SYNTHESIS, CHARACTERIZATION AND IN VITRO ACTIVITY AGAINST CANDIDA SPP OF FLUCONAZOLE ENCAPSULATED ON CATIONIC AND CONVENTIONAL NANOPARTICLES OF PLGA.

NICOLÁS SEBASTIÁN EMILIO GÓMEZ SEQUEDA

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Trabajo de Grado presentado como requisito para optar al título de Biólogo

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DEDICATORIA

A mi madre, Martha Gómez, a mi familia y a Nathaly, quienes con su infinito amor y constante apoyo me permitieron alcanzar esta meta.

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CONTENTS

LIST OF FIGURES AND TABLES

RESUMEN

TITULO: SYNTHESIS, CHARACTERIZATION AND IN VITRO ACTIVITY AGAINST CANDIDA SPP OF FLUCONAZOLE ENCAPSULATED ON CATIONIC AND CONVENTIONAL NANOPARTICLES OF PLGA*

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PALABRAS CLAVES: Doble Emulsión Solvente Difusión (Des-D); Concentración Mínima Inhibitoria, Cmi; Concentración Mínima Fungicida, Cmf; Polietilenimina (Pei); Nanoencapsulación.

DESCRIPCION:

El fluconazol (FLZ) es uno de los medicamentos de primera línea para el tratamiento de la "candidiasis" sistémica. El propósito de este estudio es desarrollar nanopartículas de ácido poliláctico coglicólico cargadas con FLZ (FLZ-NP) y nanopartículas de PLGA cargadas con FLZ recubiertas por un polímero catiónico polietilenimina (PEI) (FLZ-NP-PEI), se determinó su actividad antimicótica sobre cuatro cepas de Candida de importancia clínica. FLZ-NP y FLZ-NP-PEI fueron sintetizadas por el método de Doble emulsión solvente difusión (DES-D), su tamaño hidrodinámico, potencial zeta, morfología, perfil de liberación y eficiencia de encapsulamiento fue caracterizado in vitro. Finalmente se determinó su concentración mínima inhibitoria (MIC) y concentración mínima fungicida (MFC). Se lograron obtener FLZ-NP, con tamaño hidrodinámico promedio de 222 ± 2,4 nm, carga superficial de -11,6 ± 5,13mV, y morfología esférica. La eficiencia de encapsulamiento del 53% y liberación rápida (≥ 90% después de 3 h). También se lograron obtener nanopartículas catiónicas FLZ-NP-PEI con tamaño hidrodinámico promedio de 281 ± 6,6 nm y carga superficial de 23,5 ± 1,3 mV. Los valores de MIC para las diferentes preparaciones de FLZ (FLZ, FLZ-NP y FLZ-NP-PEI) sobre las cuatro especies de Candida mostraron mejores resultados para el FLZ nanoencapsulado, ya sea en su forma convencional FLZ-NP como catiónica FLZ-NP-PEI. Solo las FLZ-NP-PEI tuvieron actividad fungicida sobre las cepas de estudio. Las nanopartículas poliméricas utilizadas en este estudio pueden ser una alternativa viable para mejorar la actividad del fluconazol sobre Candida spp., Incluso en cepas con resistencia al antibiótico. Las nanopartículas catiónicas demostraron gran actividad fungicida sobre *Candida spp*. Estos nanocompuestos son buenos candidatos para estudios posteriores de actividad antifúngica *in vivo*.

*Trabajo de Grado

**Facultad de Ciencias, Escuela de Biología, Director: Rodrigo Torres, Codirector: Claudia Ortiz.

ABSTRACT

TITLE: SYNTHESIS, CHARACTERIZATION AND IN VITRO ACTIVITY AGAINST CANDIDA SPP OF FLUCONAZOLE ENCAPSULATED ON CATIONIC AND CONVENTIONAL NANOPARTICLES OF PLGA*

AUTHOR: GÓMEZ SEQUEDA Nicolás Sebastián Emilio**

KEYWORDS: Double Emulsion Diffusion (Des-D); Minimal Inhibitory Concentration, Mic; Minimal Fungicide Concentration, Mfc; Polyethylenimine (Pei); Nanoencapsulation.

DESCRIPTION:

Fluconazole (FLZ) is a medicine for treating systemic “candidiasis”. The aim of this study was to obtain nanoparticles of poly-lactic-co-glycolic acid loaded with fluconazole (FLZ-NP) and FLZ-N coated with the cationic polymer polyethyleneimine (PEI) (FLZ-NP-PEI), in order to improve antimycotic activity against four strains of *Candida sp* of clinical relevance. FLZ-NP and FLZ-NP-PEI were synthesized by the double emulsion solvent-diffusion (DES-D) method, and its hydrodynamic size, zeta potential, morphology, release profile and encapsulating efficiency were determined by different analytical methodologies. Finally, both minimum inhibitory concentration (MIC) and minimum fungicide concentration were determined *in vitro* by culturing these Candida strains in presence of these nanocompounds in liquid and solid media. We achieved obtaining FLZ-NP, with mean hydrodynamic sizes around 222 ± 2.4 nm, surface charge of -11,6 ± 5.13mV, and with spherical shapes. Efficiency of encapsulation around 53% and a quick release of FLZ (≥ 90% after 3 h) were obtained. We could also obtain cationic nanoparticles (FLZ-NP-PEI) with mean hydrodynamic sizes of 281 ± 6.6 nm and positive surface charge of 23.5 ± 1.3 mV. MIC values for different preparations of FLZ (FLZ, FLZ-NP and FLZ-NP-PEI) for four species of *Candida* showed best results for nanoencapsulated FLZ, either in its conventional form (FLZ-NP) or as cationic form (FLZ-NP-PEI). Nevertheless, only FLZ-NP-PEI displayed fungicide activity on the studied strains. Polymeric nanoparticles used in this study can be a promising alternative for improving antifungal activity of fluconazole against *Candida spp.*, even with antibiotic fluconazole-resistant strains. Finally, cationic nanoparticles demonstrated high fungicide activity on *Candida spp*. These nanocompounds could be good candidates for further studies of antifungal activity *in vivo.*

*Bachelor Thesis

**Facultad de Ciencias, Escuela de Biología, Director: Rodrigo Torres, Codirector: Claudia Ortiz.

INTRODUCTION

Candida spp is a microorganism constituting of both gastrointestinal and genitourinary tracts in healthy individuals(Kumamoto 2011). In cases where immune system is threatened, these microorganisms can become pathogenic, causing a disease known as Candidiasis. This can be subdivided in three main groups: cutaneous (skin and appendices), mucous and systemic (blood flow)(M. A. Pfaller and Diekema 2007). Systemic "Candidiasis" is an infectious disease with high mortality and morbidity (e.g. mortality rates range from 50 to 70%)(Blumberg et al. 2001; Bassetti et al. 2011; Kett et al. 2011; Marriott et al. 2009; Gudlaugsson et al. 2003). This health problem has gained special importance since 1980(Edwards 1991), causing nosocomial systemic infections in USA(Wisplinghoff et al. 2004) and Europe(E Bouza, Perez-Molina, and Muñoz 1999; Emilio Bouza and Muñoz 2008). In Colombia, *Candida* spp is in the five place among most frequent nosocomial disease, mainly affecting neonatal patients in Intensive Unit Cares (IUC)(Efird et al. 2005).

From 17 *Candida* species known to be etiologic agents of candidiasis, approximately 90% of cases are caused by the following five main species: *C. albicans, C. glabrata, C. krusei, C. parapsilosis a*nd *C. tropicalis*(M A Pfaller and Diekema 2004; Spellberg, Filler, and Edwards 2006; Pappas et al. 2004). However, *C. albicans* is the most common pathogenic agent (Hazen 1995; M A Pfaller and Diekema 2004). For treatment of surface and systemic "candidiasis", fluconazole (FLZ) is used as medicine, because of its both excellent bioavailability and low toxicity (Brammer, Farrow, and Faulkner 1990; Charlier et al. 2006). Nevertheless, its extensive use has caused an increasing on *C. albicans* strains with resistance to fluconazole (FLZ) and other triazoles(J. H. Rex, Rinaldi, and Pfaller 1995). This situation has caused raising on occurrence of *Candida* species with intrinsic and/or acquired resistance to fluconazole (e.g. *C. glabrata* and *C. krusei)*(M A Pfaller et al. 2001). In these cases, it is necessary to administrate large doses of the pharmaceutical and use of

multiple therapeutic agents. However, if well FLZ has an excellent tolerance profile in the dose ranges for treatment of systemic candidiasis, administration of high amounts of FLZ (over 400 mg/day) increase common side effects, such as nauseas, anorexia, hepatitis, and abdominal pain, among other side effects (Charlier et al. 2006; Grant and Clissold 1990).

Although efficiency and safety of these antifungal compounds have been proved, these can be improved through encapsulation(Pandey et al. 2005). This technology can offer several advantages such as: protection of the active agent, controlled and sustained release, increasing therapeutic effect and diminishing side effects(Nogueira de Assis et al. 2008). In this sense, several antifungal agents have been encapsulated in nanoparticles with good results in animal models and clinical therapy(Gupta et al. 2000; Di Bonaventura et al. 2004; Maheshwari et al. 2012; Prajapati et al. 2014). Among these nanoparticles, polymeric ones have captioned especial attention due to versatility of techniques that can be used for modification of their polymeric structures(Pinto Reis et al. 2006). Among the factor susceptible to be modified are size and surface charge(Pinto Reis et al. 2006), because these both properties affect nanoparticle interaction with target cells, cellular uptake and thus, its bioavailability and efficiency(McClean et al. 1998; Vieira and Carmona-Ribeiro 2008).

The selection of the polymeric matrix is essential for developing a delivery system for pharmaceuticals. Among these polymers, poly(lactic-co-glycolic acid) (PLGA) is one of the most studied polymers for drug delivery, because it is biodegradable, biocompatible, and safe; its toxicity has been evaluated in animal models and approved by FDA for use in animals(Bala, Hariharan, and Kumar 2004). Several pharmaceutical have been successfully encapsulated in PLGA nanoparticles: antibiotics(Pandey et al. 2005; Bian et al. 2013; Van de Ven et al. 2012), Hormones(Kwon et al. 2001), peptides and proteins(Li et al. 2001; N. Zhang et al. 2008), genes(Kim et al. 2005; Shau et al. 2012; Bivas-Benita et al. 2004) and anti-cancer compounds(Wang et al. 2011). Another important characteristic of PLGA

nanoparticles is the possibility to modify its surface using other compounds. For example for modification of its charge by using of cationic polymers such as polyethylenimine (PEI)(Liang et al. 2011). Cationic nanoparticles show typically a special affinity for anionic cell surfaces. However, they can also have some degree of toxicity(Chen et al. 2009). Using this strategy, antifungal pharmaceuticals as amphotericin B have been encapsulated in cationic nanoparticles with promising results for treating infections caused by *Candida albicans*(Tiyaboonchai, Woiszwillo, and Middaugh 2001).

This study was aimed for developing a delivery system for FLZ using PLGA and PLGA-PEI nanoparticles, determining their physicochemical properties, and *in vitro* activity on four *Candida* spp of medical relevance.

1. MATERIALS AND METHODS

1.1. MATERIALS AND MICROORGANISMS

Fluconazol (FLZ, ≥98%), poly(lactic-co-glycolic acid) (PLGA) 50:50 (molecular weight (MW): 38.000 Da), poloxamer 407 (POL) and polyethylenimine (PEI) in solution at 50% wt/vol (MW: 2 kD) were purchased from Sigma-Aldrich (EUA). Ethyl acetate (EtAc, 99.5+ %) was acquired from Alfa-Aesar (EUA). We used the following fungi strains: *Candida glabrata* EMLM 14 and *Candida albicans* ATCC 10231 *were gifted by* Escuela de Microbiología from Universidad Industrial de Santander, Bucaramanga, Colombia; *Candida parapsilosis* ATCC 22019 was purchased from Microbiologics® (USA) and *Candida krusei* ATCC 6258 was obtained from Laboratorio de Quimioterapia Antifúngica from Universidad de Sao Paulo, Brasil. All fungi strains were maintained in solid media using Saboraud Dextrose Agar (SDA).

1.2. SYNTHESIS OF PLGA NANOPARTICLES WITH ENCAPSULATED FLZ (FLZ-NP)

FLZ was encapsulated using PLGA utilizing the double emulsion diffusion (DES-D) methodology, according to Cohen-Sela et al. del 2009(Cohen-Sela et al. 2009) with some modifications. Two ml of 5 mM phosphate, pH=7,4, with 5mg/ml FLZ was emulsified in 4 ml of EtAc containing 3% (w/v) PLGA (50:50) using a homogenizer-disperser (IKA Ultra-turrax T-18), at 20000rpm by 30s. The resultant solution was again emulsified at 20000rpm by 30s with 10 ml of a 0.005M phosphate solution, pH=7.4, containing 2% (w/v) POL. EtAc was eliminated by evaporation at reduced pressure using a rotatory evaporator (Heidolph Hei-VAP precision). For eliminating the free FLZ and residua caused by nanoparticle synthesis, we centrifuged nanoparticles containing FLZ (FLZ-NP) at 10000 rpm in a centrifuge (Thermo Scientific IEC CL31R multispeed) during 15 min and 4°C. Then, they were re-suspended 0.005M, pH=7.4. This procedure was repeated three times.

1.3. PHYSICO-CHEMICAL CHARACTERIZATION

1.1.1. Size, zeta Potential and Morphology

Hydrodynamic Sizes of nanoparticles were performed by Dynamic Light Dispersion (DLS) and surface charge by Laser Doppler Electrophoresis (LDE), using a size analyzer based on laser diffraction (Malvern Zetasizer 1000HS, Malvern Instruments, UK). Morphology of nanoparticles was observed by Scanning Electron Microscopy (SEM) using a microscope FEI Quanta 650. 10 µL of purified FLZ-NP were taken and deposited on a gold grid (EMS, 100-400 mesh). The aqueous solvent of the sample was evaporated at room temperature and analyzed at an accelerating voltage of 30 kV with a magnification of 80000 and 160000 X. A quantitative analysis of the nanoparticle diameters was carried out from SEM images using ImageJ processing and analysis software (version 1.48, National Institutes of Health, USA)(Schneider, Rasband, and Eliceiri 2012).

1.1.2. Efficiency of FLZ encapsulation

Efficiency of FLZ encapsulation was carried out according to Rivera et al. (2004)(Rivera et al. 2004). One ml of FLZ-NP was dried using a rotatory evaporator (Heidolph Hei-VAP precision). The pellet formed was dissolved in 3ml of dichloromethane (DCM) and stirred at room temperature. This sample was analyzed spectrophotometrically at λ=260nm (Shimadzu UV-1800), and the amount of FLZ was determined using a calibration curve of FLZ dissolved in distilled water(Rivera et al. 2004). Efficiency of FLZ encapsulation was defined as ratio between encapsulated FLZ and total amount of FLZ added at the beginning of the preparation(Thomasin et al. 1996). These tests were performed by triplicate.

1.1.3. Profile of in vitro delivery of FLZ encapsulated in PLGA-Nanoparticles (FLZ-NP)

One mL of FLZ-NP was diluted in 50 mL of water Milli-Q; this sample was stirred in an orbital shaker (Thermo Scientific MaxQ 4000) at 37°C, 50 rpm. Periodically, samples from the suspension (0.5 mL) were withdrawn and centrifuged in Amicon Ultrafilter Tubes (MWDC. 30,000 Da), and spectrophotometrically analyzed at

λ=260nm(Cohen-Sela et al. 2009). This assay was performed by triplicate under sink conditions (10% of saturation concentration in the medium after release of 100% of FLZ)(Gibaldi and Feldman 1967)

1.4. MODIFICATION OF SURFACE CHARGE OF FLZ-NP WITH POLYETHYLENEIMINE (FLZ-NP-PEI)

An amount of FLZ-NP solution were mixed with polyethylene-imine (PEI) for modification of Surface charge according to protocol described by Liang et al. (2011)(Liang et al. 2011). PEI was added varying its concentration from 0.005 up to 0.1% (w/v). The pH was adjusted at 7.4 utilizing 0.1N HCl, and then were shaken under magnetic stirring during 3-4 h. FLZ-NC modified with PEI (FLZ-NP-PEI) were centrifuged at 10000 rpm by 15 min and 4°C, and then re-suspended in 5 mM phosphate solution, at pH=7.4, 3 times (3X), in order to eliminate PEI excess. Finally , measurements of surface charge and size were performed in triplicate by DLS and LDE.

1.5. DETERMINATION OF IN VITRO ANTIFUNGAL ACTIVITY OF FLZ-NP, FLZ AND FLZ-NP-PEI AGAINST *CANDIDA* SPP.

Inhibitory effect of FLZ, FLZ-NP and FLZ-NP-PEI was determined according to standard culture microdilution method M27-A3 from Clinical and Laboratory Standard Institute (CLSI)(CLSI 2008). *Candida spp.* strains were cultivated in synthetic RPMI 1640 medium supplemented with L-glutamine and 0.2% (w/v) D-glucose without sodium bicarbonate (Gibco, ICN, Oxoid, Sigma), buffered with 0.1654 M 3-(N-morpholin)-propane-sulfonic acid (MOPS) (ICN, Sigma), adjusted at pH 7±0.1. Inoculums from *Candida spp* strains were obtained from fungal cultures in Sabouraud Dextrose Agar (SDA) at 35°C/24 h. Initial concentration of *Candida spp* strains was 2-5x10^6 UFC/mL. The inoculum was adjusted in order to obtain an optical density of 0.5 in the McFarland Scale using a sterile 0.85% (w/v) saline solution. Finally, cells were suspended in RPMI 1640 medium in order to obtain a final concentration of 5x10^4 UFC/mL. For evaluation of antifungal activity, we tested, FLZ, FLZ-NP, FLZ-NP-PEI, empty nanoparticles (NP) and NP-PEI against strains of

C. parapsilosis, *C. albicans*, *C. glabrata* y *C. krusei* cultured in 96 well microplates at different concentrations at 35°C by 48h. Growth and sterility controls were also used. Fungal growth was determined in a ELISA microplate reader (Biorad, imarck) at 530 nm. Minimum inhibitory concentration ($MIC_{50)}$ was defined as the lowest concentration of FLZ, FLZ-NP and FLZ-NP-PEI that produces a reduction of 50% of the yeast growth compared to controls (in the absence of compounds).

Minimum Fungicide Concentration (MFC) was determined according to Canton et al (2003)(Cantón et al. 2003), with some modifications. We take 100µL from wells in those an apparent microbial growth was no observed, then 900µL RPMI 1640 culture medium was added and incubated at 35°C during 24 h. Subsequently, three aliquots of 10µL were taken from each of this tube was subcultured onto SDA plates in order to check absence of fungal growth. From this, we calculated Minimum Fungicide Concentration (MFC), defined as the lowest drug concentration that produced a reduction in Colony Forming Units (CFU) ≥99,9% compared to untreated inoculum.

2. RESULTS

2.1. PHYSICOCHEMICAL CHARACTERIZATION OF NANOPARTICLES

2.1.1. Size, zeta Potential and Morphology

FLZ-NPs were characterized by DLS, LDE and SEM, determining hydrodynamic size, zeta potential and morphology of NPs (Figure 1). PGLA nanoparticles loaded with FLZ (FLZ-NPs) showed mean sizes around 222 ± 2.4 nm with a poly-dispersion index lower than 0.2 (PDI=0,156) indicating a narrow particle distribution (Figure 1.A). Mean zeta potential was around -11.6 ± 5.13mV (Figure 1.B). SEM images (Figure 1.C) of the PGLA-NPs showed that the mean size of the nanoparticle was around 156±97 nm, they were mono-dispersed, with spherical morphology, and without aggregated among nanoparticles, indicating stability and low dispersion between nanoparticles.

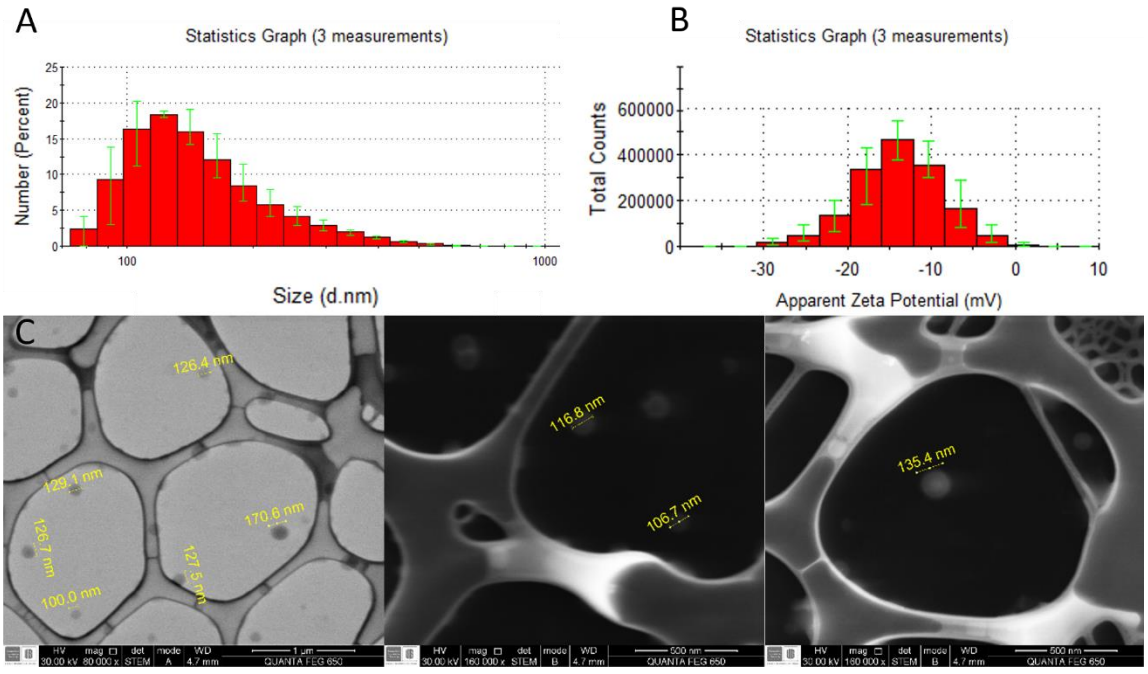

Figure 1 Characterization of FLZ-NPs synthesized by double-emulsion solvent diffusion methodology, (A) Size distribution was measured by DLS, (B) zeta Potential measured by LDE at pH=7.4. (C) Images of SEM (80.000 and 160.000 X). **Abbreviations:** FLZ-NP, Fluconazole

Nanoparticle; DLS, Dynamic Light Dispersion; LDE, Laser Doppler Electrophoresis; SEM, Scanning Electron Microscopy.

2.1.2. Efficiency of FLZ encapsulation

Set of experiments were carried out varying drug/polymer ratios, from 250 up to 2000 µg FLZ/mg PLGA (Figure 2.). Preparation of 1000 µg/mg achieved the best encapsulation efficiency (53%) with a drug loading of de 530µg FLZ/mg PLGA. In the preparation of 1500 µg/mg was obtained the maximum load of FLZ (580 µg/mg); however, a low encapsulation efficiency (38%) was observed.

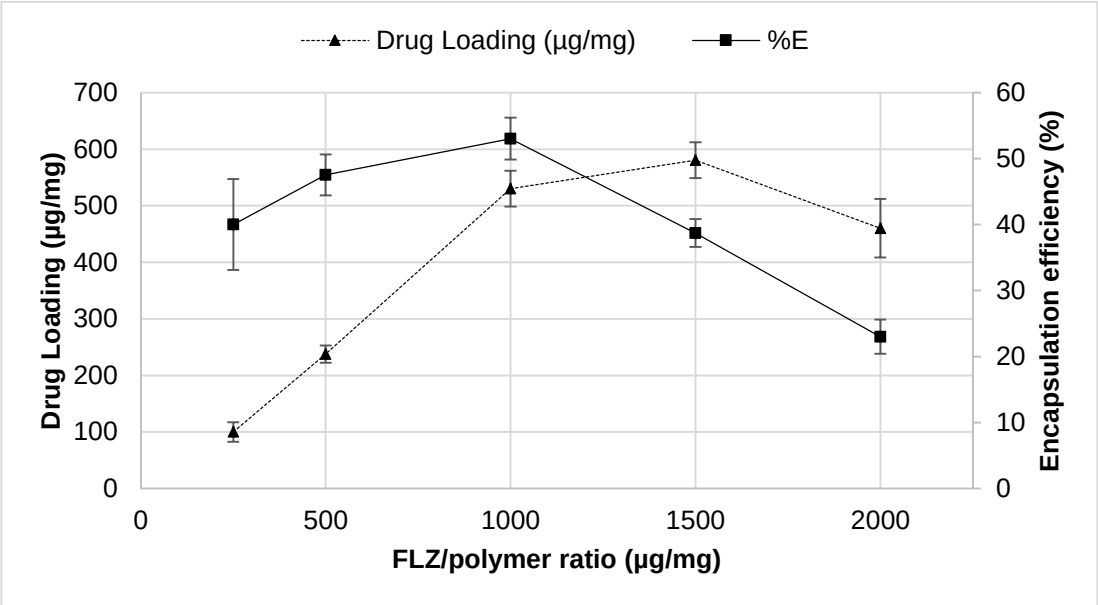

Figure 2 Effect of FLZ/PLGA ratio on encapsulation efficiency of FLZ. Data represent mean ± SD (n=3). Abbreviations: FLZ, Fluconazole; PLGA, poly(lactic-co-glycolic acid).

2.1.3. Delivery profile of FLZ from FLZ-NPs in vitro

A suspension FLZ-NPs was diluted in 50 mL of 0.05M phosphate solution adjusted at pH 7.4 and incubated at 37 °C by 4 h under sink conditions in order to avoid interference of the pharmaceutical solubility with its delivery *in vitro*(Gibaldi and Feldman 1967; Nogueira de Assis et al. 2008). A quick delivery of FLZ was observed in the first 24 min (81.5%), achieving an almost complete delivery of FLZ (≥ 90%) after 3 h (See Figure 3).

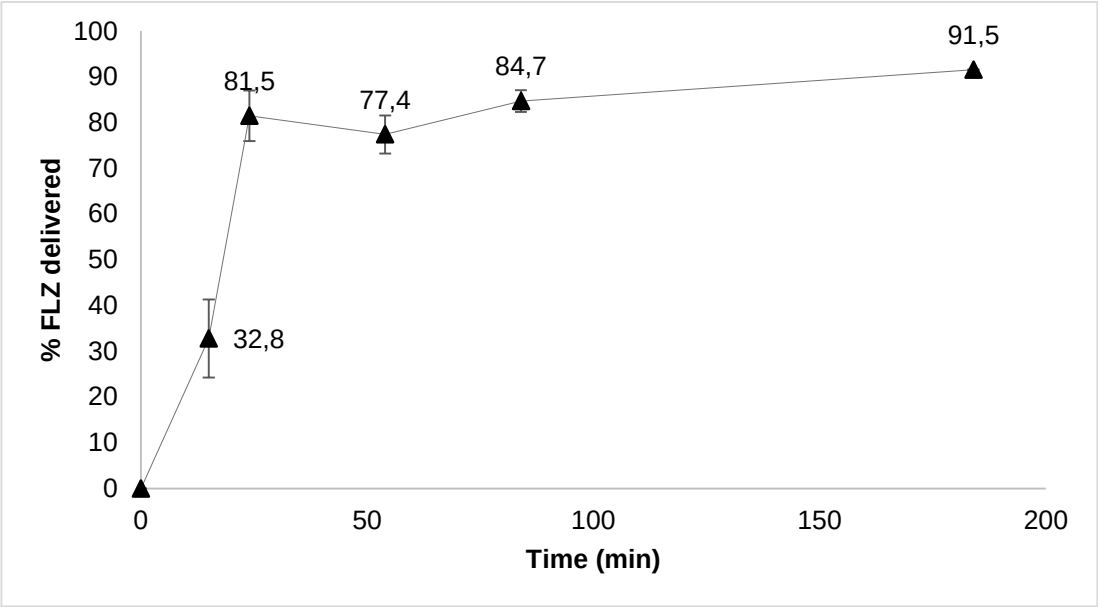

Figure 3 Cumulative Delivery of Fluconazole from PLGA-NPs in vitro (one suspension of FLZ-NPs was dilute 1:50 in 0.05M phosphate solution, pH: 7.4, 37°C).

2.2. MODIFICATION OF SURFACE CHARGE OF FLZ-NPS

Size and charge of FLZ-NPs ranged from -9.93 ± 1.5mV and 210 ± 4.4nm, respectively. An increase in size of NPs was observed when it was added poly-ethylene-imine (PEI), obtaining FLZ-NP-PEIs with mean sizes around 281 ± 6.6 nm. In addition, increasing in positive charge was significant at PEI concentrations of 0.005 and 0.01% (w/v), with values around 11.9 ± 1.2 and 23.5 ± 1.3 mV, respectively. Interestingly, increase in PEI concentration showed a diminishing in positive charge of FLZ-NP-PEI resulting nanoparticles(see figure 4).

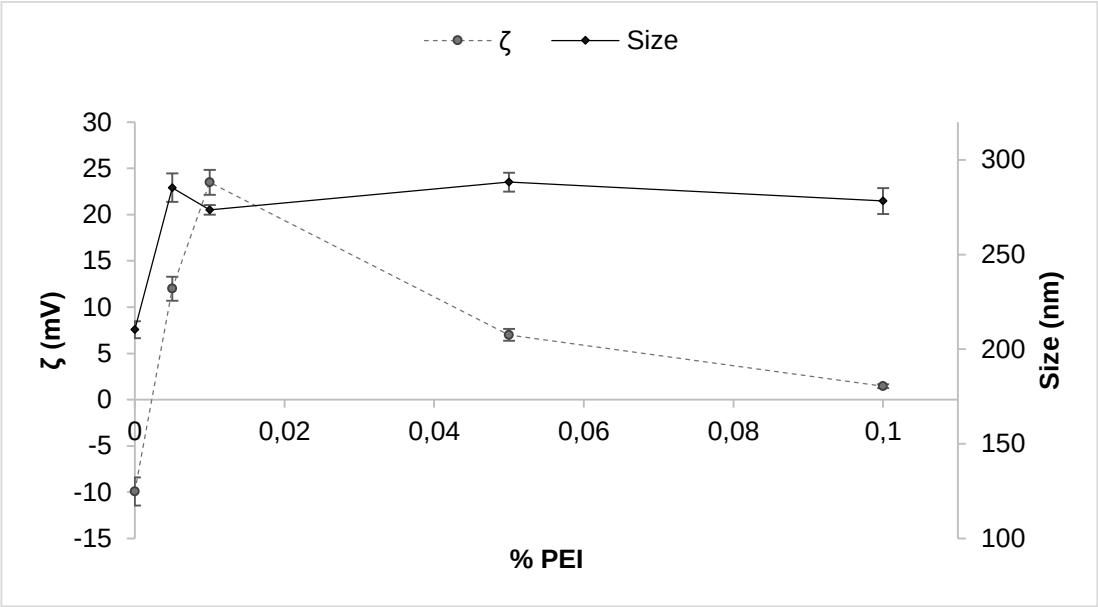

Figure 4 Effect of PEI concentration on size and zeta potential (ζ) of FLZ-NP dissolved on 5 mM Phosphate Solution, pH: 7.4.

2.3. DETERMINATION OF ANTIFUNGAL EFFECT OF FLZ-PLGA NANOPARTICLES

Evaluation of antifungal effect of FLZ-NPs was evaluated by determination of MIC and MFC values, following the methodology proposed by Canton *et al*.(Cantón et al. 2003) and CLSI from document M27-A3(CLSI 2008). We carried out respective controls for four species of *Candida,* using as negative control cultures without FLZ at 24 and 48 h. We also tested possible activity of nanoparticles without FLZ (PLGA-NP y PLGA-NP-PEI) against *Candida spp*., showing no activity at the evaluated concentrations (≤ 64 µg/ml). Finally, we tested the effect of fluconazole, as positive control, and antibiotic activity of FLZ-NPs and FLZ-NP-PEIs varying their concentrations from 0.1 up to 64 µg/mL (See Table 1). Fluconazole sensitive strains of *C. parapsilosis* ATCC 22019 and C. *albicans* ATCC 10231 strains were used as controls (M A Pfaller et al. 1995; Maebashi et al. 2001). Assays with fluconazole achieved MIC of 2 and 1 µg/mL respectively for these strains, while using FLZ-NP obtained a MIC of 0.5 and 0.1 µg/mL, for the respective strains, which means an increasing of 4 and 10 fold on antifungal activity of fluconazole. On the other hand,

FLZ-NP-PEI obtained MIC of 1 and 0.5 µg/mL for the same strains. FLZ-NP-PEIs showed a slightly lower effect on evaluated *Candida* spp compared to FLZ-NPs. We also evaluated activity of nanoparticles on *C. glabrata* and C. *krusei* ATCC 6258, strains with intrinsic and/or acquired resistant to fluconazole, and in general to azole antifungal drugs (M A Pfaller et al. 2001; M. A. Pfaller and Diekema 2007; J. H. Rex, Rinaldi, and Pfaller 1995; John H Rex and Pfaller 2002). MIC values for these strains using only fluconazole were very high, achieving 32 and 64 µg/mL, respectively. Nevertheless, nanoparticles coated with PEI showed great activity against these strains. FLZ-NPs achieved MIC values of 0.1 and 0.5 µg/mL, while for FLZ-NC-PEI was of 0.1 and 2 µg/mL, respectively. Minimum Fungicide Concentrations (MFC) for four *Candida spp strain* using free FLZ and FLZ-NPs did not show detectable activity; however, preparations of FLZ-NP-PEI were fungicide even with resistant strains, obtaining MCF of 4 µg/mL for *C. glabrata* and 8 µg/mL for *C. krusei.*

Table 1 Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC) for fluconazole, FLZ-NP and FLZ-NP-PEI Nanoparticles.

MIC and MFC range of drug (µg/mL)						
Organism	FLZ		FLZ-NP		FLZ-NP-PEI	
	MIC	MFC	MIC	MFC	MIC	MFC
C. parapsilosis ATCC 22019	2	≥ 64	0.5	≥ 64	1	4
C. albicans ATCC 10231	1	≥ 64	0.1	≥ 64	0.5	4
C. glabrata EMLM 14	32	≥ 128	0.1	≥ 128	0.1	4
C. krusei ATCC 6258	64	≥ 128	0.5	≥ 128	2	8

3. DISCUSSION

Selection of encapsulating methodology is a key step for developing new nanostructured drugs, which it depends on the physicochemical properties of pharmaceutical(Cohen-Sela et al. 2009). In spite of there are several methods for encapsulation of azoles, most of them have been focused for incorporation of lipophilic compounds(Winnicka et al. 2011; Winnicka et al. 2012; Pandey et al. 2005; L. Zhang et al. 2010). However, fluconazole is one of little hydrosoluble azoles(Goa and Barradell 1995). For this reason, synthesis of nanoparticles for transport of drugs such as fluconazole is not easily, because it needed to asses diffusion of fluconazole from the nanoparticle core to the bulk medium(Cohen-Sela et al. 2006). In our study, synthesis of nanoparticles by double emulsion solvent-diffusion (DES-D) method demonstrated to be an effective methodology for nanoparticle synthesis. This methodology shows advantages as: reduction of delivery of hydrophilic pharmaceutical during synthesis, use of pharmacologically acceptable organic solvents, and obtaining of nanoparticles with lower sizes can be achieved by this methodology(Cohen-Sela et al. 2009). This is a key factor, because nanoparticles can offer higher advantages than microparticles(McClean et al. 1998). Moreover, most nanoparticles are developed and administrated via parenteral routes, therefore, particle size, poly-dispersion index and surface charge or nanoparticles are very important properties(Tiyaboonchai, Woiszwillo, and Middaugh 2001).

By using of DES-D, we obtained nanoparticles loaded with fluconazole with mean sizes around 222 ± 2.4 nm (Fig. 1A) and spherical morphology (Fig. 1C). These NPs were both approximately 100 nm more little and efficient than other methods of encapsulation of fluconazole previously reported(Nogueira de Assis et al. 2008). Additionally, poly-dispersion index lower than 0.2 is suggesting that technique of synthesis of nanoparticles was reproducible and effective for encapsulation of fluconazole (Vij et al. 2010; Bivas-Benita et al. 2004). On the other hand, zeta potential measurements showed negative values (-11.6 ± 5.13mV), because at pH

7.4, carboxylic groups present in PLGA are highly dissociated. This surface charge is not sufficient to provide good stability of NPs(Hanaor et al. 2012), and it is possible that some aggregation or clustering among NPs could be produced at long term.

Different ratios of drug/polymer (µg FLZ/mg PLGA) used for synthesis of NPs (See Figure 2), showed a saturation curve between 1000-1500 µg FLZ per mg de PLGA, where maximum loading of fluconazole is achieved at 1000 µg FLZ/mg PLGA in terms of encapsulation efficiency. Drug release of nanoparticle was fast (≥81% of fluconazole was released in 24 min). This behavior was similar to those obtained by other authors, which it is produced due to absorption of FLZ in the nanoparticle surface (Nogueira de Assis et al. 2008; Vanessa Carla Mosqueira, Legrand, and Barratt 2006). This indicates that was no really a controlled release of fluconazole, probably because thickness of polymeric shell is not enough to influence an effective control on fluconazole release(V C Mosqueira et al. 2000).

On the other hand, superficial charge of NPs plays an important role in its potential antimicrobial activity. Positive charge higher than +15 mV can extend circulation times in bloodstream(Aoki et al. 1997). In consequences, we explored modification of charge of PLGA nanoparticles using polyethyleneimine (PEI). In Figure 4 is shown effect of different concentrations of PEI on Surface charge (ζ mV) of nanoparticles. Because of PEI is a polyelectrolyte, increase in concentration of PEI causes high ionic strength in the medium, giving a decrease on ionic interactions with the nanoparticle (PLGA contains anionic groups provided by carboxylic groups at neutral pH), which it is reflected in a low zeta potential(Boussif et al. 1995). In this study, higher cationic charges (23.5 ± 1.3 mV) were obtained at 0.01% (w/v) PEI. However, agglomeration of PEI onto nanoparticle surface increased size of NPs around 71 ± 6 nm.

MIC values for different preparations of fluconazole (FLZ, FLZ-NP and FLZ-NP-PEI) using four species of *Candida* showed best results using FLZ incorporated in nanoparticles, both in conventional form and PEI-modified NPs. This can be due to

nanometric size of particles obtained by DES-D methodology and increase in its surface area, giving them greater interaction with bacteria and a consequent high antibiotic activity(Nirmala, Mukherjee, and Chandrasekaran 2013). These findings are similar to those obtained by other authors with FLZ using different transport mechanism such as cubesomes and liposomes on *Candida sp.*(Prajapati et al. 2014; Zhao, Du, and Cao 2007; Habib et al. 2010). Analyses of MFC of free FLZ and FLZ-NPs were not effective against *Candida* sp at the assayed concentrations of fluconazole, because azoles as FLZ have a fungi-static effect on *Candida sp., due to its inhibitory activity on* the enzyme 14α-esterol demethylase is reversible(Calabrese et al. 2013; Manavathu, Cutright, and Chandrasekar 1998). Therefore, extensive use of FLZ with therapeutic goals, as prophylaxis, have favored rising of resistant strains(Rogers 2006).

On the other hand, studies with FLZ-NP-PEI achieved considerable fungicide activity, even in the fluconazole resistant strains *C. glabrata* and *C. krusei*. This fungicide effect can be produced by increase of electrostatic interactions between *Candida* and positive nanoparticles (FLZ-NP-PEI), because *Candida* sp. has a negative superficial charge envelope(Henriques, Azeredo, and Oliveira 2004). It is possible that FLZ and PEI can act synergistically, increasing antibiotic activity, because PEI can electrostatically modify surface of the fungi, increasing cationic charges of the fungi, changing from negative to positive ones(Vieira and Carmona-Ribeiro 2006). On the other hand, these cationic compounds can induce ruptures on the cell membrane at nanometric scales. This effect could facilitate uptake of the drug in the cell, causing the fungicide effect(Tao et al. 2007; Chen et al. 2009).

4. CONCLUSION

In conclusion, polymeric nanoparticles used in this study can be a viable alternative for improving antifungal activity of FLZ against *Candida sp.*, even in strains with intrinsic resistance against this antibiotic. Finally, use of PEI achieved to modify zeta potential of nanoparticles, obtaining a nanocomposite with great fungicide activity. *In vitro* activity of two types of nanoparticles (FLZ-NP and FLZ-NP-PEI) was higher compared to free fluconazole on four strains of *Candida sp*, which it is indicating that these nanocompounds represent good candidates for further studies of antifungal activity *in vivo*.

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