delivery systems may be a possible strategy to achieve this objective. Mucoadhesive drug delivery systems, like oral gels and films (Hydroxypropyl methyl cellulose, Polycarbophil, Ethyl cellulose, etc.) and polymeric nanoparticles (NPs) based on Hyaluronic acid, Alginate (Alg), Polylactic 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), oral gel (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 alginate 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 20mg/mL and Nys with a final concentration of 10mg/mL in a mixture acetone: 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 Poloxamer (Pluronic® F-127), under a constant stirring speed of 800 rpm (Fig.1). Subsequently, the organic solution was removed using a rotary evaporator at 60°C for 20 minutes until NPs dispersed in the aqueous phase were formed (10).

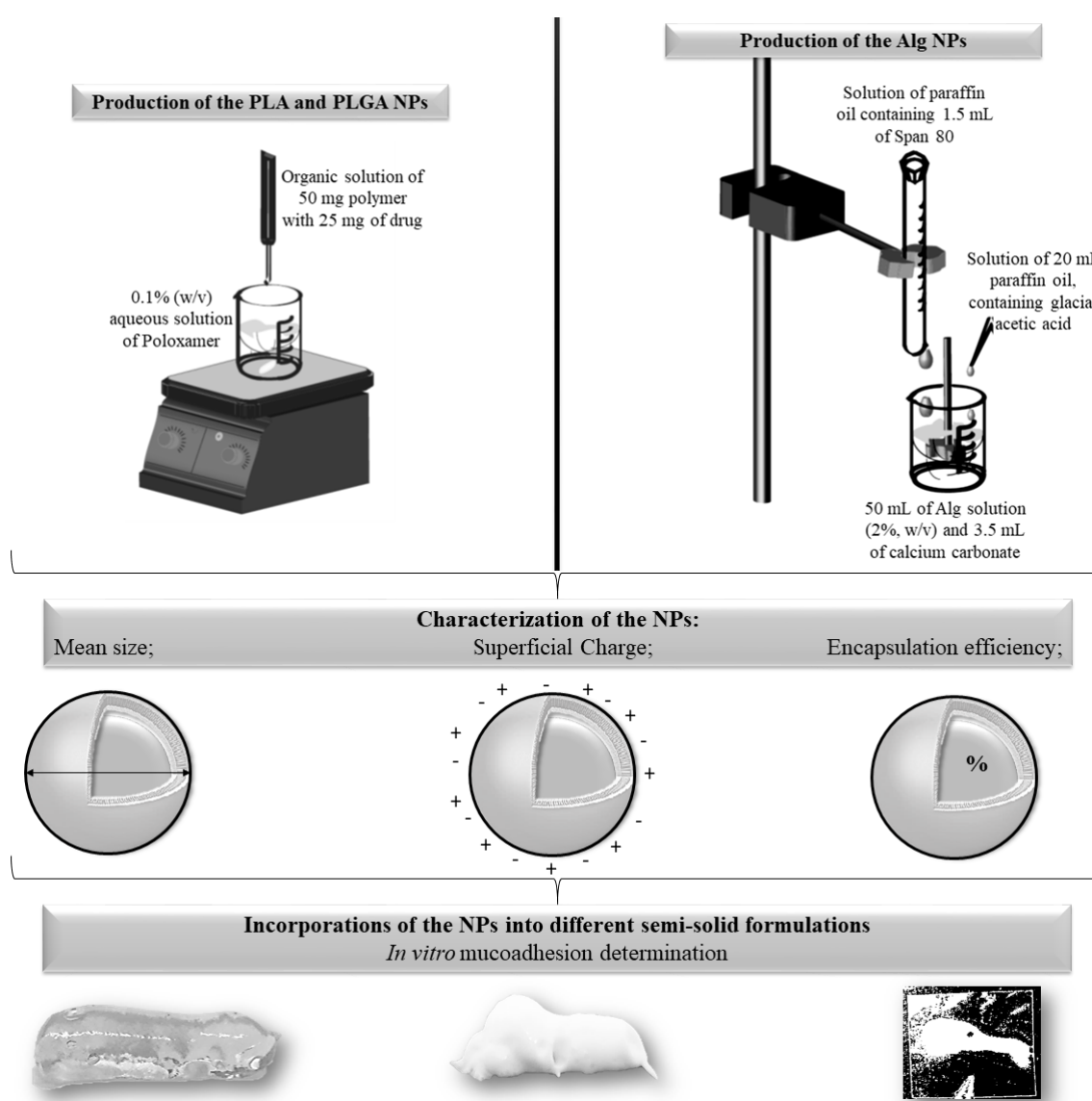


Figure 1 – Methodology of the production and characterization of the different types of NPs

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 μ L 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 nanoparticles (Fig.1). The oil-dispersed Alg nanoparticles were recovered by centrifugation at 26,230xg with a Z36-HK high speed centrifuge (Hermle Labortechnik, Wehingen, Germany) coupled with dehydrating solvents in acetate buffer to clean the nanoparticles (12).

2.2. Characterization of the Nys-loaded NPs

2.1.2. 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 (DLS) with a Coulter Nanosizer Delsa Nano™ C (Coulter Beckman, Fullerton, CA). All experiments were made at room temperature, with a detection angle of 165° and performed in triplicate (10).

2.1.3. 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 an detection angle of 90° and in triplicate (10).

2.1.4. Determination of the Encapsulation Efficiency (EE)

After the preparation of NPs, a pellet was obtained by centrifugation at 23,000xg for 15 min in the case of PLGA and PLA NPs, and at 38,000xg 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 307nm. 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 ($R^2 = 0.995$) for PLA and PLGA NPs and 0.4–50 µg/mL ($R^2 = 0.996$) for Alg NPs. For the calculation of EE, Eq. (1) was used.

$$EE (\%) = \frac{\text{Quantity of drug} - \text{Quantity of drug present in the supernatant}}{\text{Quantity of drug}} \times 100 \text{ (Eq. (1))}$$

All the measurements were made in triplicate.

2.2. 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.

2.2.1. Toothpaste (TP) 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 15g

of glycerol. The second phase is citric acid buffer (pH=5) containing sodium fluoride (13mg/mL). 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 (g/g). Optimization conditions were determined in a previous work done by our group (10).

2.2.2. Oral Gel (OG) preparation

The OG preparation involves the addition of a few drops of an aqueous solution of sodium hydroxide (NaOH) with a concentration of 0.1M 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 (g/g).

2.2.3. Oral Films (OF) 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 100mL and left for an additional 30 min stirring to ensure a homogenous distribution of the NPs in the final formulation (13–15). After preparation, the homogenous suspensions were allowed to rest for a period of 4 hours to remove all the air bubbles and poured onto a clean levelled glass plate of the Coatmaster 510 (Erichsen GmbH, 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 2x2cm and stored in desiccators to avoid moisture fluctuations. The prepared films presented a transparent and homogenous appearance, even and flexible structure.

2.3. Characterization of the semi-solid formulations

2.3.1. 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, Switzerland), 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 15cm. The mass of the upper plate was standardized at 125 g. To calculate the spreadability the following equation was used (Eq. (2)).

$$S = m \times \frac{l}{t} \text{ (Eq.(2))}$$

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 0-25mm (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 307nm wavelength.

Measurements were carried out on 10 pieces with a dimension of 2x2cm for each film.

2.4. *In vitro* assays

2.4.1. *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. The pieces of filter paper presented a dimension of 1.3x1.3cm. 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 x11 cm).

To test the OF 1x1cm squares were used whereas 0,01g 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 200g was applied for a period of 100 s, after which the upper part was raised with a speed of 0.01mm/s (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 ($F_{\max}$, g) and the work of detachment (area under the peak, AUC, g/s) 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.

2.4.2. Cell culturing

The HT29-MTX cell-line was kindly provided by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France). 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) with Streptomycin (100 µg/ml) (Sigma–Aldrich, St. Louis, USA) and Gentamicin (50 µg/ml) (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 24.000 cells/cm². 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 7 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 the solution of free Nys, respectively. (20,21).

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.

2.4.3. *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 $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 0.81 mM MgSO_4 and 4.17 mM NaHCO_3 , 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) 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 piece samples of 2x2cm 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 cells 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. Four nozzles were used, yielding a total flow of 1.6 mL/min. 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 (2.3.1.).

2.4.4. *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 1x1cm with 0.13mL 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 1mg/mL 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 (2.3.1.) 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. 0.5mL of a 15 μ M 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 appearing in the acceptors chambers were measured in triplicate using the fluorescence plate reader Victor³™ at λ_{ex} 485 and λ_{ex} 535, 0.1s, on black optical

bottom polystyrene Nunclon™ cell culture dishes, holding 200μL of solution (21). The averages of three measurements were used.

2.5. Statistics

All results are expressed as mean $\pm$ standard deviation. One-way ANOVA and two-way ANOVA analysis were applied to demonstrate statistical differences in all tested parameters. All analyses were performed using a software program (GraphPad Prism 5®, GraphPad Software, San Diego, CA) with a statistical significance level of 0.05.

3. Results and Discussion

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 into 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 polydispersity 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 $90.14 \pm 3.84\%$, $72.74 \pm 5.28\%$ and $76.16 \pm 7.95\%$ 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 of your 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).

Table 1 - Characterization of different loaded-NPs with Nys (n=3)

Polymeric NPs	Mean Size Distribution $\pm$ SD (nm)	Polydispersivity Index (PI)	Mean Zeta Potential $\pm$ SD (mV)	Mean EE $\pm$ SD (%)
Alg	886.7 $\pm$ 13.7	0.325 $\pm$ 0.013	-43.62 $\pm$ 0.25	71.78 $\pm$ 15.65
PLA	332.8 $\pm$ 172.0	0.198 $\pm$ 0.070	-26.03 $\pm$ 1.16	94.47 $\pm$ 0.41
PLGA	413.1 $\pm$ 186.8	0.189 $\pm$ 0.080	-15.65 $\pm$ 5.35	71.05 $\pm$ 9.60

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 2.2. 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 above 7.5 because the teeth actually become stronger 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 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.

Table 2 – Characterization of the Semi-solid Formulations with loaded-NPs (n=3 for the Toothpaste and Oral Gel and n=10 for the Oral Film)

	Toothpaste	Toothpaste with Nys	Toothpaste with Alg loaded-NPs
Viscosity (mPas)	1706.00 ± 16.56	3649 ± 54.31	15110 ± 20.50
pH value	7.47 ± 0.20	7.18 ± 0.10	8.58 ± 0.03
Spreadability (g.cm/s)	49.98 ± 5.47	49.90 ± 1.17	49.92 ± 2.10
	Oral Gel	Oral Gel with Nys	Oral Gel with PLA loaded-NPs
Viscosity (mPas)	83330.67 ± 8.50	66690 ± 51.96	70830 ± 50.50
pH value	6.06 ± 0.01	5.02 ± 0.05	4.17 ± 0.02
Spreadability (g.cm/s)	32.12 ± 11.89	35.20 ± 12.50	83.85 ± 31.21
	Oral Film	Oral Film with Nys	Oral Film with PLGA loaded-NPs
Weight (mg)	37.02 ± 7.67	37.83 ± 7.79	52.33 ± 6.95
Thickness (mm)	7.95 ± 0.65	8.89 ± 0.84	14.55 ± 0.76
Drug content (µg)	N/D	24.06 ± 9.12	115.51 ± 18.63

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 Fig. 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 didn't present any statistical significant differences when compared with the OG without the NPs.

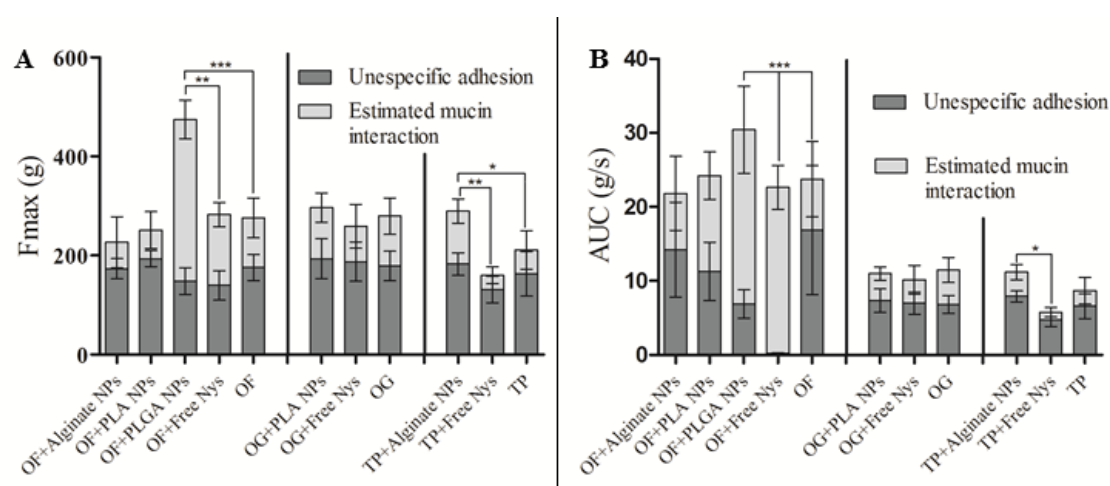


Figure 2 - General mucoadhesion assessment of the semi-solid formulations with and without the loaded NPs, where *** ($p<0.001$), ** ($p<0.01$) and * ($p<0.05$).

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 Fig. 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 (Fig. 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 3 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 2 days, 1 week, 2 weeks and in the moment of the experiments (3 weeks) were taken (Fig. 3).

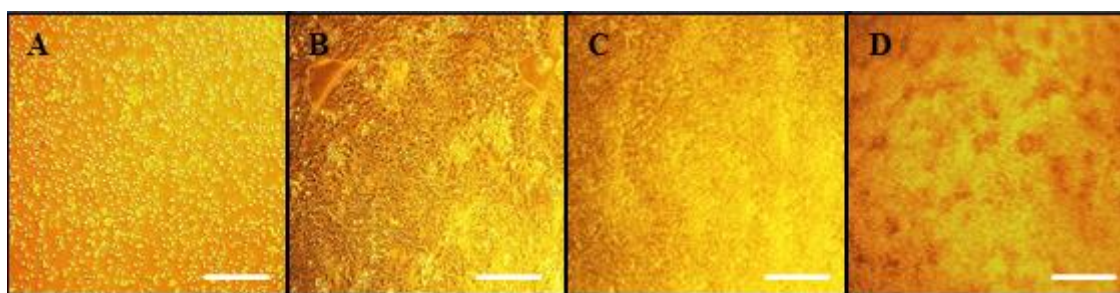


Figure 3 - Growth rate of the HT29-MTX cells after seeding (A- after 2 days; B- after 1 week; C - after 2 weeks; D - In the moment of the experiments). The images were taken under the optical microscope with obj. 20. The scale bar equals 100 μm .

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 Fig. 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 increased to $27 \pm 7\%$, $19 \pm 7\%$ and $10 \pm 1\%$ for OF, TP and OG respectively. The differences observed in Fig.4 are statistically significant ($p < 0.001$) from control from 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.

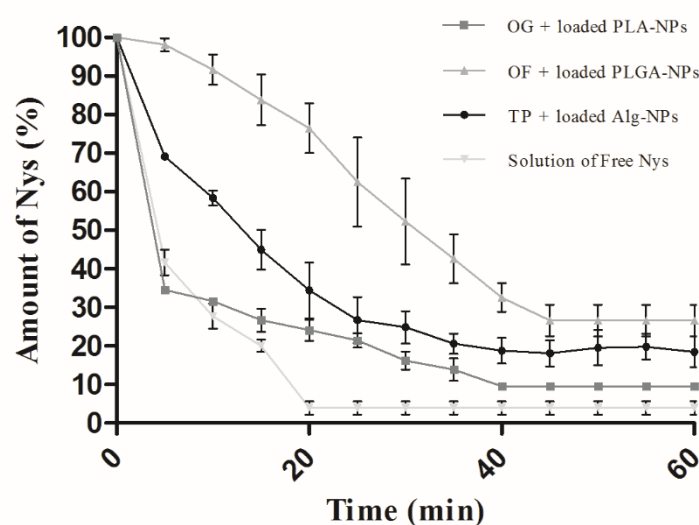


Figure 4 – Retention of Nys to the HT29-MTX cell monolayer during continuous rinsing with saliva substitute (HBSS) at 1.6 mL/min (n=3).

TEER measurement were made before and after incubation with the formulations. The results obtained are presented in Fig. 5. The values before the experiments were carried out are in the range of $200\text{--}300 \Omega \cdot \text{cm}^2$, 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 Fig. 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. Since the results obtained, in terms of 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 (Fig. 6).

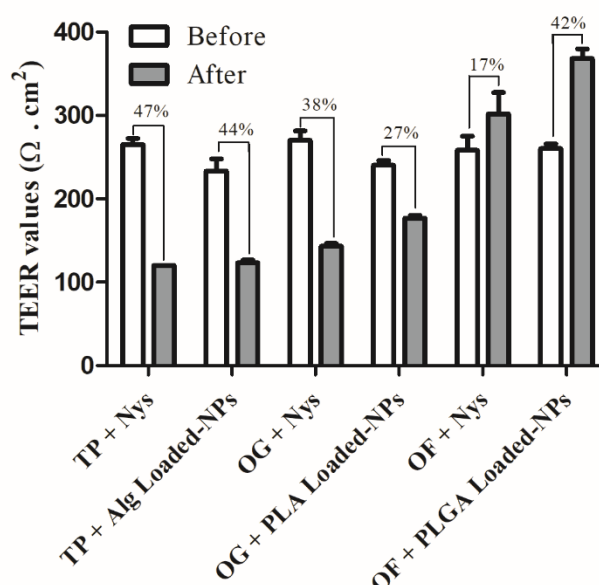


Figure 5 – TEER values of the HT29-MTX cell monolayers before (•) and after (o) the interaction with the formulations (n=3).

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% (Fig. 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 (Fig. 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.

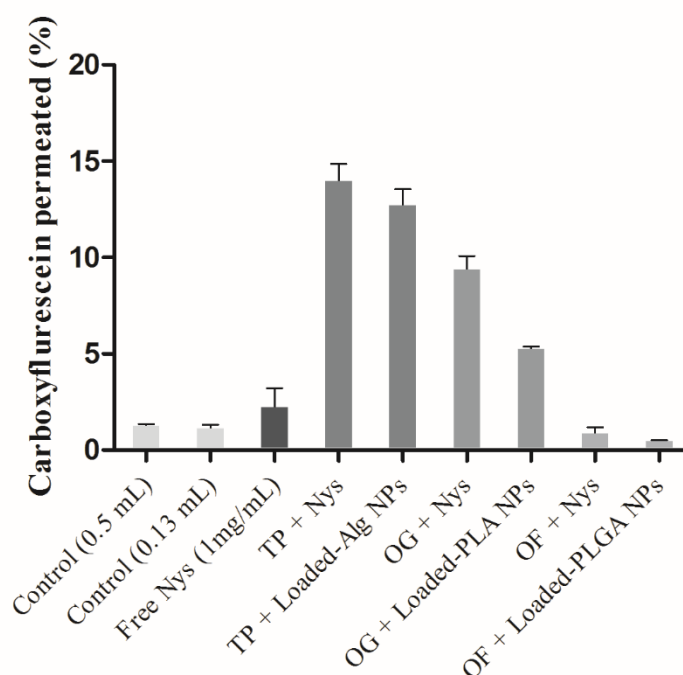


Figure 6 – Percentage of carboxyfluorescein that permeated cell monolayers during 2 hours after a 2-hour preincubation with formulations, drug or HBSS buffer as control (n=3).

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 $26 \pm 1\%$, $11 \pm 1\%$ and $17 \pm 1\%$ for the TP, OG and OF formulation, respectively, when compared to free Nys solution ($2.63 \pm 0.20\%$).

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 formulations 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.

5. Acknowledgments

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Article V

**Development of a biodhesive nanoformulation with
Glycyrrhiza glabra L. extract against *Candida albicans***

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Biofouling

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Abstract

Glycyrrhiza glabra L. is considered an important source of bioactive compounds. The objective of this study is the development of a natural solution for the treatment of oral candidiasis. Several extracts of *Glycyrrhiza glabra* L. were prepared using different solvents and their potential *in vitro* antifungal activity were assessed. Ethanolic extract showed the most promising results against *C. albicans*. This extract was incorporated into mucoadhesive loaded nanoparticles (PLA, PLGA and alginate), which were further included in oral gel, oral film and toothpaste, respectively, as nanoformulations for increased bioavailability. Results showed that ethanolic extract-loaded nanoparticles were successfully produced, presenting a mean size among 100-900 nm and a negative surface charge with high encapsulation efficiency. *In vitro* mucoadhesion showed that the most bioadhesive formulation was the oral film with extract-loaded PLGA nanoparticles followed by toothpaste with extract-loaded alginate nanoparticles and oral gel with extract-loaded PLA nanoparticles.

Keywords: Antifungal; Glabridin; *Glycyrrhiza glabra* L.; Nanoparticles; bioadhesion

1. Introduction

Over the years, medicinal plants have become an interesting source of bioactive compounds (Cragg & Newman 2013). Several studies have proven that medicinal plants have an extremely rich composition, which confers multiple therapeutic properties that can be applied in the pharmaceutical field. However, many of these natural compounds remain unexplored regarding their mechanism of action, despite being extensively studied about their synergistic and multiple therapeutic effects (Atanasov et al. 2015).

In parallel with the advances achieved with natural compounds, the microbiological field is facing several significant challenges. One of the most relevant is the development of resistance by some pathogenic microorganisms to well-known chemical drugs used in therapy, in order to survive in a hostile environment (Davies & Davies 2010; Dias et al. 2012). One of the most common opportunistic fungal infections is Candidiasis. It is caused by *Candida* spp., mainly affecting immunocompromised patients, and, more rarely healthy population. *C. albicans* is considered the most relevant species in oral candidiasis, although other *Candida* species have been pointed out, such as *C. tropicalis*, *C. glabrata*, *C. dubliniensis*, *C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*, *C. krusei*, *C. famata*, *C. guilliermondii* and *C. lusitaniae* (El-kamali 2009; Martins et al. 2015).

With the increase of fungal infections associated with the existing limited antifungal drugs, the high toxicity associated with these compounds and the development of some resistance mechanisms to the clinically used drugs, natural products have gained impact in the recent development of new antifungal treatments (Scorzoni et al. 2017; Perfect 2017). Some plants present potential antifungal activity, such as Liquorice (*Glycyrrhiza glabra* L.), which is a medicinal herb of the *Leguminosae* family and has

been used in traditional medical systems for more than 4000 years as an expectorant, emollient, anti-inflammatory, antiviral and antibacterial agent (Wang et al. 2015; Karahan et al. 2016). Liquorice roots contain many bioactive components, such as glycyrrhizin, liquiritin, liquiritenin, and glabridin (Tian et al. 2008; Fatima et al. 2009). Glabridin is a prenylated isoflavonoid compound that is found in the decayed part of its thickening roots (Lv et al. 2010; Simmler et al. 2013).

The bioavailability of natural extracts is frequently a major drawback for their use, including its administration through oral mucosa (Kesarwani & Gupta 2013). Therefore, a major challenge would be to improve the absorption of the extract into the oral mucosa to obtain better therapeutic outcomes. A possible strategy to achieve this goal, might be the encapsulation of the extract in mucoadhesive nanoformulations (Reis et al. 2012). Mucoadhesive drug delivery systems have the capacity to adhere to the oral mucosa, and thus remain in the target area for a considerable period, as compared to conventional delivery systems (Chinna et al. 2011).

The overall aim of this study was to investigate the activity of the extracts of *Glycyrrhiza glabra* L. against the most relevant species that are responsible for Candidiasis. The extracts of *Glycyrrhiza glabra* L. were prepared using the following solvents: ethanol, acetone, dimethyl sulfoxide (DMSO) and water. The extracts were tested regarding their antifungal activity and the most active was encapsulated into three different polymeric nanoparticles (NPs): alginate, polylactic acid (PLA) and poly(lactic-co-glycolic acid) (PLGA), as previously done with nystatin (Nys) (Roque et al. 2018). The NPs were embedded into three different mucoadhesive dosage forms: toothpaste, oral gel and oral film. Finally, the mucoadhesion capacity and antifungal efficacy of each formulation was assessed.

2. Materials and methods

2.1. Samples

A total of five extracts of *Glycyrrhiza glabra* L. were prepared and studied in terms of antifungal activity. Dried roots and branches were used. The applied plant scientific nomenclature is according to The Plant List version 1.1 (2013). The roots of *Glycyrrhiza glabra* L. were obtained from the Herblist of S. João de Brito (Lisbon, Portugal).

2.2. Standards and Reagents

The solvents used in the extraction were methanol, supplied by António M. S. Cruz LDA (Lisbon, Portugal), acetone from Prolabo (Fontenay-sous-Bois, France), ethanol and DMSO received from Sigma-Aldrich (St. Louis, Missouri, USA). All organic solvents presented analytical grade purity.

Sabouraud Dextrose Broth (SDB) and Agar were obtained from Biokar (Beauvais Cedex, France). Water was treated in a Milli-Q water purification system (Milli-Q® Integral Water Purification System, Billerica, MA, USA).

For the development of the NPs; sodium alginate 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-Aldrich (St. Louis, Missouri, USA) were applied as encapsulation materials and stabilizing agents.

For the preparation of the semi-solid formulations, the reagents used were sorbitol and sodium lauryl sulphate obtained from José M. Vaz Pereira S.A. (Lisbon, Portugal). Citric acid 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, Missouri, USA),

Lycoat RS 720 obtained from Roquette (Lestrem, France) and glycerol from Apotek Produksjon AS (Oslo, Norway).

Nys raw material was kindly donated by Angelini Pharmaceutical (Dafundo, Portugal) and pure glabridin powder was obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

2.3. Preparation of the extracts

All plant extracts were prepared using the ultrasound-assisted extraction method to improve extraction yield (Matias et al. 2015). Briefly, 1.0 g of dried and powdered *Glycyrrhiza glabra* L. plant was suspended in 10mL of solvent (ethanol, acetone, DMSO, methanol, ethanol/water (70:30 v/v) and water) and sonicated for one hour using an ultrasonic bath (Bandelin SONOREX RK 510H, Germany) with a working frequency of 35 kHz (Matias et al. 2015).

The bulk extract was separated from the remaining plant material by filtration and evaporated under reduced pressure at 50 °C in a rotary evaporator (IKA RV06-ML 1-B, Staufen, Germany) until it was dry. This procedure was repeated until exhaustive extractions were achieved. For the antifungal activity tests, the dry extract was resuspended in DMSO, obtaining a final concentration of 10 mg/mL.

2.4. Antifungal activity and characterization of the extracts

2.4.1. Agar diffusion test

A preliminary antifungal activity screening of the extracts was carried out using *C. albicans* ATCC 10231, with a well diffusion method described by Rijo et al. 2014 The yeast was cultured on *Sabouraud* agar by the following method; 100 µL of a standardized microorganism suspension, corresponding to 0.5 McFarland, to inoculate a Petri dish of solid *Sabouraud* agar. The suspension was spread over the medium surface using a sterile

swab. Subsequently, agar wells were made with approximately 5.0 mm in diameter. Then, 50 μ L of each sample (extract), negative control (DMSO) and positive controls (Nys) were added to separated wells (Neto et al. 2016). The plates were incubated at 37 °C for 24 hours. After this period, the diameters of the inhibition zones were measured and the results were expressed in millimetres (mm). All the measurements were made in triplicate.

2.4.2. Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) was determined on the most active extract identified in the agar diffusion (ethanolic extract). This assay was carried out using the microplate broth microdilution method according to Clinical and Laboratory Standards Institute (2006) (Rijo et al. 2014). Briefly, various species of *Candida* (*C. krusei*, *C. guilliermondii*, *C. tropicalis* and *C. glabrata*) were cultured on *Sabouraud* agar. Following the fungal growth, a standardized fungal suspension, corresponding to 0.5 McFarland, was prepared and used to inoculate 96 well plate with increasing concentrations, starting at 78 μ g/mL and ending at 5000 μ g/mL of the extract under analysis. After incubation, turbidity of the broth in the wells was observed and the MIC was defined as the lowest concentration (μ g/mL) of extract at which no visible growth of the microorganisms could be detected after 24h of incubation at 37 °C. Microbial growth was measured at 620 nm using an Absorbance Microplate Reader (Thermo scientific Multiskan FC, USA). All assays were performed in triplicate, and positive (containing Nys) and negative (DMSO) controls were included (Neto et al. 2016).

2.4.3. Minimal fungicidal concentration (MFC)

The MIC end-point criterion was the lowest extract concentration at which there was no visible growth after 24 h incubation at 37 °C (Neto et al. 2016; Siopa et al. 2016).

To obtain the MFC, each serial dilution was taken from each well and spread on *Sabouraud* agar plate. These plates were then incubated at 37 °C for 48 h. The MFC was defined as the lowest extract concentration that yielded three or fewer colonies (ie 99 % of the inoculum was killed).

2.4.4. Time-killing test

After the determination of the antifungal activity, the extract at the MIC value obtained against *C. albicans* was tested in terms of the capacity to influence the natural growth of *C. albicans*. A time-killing test protocol was adjusted to achieve this purpose. Briefly, a strain of *C. albicans* (ATCC 10231) was prepared at the starting inoculum of 106 CFU/mL. The amount of extract corresponding to the MIC value (625 µg/mL) was immersed in the suspension of *C. albicans* for 24 h at 37 °C. The microorganism growth was monitored spectrophotometrically over time (each hour) and quantified by absorbance measurements at optical density of 620 nm. Also, a control containing only the strain was analysed to determine the natural growth of the *C. albicans* (Siopa et al. 2016).

2.4.5. Liquid Chromatography and Mass Spectrometry (LC-MS) analysis

LC-MS/MS analyses were performed on a Waters® Alliance 2695 HPLC Separation Module connected to a Micromass® Quattro Micro triple Quadropole (Waters®, Ireland) Mass Spectrometer equipped with a Waters® electrospray ionization (ESI) source.

Separation of the target compound was achieved using a reverse phase column Waters® Sunfire (5 µm, 2.1 x 100 mm) with an injection volume of 10 µL. The mobile phase consisted of 0.5 % formic acid in Milli-Q water (eluent A) and acetonitrile (eluent B) at a constant flow rate of 0.2 mL/min, with the following elution program 0–15 min

from 60 to 10 % A, 15–25 min at 60 % A. The column oven temperature was set at 20 °C. Mass spectrometer was operated in positive mode and spectra were recorded in the m/z 100 to 1000 Da range. For the selection of the best operating conditions in MRM mode, individual standard solution of glabridin (100 ppm) was infused in the mass spectrometer and capillary and cone voltages, and collision energy were tuned to maximize [precursor ion > product ion] transition signal. ESI capillary voltage was optimized to 3kV, cone voltage was set on 30 V. Source and desolvation temperature were adjusted to 120 °C and 350 °C, respectively. High purity nitrogen (N₂) was used as drying gas and as a nebulizing gas. Ultra-high purity argon (Ar) was used as collision gas. Micromass Mass Lynx Version 4.1 software (Waters) was used for instrument control, data acquisition and data processing.

Standard stock solution (1000 ppm) of glabridin was prepared with MeOH. Calibration solutions, over the concentration range 0.01–100 ppm, were obtained from the stock solution. Stock solutions and calibration standards were stored below 4 °C. Injections of sample of the extract were bracketed by injections of MeOH to assess carryover. Glabridin amounts were calculated from linear calibration curves based on the quantification MRM1 transition. Ratios of (MRM1:MRM2) were within the defined limits (maximum deviation of 10%).

2.5. Development of bioadhesive nanoformulation

2.5.1. Encapsulation into polymeric nanoparticles

After the determination of the amount of glabridin, the extract was encapsulated into polymeric NPs. Three types of polymeric NPs were selected based on previous work to encapsulate the extract: in this case, alginate, polylactic acid (PLA) and poly lactic-co-glycolic acid (PLGA) NPs. PLGA and PLA NPs were prepared according to previous work by our group (Roque et al. 2017), using the same method. Briefly explained, an

emulsion was prepared dissolving 50 mg of PLGA or PLA with 25 mg of extract in a mixture of acetone:ethanol (8:2, v/v). This solution was then placed in an ultrasonic bath for a period between 15-20 min. Then the solution was added dropwise to 0.1 % (w/v) aqueous solution of Pluronic® F-127, under a constant stirring speed of 800 rpm. Subsequently, the organic solution was removed using a rotary evaporator at 60 °C for 20 minutes until the NPs suspension was formed (Roque et al. 2017).

The alginate NPs were prepared according to an emulsification /internal gelation method, also used by our group earlier (Reis et al. 2008). In short, the aqueous solution was made by 50 mL of sodium alginate solution (2 %, w/v), 3.5 mL of 5 % calcium carbonate (w/v) and 200 mg of extract. The resulting solution was emulsified within a solution of paraffin oil containing 1.5 mL of Span 80 by impeller-stirring homogenisation (1600 rpm). After 15 min of emulsification, 20 mL of paraffin oil containing glacial acetic acid was added to the mixture and the stirring continued for 60 min to trigger the solubilisation of the calcium from the carbonate complex, and concomitant gelation of the alginate NPs (Reis et al. 2016). After the preparation of the NPs, they were isolated using centrifugation at 23,000xg for 15 min in the case of the loaded PLA and PLGA NPs and at 38,000xg for 15 min in the case of alginate NPs.

2.5.2. Characterization of the extract-loaded nanoformulation

The NPs were then characterized in terms of size and superficial charge. The three types of NPs were assessed as hydrated particulate systems with a Coulter Nanosizer Delsa NanoTMC (Coulter Beckman, Fullerton, CA). The samples were diluted with purified water to 1:20 aqueous solutions for appropriate concentration range, so polydispersity index could be determined. A low value of polydispersity index (PI) indicates a more uniform and less dispersed distribution in size.

All experiments were made at room temperature, with angle detection of 165° and in duplicate. Zeta potential was measured in the same equipment by electrophoretic mobility technique. Each analysis was carried out at 25 °C with a 90° detection angle and in triplicate (Roque et al. 2017).

The encapsulation efficiency of the glabridin in the nanoparticles was determined through an indirect method. The quantity of extract present in the supernatant (QS) and the quantity used in the preparation of the NPs (QNPs) was analysed using the method of LC-MS described previously (section 2.4.5). The encapsulation efficiency was determined following Equation 1.

$$\text{Encapsulation efficiency} = \frac{QNPs-QS}{QNPs} \times 100 \text{ (Equation 1)}$$

2.5.3. MIC and MFC determination of the nanoformulations

Using the same methods as described before (sections 2.4.2 and 2.4.3), the MIC and the MFC values were determined for the NPs and were compared with the same concentration of free glabridin. The concentrations used were determined according with the values obtained in the encapsulation efficiency. In this case, they were 23.62 µg/mL, 17.49 µg/mL and 4.22 µg/mL for the PLA, PLGA and alginate NPs, respectively.

2.5.4. Biocompatibility study with mucus-producing cells (HT29-MTX)

The HT29-MTX cell line was kindly provided by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France). 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 (Lesuffleur et al. 1993, J. Cell Science, 106: 771-783). They do not need to be maintained in media containing MTX to differentiate after confluency.

In this assay, a Transwell® setup (12 well plate, with a pore size of 0.4 µm) was used. The cell monolayers, with a growth time of 3 weeks and with the passage number 27, were washed with HBSS and incubated on the donor side with 0.5 mL of sample; the extract-loaded PLA, PLGA and alginate NPs, respectively. Additionally, two samples consisting of 0.5 mL and 0.13 mL of HBSS were used as control. 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 at 37 °C), the samples present in the donor chamber and the HBSS buffer present in the receptor chamber were removed, and the two chambers were washed with HBSS. The integrity of the cell monolayer was assessed by measuring the paracellular permeability of carboxyfluorescein, 0.5mL 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, 200 µL of solution from each acceptor chamber was removed and the amount of permeated carboxyfluorescein appearing in the acceptor chambers was measured in triplicate using the fluorescence plate reader Victor3™ at λ_{ex} 485 and λ_{em} 535, 0.1s, on a black optical bottom polystyrene Nunclon™ cell culture dish, (Adamczak et al. 2016). The average of these three measurements was used. Additionally, the transepithelial electrical resistance (TEER) was monitored with a Millicell ERS-2 Voltohmmeter, before and after the experiments were carried out. TEER was measured to verify the integrity of the cell monolayer.

2.6. Mucoadhesion assessment of extract-loaded nanoformulations

After the preparation and characterization, the extract-loaded NPs were further included into different formulations. More specific, the alginate NPs were incorporated into a toothpaste formulation (0.91 % w/v) and the PLA NPs were incorporated into an oral gel formulation (5.00 % w/v). Both formulations were based on our previous work

(Reis et al. 2016). The viscosity of the semi-solid formulations was then assessed using a Rotating Viscometer Brookfield (Middleboro, MA, USA). pH values (827 pH Lab, Metrohm, Switzerland), organoleptic and tactile aspects were also assessed. Finally, the PLGA NPs were embedded into an oral film. The selection of the type of polymeric NPs and the type of semi-solid formulation was optimized by our group (Roque et al. 2018). The oral film was formulated using Lycoat as the film-forming polymer and by using the solvent casting method (Moura et al. 2012). Briefly, an aqueous preparation containing 17 % of Lycoat and 4 % of glycerol (w/w) was prepared and 7 % (w/w) of hydrated PLGA extract-loaded NPs were added. Then, the final volume was adjusted with water to 100 g and left to stir for an additional 30 min to ensure a homogeneous distribution of the NPs in the final formulation. After the preparation, homogeneous suspensions were allowed to rest for a period of 4 hours to remove all the air bubbles and then they were poured onto a clean levelled glass plate of the Coatmaster 510 (Erichsen GmbH, Germany). The suspension was spread evenly by a glass rod knife with a gap-opening of 1000 μm and allowed to dry overnight at room temperature to obtain a larger film sheet (Alayoubi et al. 2017). After complete drying, the film sheet was peeled off the glass plate, cut into pieces of single dose units of 2x2 cm and stored in desiccators to avoid moisture fluctuation. In addition to the extract-loaded NP-containing film, one extract-loaded film without NPs and one blank (no extract or NPs) film were prepared as references. The prepared films showed a transparent and homogeneous appearance, even and flexible structure. The oral film was characterized for thickness, weight, and water content. The water content was determined using a Moisture Analyzer MA30 (Sartorius, Netherlands).

In all the assays, the formulations were compared with a control of the formulation by itself.

2.6.1. *In Vitro mucoadhesion test*

Mucoadhesion was estimated from the interaction of the samples and a 3 % (w/v) dispersion of mucin in phosphate buffered saline (PBS) pH 7.4. Briefly, the mucin dispersion was evenly spread on the top of two different pieces of filter papers (25 μ L on each) with an inert backing layer (Whatman® Benchkote, Germany). The pieces of filter paper presented a dimension of 1.3x1.3 cm. Both pieces were attached with double sided adhesive tape; one of them was placed on 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 x11 cm).

To test the oral film 1x1 cm squares were used whereas 0.01 g was the amount taken for the oral gel and toothpaste assessment. The samples were placed between the stationary and the movable part. 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 (Hagesaether et al. 2009). The measurement was repeated ten times for each formulation and compared to the respective controls, ie, formulation without NPs and formulation with only extract (not encapsulated). In addition, the intermediate precision was tested by repeating the measurements the following day. The measurements were also repeated for the filter paper soaked with pure buffer instead of mucin dispersion to allow separation of the unspecific adhesion of the formulation from the interaction with mucin. Displacement and force of detachment were recorded. Based on the obtained force versus time curve, peak force (F_{max} , g) and the area of the peak (AUC, g/s) were obtained. The values obtained from testing the samples versus the mucin dispersions were designated as the general mucoadhesion, and the values obtained from testing samples versus PBS were designated as the unspecific adhesion. To estimate the mucin interaction for the semi-

solid formulations, the unspecific adhesion was deducted from the general mucoadhesion (Hagesaether et al. 2009).

2.7. Statistics

All results are expressed as mean $\pm$ standard deviation. One-way ANOVA analysis was applied to demonstrate statistical differences in all tested parameters. All analyses were performed using a software program (GraphPad Prism 5®, GraphPad Software, San Diego, CA) with a statistical significance level of 0.05.

3. Results

3.1. Selection of the extract

In order to select the extract with better antifungal activity several solvents were used and different extracts were obtained using the ultrasound-assisted extraction method (Table 1). Preliminary antifungal activity was determined by the well diffusion assay. This assay intended to identify the most active antifungal extract against *C. albicans* compared with Nys used as positive control. Results are presented in Table 1. The most active extract was the ethanolic and it was also one of the most efficient solvents regarding the extraction yield percentage achieving a value of 18%.

Table 1. Extraction yields and inhibition zone of *Glycyrrhiza glabra* L. extracts with different solvents

Extracting solvent	Extraction yield (% w/w)	Inhibition zone (mm)
Ethanol/Water (70/30)	19%	9
Acetone	4%	10
Ethanol	18%	11
Water	11%	No Inhibition
DMSO	17%	7
Negative Control (DMSO)	Not applicable	No Inhibition
Positive Control (Nys)	Not applicable	25

Further studies included MIC evaluation by the microdilution method against different species of *Candida* (*C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. guilliermondii*). The results demonstrated that the ethanolic extract of *Glycyrrhiza glabra* L. have a great inhibition capacity achieving a MIC value of 625 µg/mL against *C. albicans* (Table 2).

Table 2. Antifungal activity of the *Glycyrrhiza glabra* L. ethanolic extract.

Sample	MIC (µg/mL)				
	<i>C. krusei</i>	<i>C. guilliermondii</i>	<i>C. tropicalis</i>	<i>C. glabrata</i>	<i>C. albicans</i>
Extract	5000	1250	2500	2500	625
Nys	78.13	156	78.13	78.13	156

Concerning the MFC assessment, the effect on the fungal growth over time were assessed using only *C. albicans* since this species is considered the most relevant yeast pathogen in oral candidiasis. The MFC value (1250 µg/mL) was two times higher than the MIC value of the extract. Subsequently, the effect of the extract on the fungal growth over time was investigated for *C. albicans* (ATCC 10231). The results are showed in Figure 1, demonstrating a difference of 57.27% when comparing the natural growth of the fungus in the absence and in the presence of the extract.

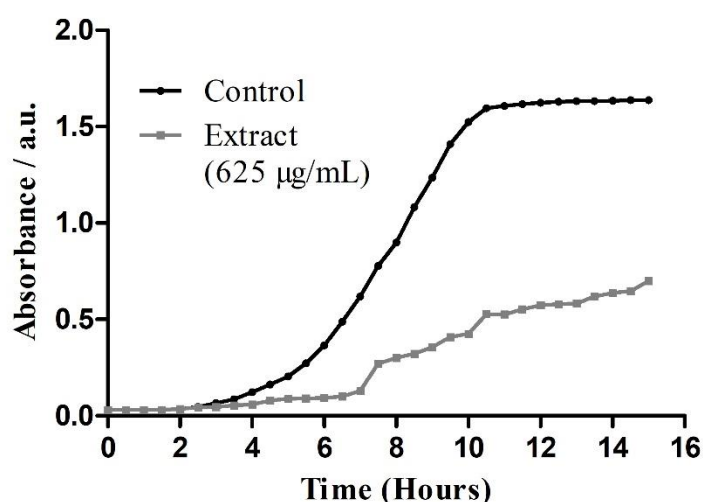


Figure 1. Growth curves of fungal strains of *C. albicans* (ATCC 10231). Fungal growth was assessed in the absence of extract (*C. albicans*) and in the presence of the extract of *Glycyrrhiza glabra* L. at the MIC.

3.1.1. Quantification of glabridin present in the ethanolic extract

The next step consisted on the identification and quantification of glabridin, the main compound considered responsible for the antifungal activity of *Glycyrrhiza glabra* L., in the ethanolic extract (Tian et al. 2008). LC-MS studies started with the direct infusion of a standard solution of glabridin (100 ppm) in positive scan ionization mode. Capillary and cone voltages of ionisation source were tuned in order to maximise the precursor ion signal $[M+H]^+$. The collision energy was also optimised to produce characteristic product ions from the precursor ions. The best conditions were found to be 3.0 kV, 30 V and 15 eV for the capillary and cone voltages and collision energy, respectively. Figure 2 shows the MS and MS/MS spectra obtained after the fragmentation of the protonated molecule. The MS/MS spectrum of glabridin displayed fragment ions characteristic of the compound at m/z 189 (base peak), 149 and 123.

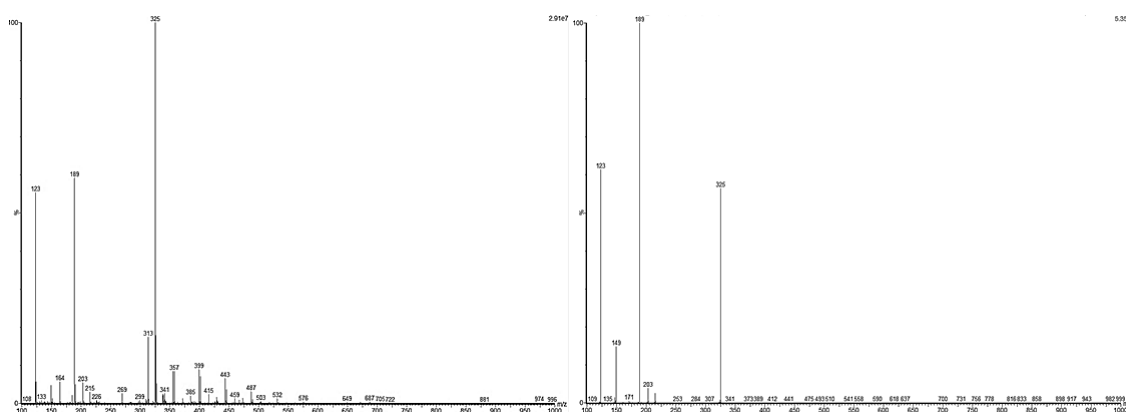


Figure 2. MS and MS/MS spectra of glabridin.

Mass spectrometry in combination with liquid chromatography provided positive, unambiguous confirmation of the presence of glabridin in sample extracts and formulations (van Breemen 2015). A significant increase in the sensitivity and selectivity of MS analysis can be achieved by using Multiple Reaction Monitoring (MRM)

acquisition mode. Three MRM transitions ($325 > 189$; $325 > 149$ and $325 > 123$) were monitored from glabridin, although only two were chosen for quantification and confirmation purposes (Table 3). The transition corresponding to the most intense signal for the product ion (MRM1: $325 > 189$) was chosen as quantification transition and that corresponding to the second transition was used as a qualification/confirmation transition (MRM2: $325 > 123$) (Table 3 and Figure 3). MRM1/MRM2 ratio transition intensities must be of the same range and have a maximum deviation of 10 %, ensuring that the compound detected in the samples correspond to the peak of the standard compound detected at the same retention time.

Table 3. Optimized MRM conditions for glabridin.

Parameter	
Protonated molecule (m/z)	325
Product ions	123, 149, 189
Collision energy (eV)	15
Transitions	$325 > 189$ (MRM1, quantification transition) $325 > 123$ (MRM2, confirmation transition)

Five standard solutions ranging from 0.01 to 100 ppm were analysed in MRM mode to assess linearity. Linear regression curve was fitted to the average integrated area values corresponding to peaks as a function of the concentration of the standards. All experiments were performed in triplicate. For the concentration range of 0.01 to 100 ppm the best fit for the calibration curve ($y = 88216x + 73393$) presents a correlation coefficient $r^2 > 0.9986$.

Glabridin was successfully identified in the ethanol extract based on the following criteria: a chromatographic peak at 11.68 min (mean value), the presence of two monitoring MRM transitions (MRM1: $325 > 189$ and MRM2: $325 > 123$), and the ratio of MRM1/MRM2 transition intensities on the same range and with a maximum deviation of 10%.

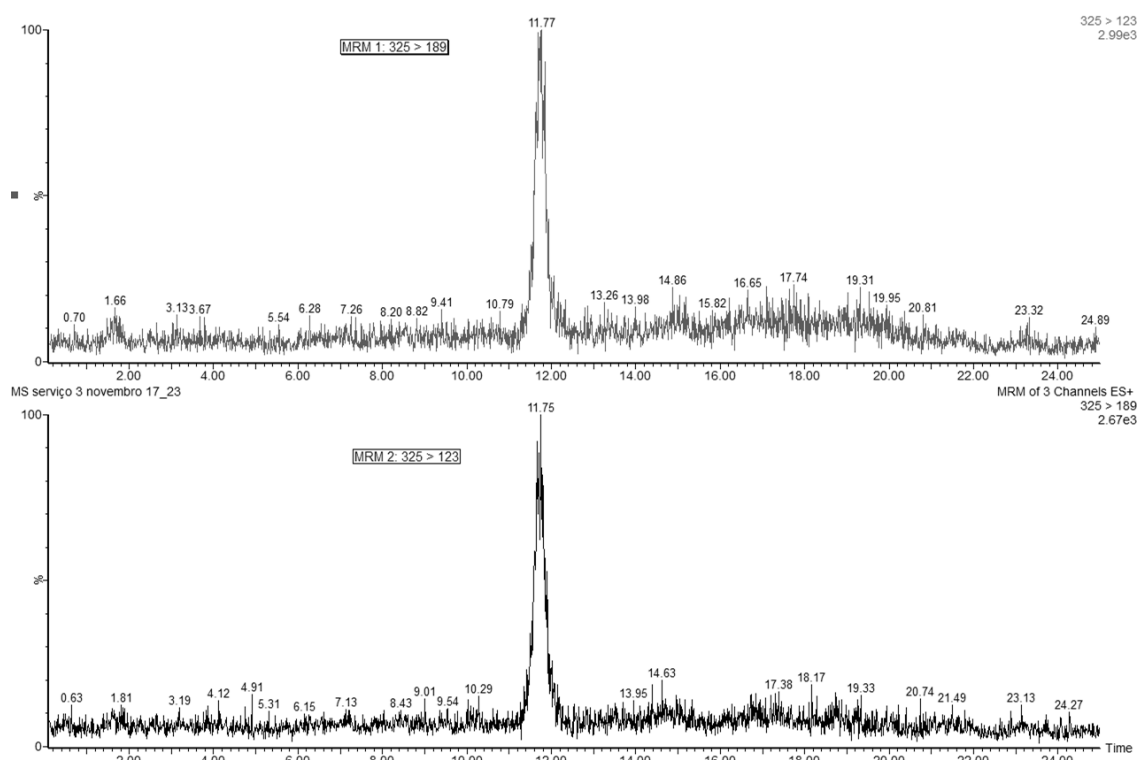


Figure 3. MRM chromatograms of glabridin standard solution (0.01 ppm)

A solution with a concentration of 10mg/mL of ethanolic extract contained a concentration of 47.71 ppm of glabridin based on the calibration curve. Therefore, there is 4.77 μ g of glabridin per mg of extract, these results were similar to those obtained in other works that quantify glabridin in different extracts of *Glycyrrhiza glabra* L. (Tian et al. 2008; Xu et al. 2009).

3.2. Characterisation of the nanoformulation

The ethanolic extract was encapsulated into three different polymeric nanoparticles PLA, PLGA and alginate. The results obtained regarding the production of the extract-loaded NPs showed that they were successfully produced, presenting a mean particle size between 100-900 nm and a surface charge between -5.47 and -16.73 mV (Table 4). Also, the results regarding the encapsulation efficiency by indirect determination showed that the PLA, PLGA and alginate NPs were capable of entrapping

99.00 %, 73.35 % and 97.00 %, respectively, of the glabridin initially added, which can be also observed in Table 4.

Table 4. Characterization of the extract loaded-NPs

Samples	Mean Size $\pm$ SD (nm)	IP $\pm$ SD	Zeta Potential $\pm$ SD (mV)	Encapsulation efficiency $\pm$ SD (%)
PLA NPs	142.57 $\pm$ 5.70	0.073 $\pm$ 0.044	-16.73 $\pm$ 4.96	99.00 $\pm$ 1.00
PLGA NPs	135.67 $\pm$ 2.04	0.079 $\pm$ 0.022	-15.53 $\pm$ 0.66	73.35 $\pm$ 1.50
Alginate NPs	846.73 $\pm$ 39.39	0.292 $\pm$ 0.067	-5.47 $\pm$ 4.69	97.00 $\pm$ 2.55

Aiming to compare the antifungal activities of NPs with those of the free extract and of free glabridin solution, the MIC and MFC values were determined, as can be observed in Table 5. The NPs showed the same value of MIC (125 μ g/mL) as free glabridin solution with the same concentration of glabridin inside the NPs (23.62 μ g/mL, 17.49 μ g/mL and 4.22 μ g/mL for the PLA, PLGA and alginate, respectively).

Table 5. Characterization of the extract loaded-NPs

Samples	MIC (μ g/mL)	MFC (μ g/mL)
PLA NPs	125.0	No Inhibition
PLGA NPs	125.0	No Inhibition
Alginate NPs	125.0	No Inhibition
Concentration of Glabridin entrapped in PLA NPs	125.0	250.0
Concentration of Glabridin entrapped in PLGA NPs	125.0	125.0
Concentration of Glabridin entrapped in alginate NPs	125.0	250.0
Free Glabridin at 1mg/mL	20.8	20.8
Extract at 1mg/mL	62.5	125.0
Nys at 1mg/mL	20.8	20.8

3.2.2. Preliminary cell interaction with mucus-producing cells (HT29-MTX)

In order to study the interaction of extract-loaded NP with a cell monolayer able to produce mucus, TEER measurements were performed before and after incubating the HT29-MTX cell monolayers with the NPs. The results obtained are presented in Figure

4. Before and after incubation, the cell monolayers showed TEER values of around 250 Ω/cm^2 . These results were also confirmed with the paracellular permeability of carboxyfluorescein after the incubation process (Figure 5). In general, the different types of nanoparticles showed a high degree of biocompatibility, indicating low toxicity, since the amount of carboxyfluorescein that permeated is in all cases below 10 %. These results indicate that the nanoformulations most likely do not have a potential toxic effect on cells, but further studies will be conducted.

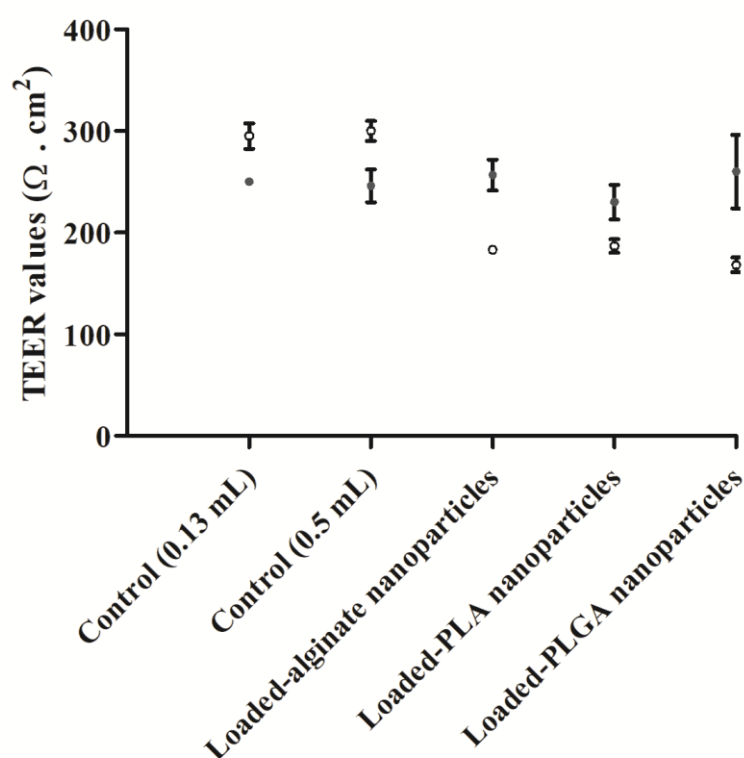


Figure 4. TEER values of the HT29-MTX cell monolayer before (•) and after (o) the interaction with the NPs (n=3). Controls are HBSS buffer in different quantities to simulate the lowest and highest amounts of NPs solution used (μL)

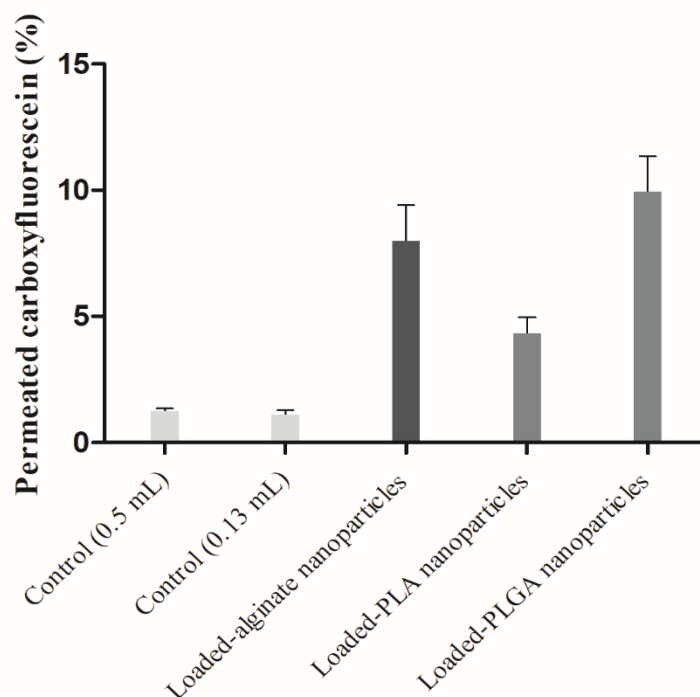


Figure 5. Carboxyfluorescein permeation percentage through a HT29-MTX monolayer with and without preincubation with NPs (n=3). Controls are HBSS buffer in different quantities to simulate the lowest and highest amounts of NPs solution used (μL)

3.3. Characterization of the mucoadhesive nanoformulations

NPs were implemented in semi-solid formulations. The viscosity values of toothpaste and oral gel with extract-loaded NPs (45417.00 ± 1805.78 and 53190.00 ± 2213.32 mPas respectively). When compared with the same formulation without the NPs but with extract (3773 ± 30.02 and 9167 ± 136.50 mPas, respectively). In addition, it was observed that the inclusion of the NPs in the toothpaste exhibited a slight change in the pH value when compared with the toothpaste without the NPs: 8.45 ± 0.15 vs 7.47 ± 0.20 respectively. Concerning the oral gel, the pH value was 4.03 ± 0.09 for the formulation with NPs and 6.06 ± 0.01 for the oral gel only.

The different oral films were also characterized in terms of chemical and physical properties. The films with NPs were slightly different in weight and thickness, since in the case of the films with the NPs the weight was 92.18 ± 6.47 mg and they were 17.8 ± 0.89 μm thick. As for oral films with only free extract, and the oral film by itself, the

results were 80.43 ± 9.76 mg and 73.83 ± 9.44 mg, respectively, for the weight and 15.95 ± 0.89 μ m and 11.75 ± 0.91 μ m, respectively, for the thickness. The residual water content of all films was less than 20 % of the total film weight.

3.3.1. *In Vitro* mucoadhesion test

The mucoadhesion capacity of the formulations was tested using a texture analyser. Maximum peak force and AUC were measured. Results are presented in Figure 6. The oral films with PLGA NPs exhibited the highest value of general mucoadhesion.

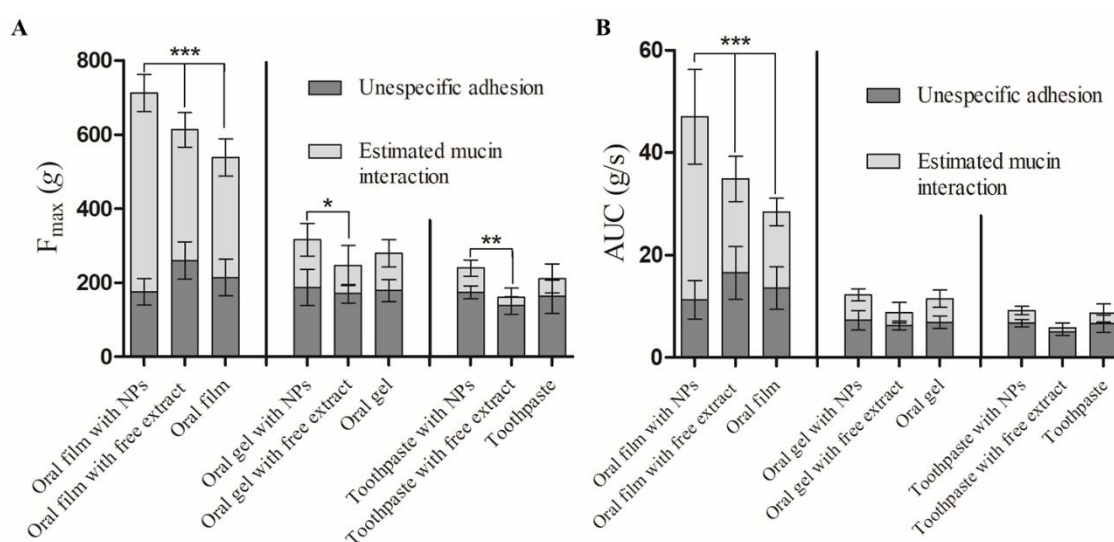


Figure 6. General mucoadhesion assessment of the semi-solid formulations with and without the loaded NPs, where *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$)

The real value of the mucin interaction consists of the deduction of the unspecific adhesion of the formulations (obtained from testing formulation versus buffer), from the general mucoadhesion (testing the formulations versus the mucin). As can be observed in Figure 6 A and B, the general mucoadhesion was higher than the unspecific adhesion in all cases, indicating interactions between the formulations and the mucin. Maximum peak force values correlated with the AUC values (Figure 6-B). The formulations with the NPs had a high interaction with mucin, compared with the corresponding formulations without

NPs, presenting values for the general mucoadhesion of maximum force of 239.37, 316.88 and 712.84 g, for the toothpaste, the oral gel and the oral films, respectively.

4. Discussion

Medicinal plants have been used as a source of bioactive compounds, some of them with great potential to be applied in the market (Cragg & Newman 2013). *Glycyrrhiza glabra* L. is one of the oldest herbs that has an ancient medical history described and it can be used as a medicine or as an excipient to disguise the unpleasant flavour of other products (Fatima et al. 2009; Pan et al. 2013). Glabridin is one of the major isoflavonoids of *Glycyrrhiza glabra* L. It is described to have several biological activities, including antifungal activity (Simmler et al. 2013). Tian et al. 2008 and Simmler et al. 2013 described that this compound can be extracted from the plant using various types of organic and non-organic solvents .

In this work, ethanol was selected as the best solvent to perform further studies since it showed the highest activity against *C. albicans* in a preliminary well diffusion assay. Moreover, the ethanolic extract presented higher inhibitory activity against *C. albicans* compared to the other species with a MIC value of 625 µg/mL, showing as well an fungistatic capacity. These results are in accordance with previous studies, which showed that ethanolic extracts of *Glycyrrhiza glabra* L. presented MIC values normally lower than 1.5 mg/mL, and are independent of the origin of the fungus (Irani et al. 2010; Martins et al. 2016). When focusing on the data obtained in the MFC analysis, the values were an indication that the extract can be considered as fungicidal, since the MFC ratio should be lower than three times its MIC value (Neto et al. 2016). The study of fungal growth over time showed that the extract, at its respective MIC value, was responsible for a strong delay and decrease in the growth rate when compared to the control (cell growth in the absence of the extract) (Neto et al. 2016). This is very important since *C.*

albicans is one of the most important agents involved in candidiasis present in humans and is strongly associated with denture stomatitis. Also, this fungus holds various virulence features, like the ability to form biofilms and to switch from yeast to hyphae form, both characteristics may represent potential targets for new antifungal treatments (Messier & Grenier 2011).

Nevertheless, the major challenge is to improve the absorption of the extract at the site of action, which in this case is the oral mucosa, to improve therapeutic outcomes. To achieve this goal, different systems of NPs were adapted from previous work, in order to improve the absorption of the extract (Roque et al. 2018). The mean size of the NPs obtained, showed that they can be useful, since for NPs to permeate through oral mucosa, they would have to present a mean size around, or lower than 500 nm. This size range particles can diffuse between the mucin fibres in the microarchitecture and mesh spacing of the mucin network (Roque et al. 2018). Also, the results regarding the encapsulation confirmed that the association established between the polymers and glabridin is very high, increasing the possibility of achieving an efficient therapeutic effect. The NPs showed an inhibition activity lower than that observed by the free glabridin and the free extract at 1 mg/mL. Even though no MFC values were identified for the NPs, indicating no fungicidal activity, it suggests that glabridin is efficiently entrapped inside the NPs, but there is still a percentage of glabridin absorbed on the NP surface.

The TEER values obtained in the beginning of the cell studies are in accordance with the standard range (Srinivasan et al. 2015), and no significant differences were observed between the units. The TEER values decreased after incubation with NPs, which may suggest a weakening of the monolayer caused by the presence of NPs of the extract on monolayer surface. A higher amount of carboxyfluorescein permeated the cell layer after pre-treatment with NPs compared to control, indicating some interaction between

the cell layer and the NPs. These results also indicate a correlation between the amount of glabridin and the paracellular permeation, since it was observed that different amounts of glabridin present in the NPs caused different results. But there is no direct correlation to solely amount of glabridin, since PLA NPs, which had the highest amount of glabridin, caused a lower value of paracellular permeation than alginate and PLGA NPs. The alginate NPs had the lowest amount of glabridin, but resulted in the second lowest value of paracellular permeation. Although permeability enhancement is not the same as toxicity, these two properties are usually associated.

The viscosity effect can have a strong influence on the residence time of the extract on the oral mucosa since the viscosity can be directly correlated to the mucoadhesion capacity of the formulations. The pH-values of the toothpaste varied, but were in all cases within the recommended range, which is above 7.5. The teeth become stronger and re-mineralize when the mouth is alkaline (Roque et al. 2018). In the case of the oral gel with the NPs, the formulation presented a good value of pH since it should be between an interval of 4 and 5.5 so that it does not influence the development of teeth in children (Soares et al. 2016).

The general mucoadhesion is a function that combines both the unspecific adhesion and the mucin interaction (Donnelly et al. 2011). 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 toothpaste with loaded alginate NPs, the oral gel with loaded PLA NPs and the oral film with loaded PLGA NPs had a high interaction with mucin, compared with the corresponding formulations without nanoparticles. Moreover, the oral film with loaded-PLGA nanoparticles presents a significantly higher interaction with the mucin compared to all the other formulations.

5. Conclusions

In this study it can be concluded that NPs implemented into three different mucoadhesive formulations can be a promising approach to increase the time residence of the ethanolic extract of *Glycyrrhiza glabra* L. for buccal administration and for the treatment of yeast infections in the oral mucosa. It was observed that PLGA, PLA and alginate NPs could efficiently encapsulate glabridin, that was shown to be an active compound present in the extract in terms of inhibition capacity against *C. albicans*, and to improve the interaction of this extract with mucin and HT29-MTX cells. This study also suggested that these formulations may be non-toxic/non-irritating, but further studies will be performed in the near future. Furthermore, all the formulations developed exhibited promising results for a possible industrial scale-up and to be applied in the market as an alternative solution for the treatment of oral candidiasis.

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Annex I

Design of Finasteride-Loaded Nanoparticles for Potential Treatment of Alopecia

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Design of Finasteride-Loaded Nanoparticles for Potential Treatment of Alopecia

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Keywords

Alopecia · Nanotechnology · Drug · Prolonged drug release · Toxicity · Efficacy and safety testing

Abstract

Background/Aims: Androgenetic alopecia is an extremely common dermatological disorder affecting both men and women. Oral finasteride (FNS), a synthetic 4-aza-3-oxo-steroid compound with poor aqueous solubility, blocks the peripheral conversion of testosterone to dihydrotestosterone (DHT) in a significant reduction in DHT concentration, achieving satisfactory results in alopecia treatment. However, its oral intake generally causes severe side effects. Considering that there is currently no scientifically proven treatment, new drug delivery systems able to improve alopecia therapy are urgently required. **Methods:** In this study, polymeric nanoparticles have been proposed as a new carrier for topical delivery of FNS in hair follicles. **Results and Conclusions:** Polymeric nanoparticles, prepared by using a modified method of the emulsification/solvent diffusion, showed a mean particle size around 300 nm, which may be sufficient for reaching the dermis and hair follicles and

negative zeta potential values. Scanning electron microscope measurements showed that all the polymeric nanoparticles exhibited a spherical shape and a smooth surface regardless of their composition. A high encapsulation efficiency was achieved for FNS ($79.49 \pm 0.47\%$). In vitro release assays in physiological conditions demonstrated that nanoparticles yielded a prolonged release of FNS for 3 h. Skin assays through an in vitro permeation study demonstrated that nanoparticles had low levels of penetration of FNS, improving its time residence onto the skin. All excipients used in nanoparticle composition and in 3 different vehicles were safe. These results suggest that the proposed novel formulation presents several good characteristics indicating its suitability for dermal delivery of FNS for alopecia treatment.

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Introduction

Androgenetic alopecia is an extremely common dermatological disorder affecting both men and women. This disease is characterized by a reduction of frontal hair

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in temporal region, resulting in a gradual decrease in the hair diameter [1].

Some of these follicular disorders involve changes in the protein synthesis of follicular cells [2]. These changes occur because of dihydrotestosterone (DHT) or 5 α -DHT, which is a derivative of testosterone, and causes change in the synthesis of proteins in the follicular cells. Testosterone is an endogenous hormone produced by the adrenal gland and is converted into the skin, liver, and prostate DHT by the action of 5 α -reductase. In human tissue, there are 2 types of 5 α reductase, type I and type II. Type I is most evident on the skin and in the outer sheath of the hair follicle root. Type II is most apparent in the prostate and seminal vesicle. In the hair follicle, type II is more active in the dermal papillae and the outer sheath of the hair follicle root, which has been described as causes of androgenic alopecia [3].

Finasteride (FNS), a synthetic azosteroid with potent selective antagonist action on 5 α -reductase type II enzymes, acts by irreversibly binding to the enzyme, preventing the conversion of testosterone to DHT, its active metabolite. FNS was initially approved for the reduction of prostate size associated with urinary obstruction (benign prostatic hyperplasia), specifically 1 mg/day for the treatment of benign prostatic hyperplasia and 5 mg/day for prostate cancer. In recent years, it was found that oral administration of FNS was useful in the treatment of various dermatological and follicular disorders, particularly in androgenetic alopecia [2].

Taking into account the synthesis of DHT, the use of FNS may be very useful because it will lead to an inhibition of the conversion of testosterone into DHT, thereby reducing the effects on the protein synthesis in follicular cells. However, the oral intake of FNS causes some adverse side effects, such as impotence, erectile dysfunction, and impaired reproductive function. Therefore, it is necessary to find another route to administer FNS into the system without those adverse side effects. On the other hand, most topical products available in the market for alopecia are formulated with the actives dissolved in water-alcohol solutions. However, due to the low permeability through the keratin layer, only a fraction of the applied drug dose actually reaches the target site, penetrating the pores and hair follicles. As a result, those products do not exceed consumer expectations, leading in some cases to lack of adherence to treatment.

Thus, recent studies have confirmed the hypothesis that nanoparticles can penetrate effectively and remain in the pilous follicles for longer periods [4]. Besides, nanoparticles may have the advantage of achieving solubilization

of hydrophobic drugs, reduction of a drug dose used and, in some cases, prevention or elimination of side effects [5–8].

In this study, PLGA nanoparticles loaded with FNS were produced by the modified method of spontaneous emulsification with diffusion of the solvent and characterized. PLGA is one of the most used polymers, mainly because of its biodegradability, biocompatibility capacity, and sustained drug release [9–11]. A second objective of this study was to incorporate these nanoparticles in 3 different types of topical formulations: lotion, shampoo, and the solution. All topical formulations were fully characterized including the human safety testing of the used excipients and nanoparticles, but always without FNS.

Materials and Methods

FNS and the Pluronic® F68 were obtained from Sigma-Aldrich (St. Louis, MO, USA). PLGA (50/50) was obtained from Purac (Gorinchem, The Netherlands). Sodium polyacrylate or Carbopol®940 was obtained from Fagron (Barcelona, Spain). All other reagents were of analytical grade.

Preparation of PLGA Nanoparticles of FNS

PLGA nanoparticles with FNS were prepared using a modified method of the emulsification/solvent diffusion [12]. Briefly, PLGA and FNS were dissolved in a mixture of acetone:ethanol. This ratio between FNS and PLGA was determined by previous works [13, 14]. The previous solution then was added drop by drop to an aqueous solution of Pluronic® F68 at 0.1% (w/v), with a constant stirring speed of 800 rpm, using the RT15 power IKA® WERKE (IKA, Königswinter, Germany), at room temperature. Nanoparticle suspension was formed instantly and the aqueous layer became milky. After the last drop was added, the stirring continued for additional 15 min. Subsequently, the organic solution was removed using a rotary evaporator at 40°C for 20 min. After evaporation of the solvent, there was a colloidal suspension of PLGA nanoparticles with FNS in water. Empty nanoparticles were also prepared, i.e., without the FNS.

Preparation of the Topical Formulations

A typical formulation of shampoo can usually contain up to 30 compounds, which include cleaning agents (surfactants), conditioning agents, additives that modify the effect of surfactant, preservatives, scents, dyes, and additives [15–17]. Throughout the development of the formulation, different formulations were prepared and tested. All compositions were firstly optimized. This formulation resulted from the mixture of 2 phases where the first phase included 0.2 g of methylparaben in 15.68 mL of glycerol with 35 g of sodium lauryl ether sulfate and 0.5 mL of essence of menthol. The second phase contained 0.48 mL of citric acid buffer in 46.64 mL of water.

In case of the lotion, the first phase contained a homogeneous mixture of 0.05 g of Carbopol® 940 dissolved in 70 mL of water. The second phase included 0.5 mL of essence of menthol and 6.45 g of propylene glycol in 23 mL of isopropyl alcohol.

Finally, a solution was made of 16.3 mL of water, 20 g of propylene glycol and 63.2 mL of ethanol.

Characterization of the Formulations

Viscosities of the formulations were accessed using a Brookfield Rotating Viscometer (Middleboro, MA, USA) with a spindle 2 with a rotation speed of 20 rpm and at a temperature $22.0 \pm 2.0^\circ\text{C}$. Mean viscosity was recorded in triplicate. The pH value and the organoleptic and tactile aspects were also accessed [18].

Particle Size Analysis

Mean particle size and polydispersity index (PI) of the nanoparticles were measured in hydrated particulate systems (diluted aqueous solutions, 1:1) with a Coulter Nanosizer Delsa Nano™ C (Coulter Beckman, Fullerton, CA, USA). A low value of PI factor indicates a more stable and less dispersed nanoparticle distribution in size. All experiments were made at room temperature, with angle detection of 165°C and in triplicate.

Zeta Potential Analysis of the Nanoparticles

Zeta potential was measured in Delsa Nano™ C (Coulter Beckman, Fullerton, CA, USA) by electrophoretic mobility technique. Each analysis was carried out at 25°C with an angle of detection of 90°C and in triplicate.

Particle Morphology and Surface

Morphology of the particles was assessed by scanning electron microscopy before the centrifugation procedure. An aliquot of each sample was placed in a glass coverslip and left to dry in a desiccator for scanning electron microscopy analysis [19]. The material was then coated with a thin layer of gold (with 500 nm of thickness) and observed on a JEOL 5200 LV scanning electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV. Images were digitally recorded.

Determination of FNS Encapsulation Efficiency

After the preparation of the nanoparticles, the supernatant was centrifuged 13,158 g for 45 min and the amount of free FNS present in the supernatant was analyzed by a spectrophotometric method at 210 nm (UV-visible spectrophotometer, Evolution 300, Hertfordshire, England). Measurements were made in triplicate and a standard calibration curve was made with a FNS standard solution. The established linearity range was 5–25 $\mu\text{g/mL}$ ($R^2 = 0.9995$). The precision, accuracy, and repeatability of the method were also determined [20, 21].

In vitro Drug Release

An amount of nanoparticles containing 5 mg of FNS were placed in a recipient containing 10 mL of PBS buffer with a pH 7.4 and without enzymes and following the sink conditions (USP XXX), respecting FNS solubility. The assay was maintained at $25.0 \pm 2.0^\circ\text{C}$ and in a constant stirring (130 rpm) in a multipoint laboratory magnetic stirrer (Thermo Scientific Variomag Telesystem Stirrers; Langenselbold, Germany). In appropriated time intervals, aliquots of the release medium were taken and replaced immediately with fresh PBS buffer. The aliquots were then centrifuged at 13,158 g for 10 min and the FNS concentration was determined in triplicate using the UV-visible spectrophotometer and using a standard calibration curve determined before ($R^2 = 0.9995$). Possible interferences were taken into account.

In vitro Permeation Test

After incorporation of the nanoparticles in the 3 different formulations (1:1), in vitro diffusion studies with Franz cells were conducted using a PDMS membrane as a simplified model of human epidermis [22]. The effective diffusion area was 0.95 cm^2 and the mean volume of the receptor chamber was, approximately, 4.0 mL. The receptor chamber was filled with PBS at pH 7.4 and stirred constantly at 130 rpm. The receptor compartments of the diffusion cells were maintained at 32°C using a recirculating water bath. Donor compartments were covered by glass slips. Samples were taken at regular intervals for UV spectrophotometry assay and replaced immediately with an equal volume of fresh PBS. A standard calibration curve was performed with the FNS solution in PBS at pH 7.4. All experiments were performed 5 times. The multiple steady-state fluxes of FNS and the percentage of drug permeation were determined.

In vitro Safety Assessment

Preliminary cytotoxicity studies were first performed using *Saccharomyces cerevisiae*. The study consisted of exposition of test substances to yeast cells growing in cuvettes containing complete YPD medium (1% yeast extract, 0.5% peptone, 2% glucose, and 1.5% agar). Cell cultures of *S. cerevisiae* were prepared by transferring a cell colony growing on a Petri dish into a 50-mL sterile Erlenmeyer with 20 mL of YPD medium and incubated at 30°C with agitation (80 stirring cycles per min) for 24 h, which leads to approximately 1.0×10^7 cells/mL. Afterwards, an equal volume of the yeast suspension (20–50 μL) was diluted with YPD medium in disposable cuvettes in order to obtain an initial absorbance for the tested samples and control, not higher than 0.15 at 525 nm.

The final volume in all cuvettes containing the substances to be tested together with YPD medium and yeast cell cultures was 2 mL. The control groups were prepared with medium without any exposing agent. Growth curve in the logarithmic phase for each tested group as well as for the control group was conducted by correlating the logarithm of the cell concentration (number of cells per mL) against the time of incubation. Each group was tested in 4 replicates. Cell concentrations were calculated by equation 1 at 525 nm [23–27].

$$C = \frac{A - 0.0327}{6.8219 \times 10^{-8}} \quad (1)$$

The growth inhibition in percent was calculated from the linear slope in the logarithmic phase for each tested substance and concentration for the cytotoxicity evaluation toward *S. cerevisiae*.

In vivo Safety Assessment

Safety of the formulation excipients was made by the Occluded Patch Test method [28–30]. A convenience sample of 10 healthy volunteers was selected, all female, between 21 and 27 years of age (23 ± 2). Volunteers were selected after informed consent, according to inclusion criteria previously established and in full compliance with all ethical standards from the Declaration of Helsinki and its amendments. Noninclusion criteria were being pregnant and/or in lactation, nonsmokers (more than 2 years of not smoking), presence of cutaneous signs in the experimental area, existence of any type of topical treatment in the 8 days preceding the study, intense exposure to UV radiation,

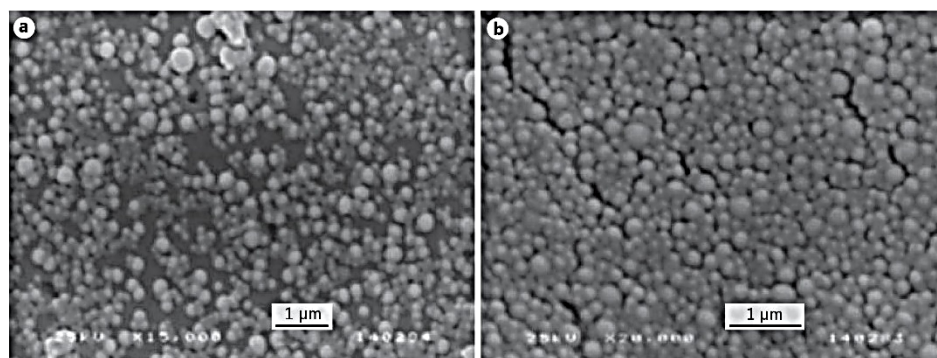


Fig. 1. Scanning electron microscopy image of PLGA nanoparticles before loading (**a**, magnification 15,000 $\times$) and after loading (**b**, magnification 20,000 $\times$).

and regular practice of physical exercise. Some restrictions were imposed, in particular, not to wash the anatomical area subject to measurement with soap or shower gel and no application of cosmetics in these areas in the 24 h preceding the measurements. Evaluations were carried out in the laboratory under controlled temperature and humidity ($22.0 \pm 1.0^\circ\text{C}$; RH $60 \pm 3\%$) in the absence of heat and forced convection according to previously published methods [29, 30]. In this study, we aimed to make the assessment of excipients present in all 3 formulations: shampoo, lotion, and solution with and without empty nanoparticles, but in all cases without FNS. Two adhesives were applied in the lumbar area of each of the volunteers during 24 h. These adhesives had semiocclusive devices which contained 100 μL of each formulation (lotion with empty PLGA nanoparticles, lotion without nanoparticles, solution with empty PLGA nanoparticles, solution without nanoparticles, shampoo with empty PLGA nanoparticles, and shampoo without nanoparticles) in the respective areas. FNS was not included in any situation. Distilled water was used as a negative control. The location of each sample was randomized among volunteers and visual observations were carried out for 2 h after removal of the adhesives. Noninvasive measurements were performed using skin biometrics equipment to assess erythema through a Chromameter CR400 (Minolta, Japan) and the transepidermal water loss (TEWL) using Tewameter TM300 (CK Electronics, Germany). For both analysis, especially TEWL analysis, an acclimation period of at least 15 min to 1 h was required. During this analysis, any thermally or emotionally induced sweating was completely avoided.

All the tests were based on previous studies [31–33] and received the approval of the local ethical committee.

Statistics

All results are expressed as means $\pm$ SD. One-way ANOVA analysis was applied to demonstrate statistical differences in all tested parameters. All analyses were performed using a software program (GraphPad Prism 5[®], GraphPad Software, San Diego, CA, USA) with a statistical significance level of 0.05.

Results and Discussion

Spherical PLGA particles were obtained using standardized and reproducible conditions (Fig. 1). Size particle range was around 316.5 ± 14.44 nm (PI 0.114) and 185.3 ± 9.32 nm (PI 0.122) for the nanoparticles with and without FNS, respectively. According to previous studies, particle size should be between 200 and 400 nm in diameter to penetrate through the hair follicles [34]. On the other hand, the zeta potential of FNS-loaded nanoparticles was negative (-5.71 ± 0.431 mV), probably due to the presence of terminal carboxylic groups of PLGA. Concerning this subject, it is well-known that the investigation of nanoparticle interactions with skin is a complex process [35]. The behavior of the skin surface seems to be similar to the functioning or artificial ion exchange membranes, designed to attract positively or negatively materials to their pores [36]. Hence, there are both indiscriminate and specific charge adsorption processes on the skin surface [36, 37]. In a dry state and neutral pH, the skin surface is negatively charged because of the carbohydrate content, residues of proteins in the outer membranes of the cells, and active ions pumps. Consequently, it is expected to observe a great adsorptivity of cationic solutes to the skin surface, but curiously anionic nanoparticles also strongly interact with the skin [37–39]. Here, the composition of the skin may confer some positive charge because of some surface lipids covering the stratum corneum. Previous studies have shown that negatively charged carriers like NLC or SLN generally interact with lipids in the outermost skin layers, resulting in an occlusive effect [37, 40]. Those negatively charged

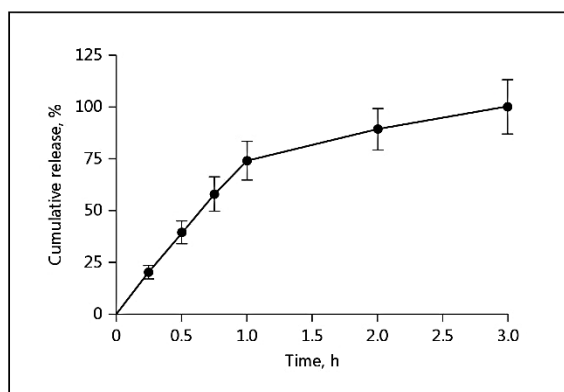


Fig. 2. Release profile of FNS from the nanoparticles.

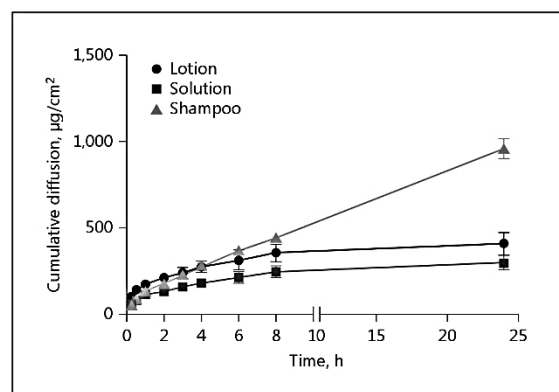


Fig. 3. Permeation profiles of the FNS in the different vehicles: lotion, solution, and shampoo.

NLC and SLN carriers may increase the drug penetration in the stratum corneum and retention in the skin, forming an intact film on the skin surface [40]. Other nanoparticles like polymeric nanoparticles may accumulate in skin appendages such as hair follicles, which can act as a long-term reservoir. We suspect that our negatively charged nanoparticles may retain FNS in the target area, i.e., within the hair follicles [36, 41]. The encapsulation efficiency of FNS into PLGA nanoparticles was high, around $79.49 \pm 0.47\%$, suggesting that the interaction established between PLGA and FNS was very probable. It has been documented by a number of investigators that highly lipophilic drugs (FNS) feature a good affinity with PLGA, leading to satisfactory encapsulation efficiency and great interest in the pharmaceutical industry [42, 43]. An in vitro release study demonstrated that the PLGA nanoparticles led to a prolonged release profile of FNS. Figure 2 showed that 100% of FNS was released after a period of 3 h. All FNS was released from PLGA nanoparticles.

Moreover, in this study, pH values, viscosity, and the organoleptic and tactile aspects of the developed formulations were evaluated and the results were present in Table 1.

Regarding the value of pH, the values obtained for the 3 formulations were compatible with human skin, which presents a pH within the range of 4.5 and 6.5 [44, 45]. Viscosity of all formulations was adequate. In case of shampoo, its viscosity allows the effective removal of the formulation and at the same time it must be easy to apply on the hair.

Table 1. Parameters of the developed topical formulations (mean values $\pm$ SD)

Formulations	Organoleptic parameters	Viscosity, cps	pH values ($n = 3$)
Shampoo	Translucent Homogeneous Very good foaming ability	$2,650 \pm 15$	5.66 ± 0.01
Lotion	Colorless formulation Homogeneous	135 ± 5	5.31 ± 0.01
Solution	Colorless formulation Clear No particles in solution	<50	5.96 ± 0.02

During the in vitro skin permeation test, it was found that the higher diffusion rate of the FNS was observed with the shampoo and the lowest was observed in the solution (Fig. 3). It should be noted that all experimental variables, including the diffusion area (0.95 cm^2), cell volume ($4.09 \pm 0.16 \text{ mL}$), thickness (75 microns), and type of membrane (hydrophobic membrane of PDMS) were constant for all Franz cells. The steady state flows in the different formulations of FNS were also calculated using the slope obtained by the diffused amount of drug over time, after steady state was achieved. No significant differences were observed ($p = 0.4793$) (Table 2). In the case of the shampoo, a flux in steady state of $40.01 \pm 2.4 \text{ µg/cm}^2 \cdot \text{h}^{-1}$ was obtained. Shampoo composition with sur-

Table 2. Permeation profiles of the tested formulations ($n = 5$)

Time, h	Mean cumulative diffused FNS $\pm$ SD, $\mu\text{g}/\text{cm}^2$		
	lotion	solution	shampoo
0.25	99.27 $\pm$ 17.38	61.11 $\pm$ 28.34	47.50 $\pm$ 18.77
0.50	141.48 $\pm$ 24.31	87.11 $\pm$ 25.72	82.41 $\pm$ 19.26
1.00	173.20 $\pm$ 20.75	110.66 $\pm$ 26.74	129.86 $\pm$ 19.23
2.00	209.93 $\pm$ 28.78	133.33 $\pm$ 26.12	179.53 $\pm$ 21.55
3.00	241.41 $\pm$ 31.96	158.20 $\pm$ 27.13	228.95 $\pm$ 24.29
4.00	270.55 $\pm$ 32.57	181.73 $\pm$ 26.43	284.51 $\pm$ 25.93
6.00	313.75 $\pm$ 55.77	213.12 $\pm$ 31.09	369.17 $\pm$ 21.45
8.00	354.13 $\pm$ 51.05	247.07 $\pm$ 33.99	448.99 $\pm$ 26.59
24.00	409.22 $\pm$ 64.86	300.22 $\pm$ 42.22	960.24 $\pm$ 56.87

Table 3. Comparison of the parameter a^* (erythema) and the TEWL of the different formulations ($n = 10$)

Formulations	Comparison of the parameter a^* , AU	Comparison of the TEWL, g/m^2
Lotion with NP	0.582	0.376
Lotions without NP	0.903	0.221
Solution with NP	0.153	0.107
Solution without NP	0.800	0.077
Shampoo with NP	0.372	0.329
Shampoo without NP	0.746	0.023
Negative control	0.898	0.623

TEWL, transepidermal water loss; NP, nanoparticles.

factants such as sodium lauryl ether sulfate may be responsible for those high flux values. Solution and the lotion showed similar values ($12.51 \pm 1.8 \mu\text{g}/\text{cm}^2 \cdot \text{h}^{-1}$ and $17.05 \pm 2.7 \mu\text{g}/\text{cm}^2 \cdot \text{h}^{-1}$, respectively). Those results can be explained with the particular characteristics of each formulations, such as the high water content and low viscosity of the lotion and solution, which influence the permeation capacity of the nanoparticles through the synthetic and hydrophobic membrane [46, 47].

The cytotoxicity profile of the FNS-loaded PLGA nanoparticles was characterized using the *S. cerevisiae* model. FNS was tested at different concentrations of 15, 30, and 45 $\mu\text{g}/\text{mL}$. Results showed a linear relationship between FNS concentrations and the magnitude of cytotoxic effects. The decrease in cell viability was probably related to an occlusive effect of the nanoparticles on *S. cerevisiae*. In fact, the cytotoxicity testing of nanoparticles on the *S. cerevisiae* model is limited by the amount of nanoparticles in suspension. Cytotoxicity profiles for nanosystems are not yet well understood. Other examples

of our group such as polycaprolactone nanoparticles with betamethasone-21-acetate confirmed the occlusive effect of nanocarriers [48]. In this case, betamethasone-21-acetate showed an IC_{50} of 168.8 $\mu\text{g}/\text{mL}$ and betamethasone-21-acetate-loaded nanoparticles showed an IC_{50} of 14.5 $\mu\text{g}/\text{mL}$. These empty nanoparticles also demonstrated a concentration-dependent toxic effect on the human keratinocyte HaCaT cell line [42, 49, 50]. In addition, our empty PLGA nanoparticles was already tested using the human keratinocyte HaCaT cell line [48] and corroborates with the present study.

In human safety testing, all excipients used in those formulations did not cause any erythema or other adverse reaction in any of the volunteers. All excipients are included in approved substance list of the European Commission and all are used in lower concentrations [15]. In this study, erythema effects were determined by comparing a^* values of the treated site with control sites. In this study, there were no significant differences between the values of the parameter a^* after application (Table 3) which means that PLGA or other reagents used in all formulations, such as surfactants used in shampoo or solvents, did not cause any change [51]. In this technique, the increase in skin redness was calculated by a difference between baseline skin redness and skin redness resulting from the effect of an irritant. This technique of reflectance spectrophotometry utilizes the fact that hemoglobin present in the blood vessels in the upper dermis is the main cutaneous chromophore of green light. Blood flow increases have been shown to correlate positively with increased erythema, TEWL, and skin thickness [15]. Surfactants like sodium lauryl ether sulfate generally increase cutaneous blood flow. Moreover, other surfactants have been shown to cause changes in skin temperature. Exposure of skin to surfactants may induce irritation, but in our case this fact was also not observed. The type and degree surfactant irritancy can be related to the type and concentration and also some other factors such as skin type, color, and age [1, 32, 51].

For TEWL analysis, experimental conditions were similar for all formulations. TEWL evaluation only showed that there were significant differences ($p < 0.05$) for solutions with nanoparticles and shampoo, probably due to the rheological properties of such formulations. High-viscosity values were observed after inclusion of nanoparticles in each vehicle [52]. The increase of TEWL values after application of the nanoparticles may be related to some destabilization of skin surface and follicular ducts, causing a modification on the stratum corneum as previously observed [46].

Conclusion

The success of topical products in alopecia therapy depends on the capability of the drug to penetrate through the skin in sufficient amounts to achieve the desired therapeutic effect. In this study, PLGA nanoparticles were chosen as carrier systems with the ability to locally incorporate FNS into hair follicles. The overall conclusion confirms that PLGA is a useful and safe polymer for drug delivery to the skin surface and for the potential application of nanotechnology in transdermal delivery systems. The resultant PLGA nanoparticles were shown to be an efficient encapsulation system for FNS with potential interest for pharmaceutical industry. No toxic effects of the FNS-loaded PLGA nanoparticles were observed in the *S. cerevisiae* model. Safety testing of the excipients using hu-

man volunteers demonstrated that formulation excipients were compatible with the skin. Future studies should include the study of the efficacy and safety of the previous FNS-loaded formulations.

Statement of Ethics

Volunteers were selected after informed consent, in full compliance with all ethical standards of the Declaration of Helsinki and its amendments.

Disclosure Statement

The authors declare no conflicts of interest or any sponsorship or funding arrangements relating to their research.

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Conclusions

4

Conclusion

This thesis aims the development and characterization of bioadhesive NPs and semi-solid formulations (toothpaste, oral gel and oral film) to be the carrier of two different antifungal agents in the treatment of OC: a classic antifungal agent like Nys and an extract from plants. Briefly, seven types of polymeric NPs were designed but only three were selected to encapsulate the antifungal drug, Nys. On the other hand, several extracts were prepared but only the ethanolic extract of *Glycyrrhiza glabra* L. was further assessed. The results obtained allowed us to achieve the following points and conclusion:

1. Spherical polymeric particles loaded with Nys were successfully prepared and the microbiological studies revealed that the encapsulation of the drug resulted on an enhanced of the antifungal effect when comparing with a commercial suspension of Nys. Also, it was observed that the particles achieved the lowest minimal inhibitory concentration and a high efficiency during a period of one year, when compared the commercial Nys.
2. Bioadhesive NPs proved to be a promising approach to increase the efficacy of locally administered of Nys in the treatment of OC. After the preparation of several types of bioadhesive NPs, the PLGA, PLA and Alginate NPs successfully encapsulated Nys. The particle size ranged between 300-900 nm. Also, the chosen polymers were bioadhesive and NPs stable along 3 and 6 months and no preliminary toxicity was observed in empty NPs. On the other hand, a high adhesion to buccal mucosa was achieved with those NPs.
3. At the same time, these bioadhesive NPs were implemented in three different semi-solid formulations (toothpaste, oral gel and oral film) and proved to be excellent dosage form to be apply in the treatment of yeast infections of the oral mucosa. We observed that PLGA, PLA and Alginate NPs implemented in an oral film, oral gel and a toothpaste could improve the interaction of this antifungal drug with mucin and HT29-MTX cells. These nanoformulations increased the amount of Nys attached to the superficial area of the buccal mucosa allowing the increasing of the contact time of the drug with the site of action. This study also confirmed that these formulations were non-toxic/non-irritating.

4. Regarding the extracts from plants and the screening their antifungal activities against *C. albicans*, different extracts from *Glycyrrhiza glabra* L. were prepared and generally characterized. The *in vitro* studies showed the ethanolic extract of *Glycyrrhiza glabra* L. the most active against *C. albicans*. These results were also confirmed by the value of the minimal inhibitory concentration and the minimal fungicidal concentrations, but also by the growth curve of *C. albicans* in the presence and in the absence of the extract. Thus, the ethanolic extract of *Glycyrrhiza glabra* L. was then encapsulated into the optimized NPs previously described. The used of the NPs also improved the interaction of the extract with mucin and HT29-MTX cells. Additionally, these nanoformulations were non-toxic/non-irritating.
5. Finally, independently of the source of antifungal agent (as isolated compound like Nys or extract), this thesis confirmed that bioadhesive NPs can be considered a promising strategy to locally deliver therapeutic agents. Moreover, this thesis also confirmed that NPs when included in semi-solid formulations improved the time residence of the antifungal agent in the target area by providing high adhesion forces; they can be easily adapted in the patient daily routine; they did not have alcohol content and they can be applied in case of ulceration of the oral mucosa.