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**Research Article**

# EVALUATION OF THE ANTIBIOFILM EFFECT OF FLUCONAZOLE LOADED PLGA NANOPARTICLES PREPARED USING RHAMNOLIPID ON *Candida albicans*

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**Abstract:** In this study, fluconazole (FLZ) loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) were prepared with two different formulations consisting of polyvinyl alcohol (PVA) and PVA-rhamnolipid (R) in order to improve antibiofilm activity against *Candida albicans* ATCC 90028. The encapsulation efficiency, drug loading capacity, in-vitro release, characterization and antibiofilm activity of these formulations were compared. Characterization of NPs were analyzed by scanning electron microscopy (SEM) and Zetasizer. Drug loading capacity and encapsulation efficiency percentages were measured by spectrophotometric method. PLGA-NPs were spherical in shape with mean sizes of ~300 nm and surface charge of FLZ loaded PVA and PVA-R-PLGA NPs -25,9±1.99, -48,1±2.46, respectively. Sustained release of FLZ (≥60% after 6 h) were obtained in PVA-R PLGA-NPs. The encapsulation efficiency percentages of PVA-FLZ-PLGA and PVA-R-FLZ-PLGA were 50% and 85%, respectively. Antibiofilm inhibition percentages are 55% and 63%, respectively. These results show that the PVA-R-FLZ-PLGA drug delivery system is a new therapeutic approach that can be used in infections caused by *C. albicans.*

**Özet:** Bu çalışmada, flukonazol (FLZ) yüklü poli(laktik-ko-glikolik asit) (PLGA) nanopartikülleri (NP'ler), *Candida albicans* ATCC 90028'e karşı antibiyofilm aktiviteyi artırmak için polivinil alkol (PVA) ve PVA-rhamnolipid (R)'den oluşan iki farklı formülasyon ile hazırlandı. Bu formülasyonların enkapsülasyon etkinliği, ilaç yükleme kapasitesi, in vitro salınım, karakterizasyonu ve antibiyofilm aktivitesi karşılaştırıldı. NP'lerin karakterizasyonu, taramalı elektron mikroskobu (SEM) ve Zetasizer ile analiz edildi. İlaç yükleme kapasitesi ve enkapsülasyon etkinliği yüzdeleri, spektrofotometrik yöntemle ölçüldü. PLGA-NP'ler, ortalama büyüklükleri ~300 nm, küresel şekilli ve FLZ yüklü PVA ve PVA-R-PLGA-NP'lerin yüzey yükü sırasıyla -25,9±1.99, -48,1±2.46'dır. PVA-R-PLGA-NP'lerde sürekli FLZ salınımı (6 saat sonra >%60) elde edildi. PVA-FLZ-PLGA ve PVA-R-FLZ-PLGA'nın enkapsülasyon etkinliği yüzdeleri sırasıyla %50 ve %85'tir. Antibiyofilm inhibisyon yüzdeleri sırasıyla %55 ve %63'tür. Bu sonuçlar, PVA-R-FLZ-PLGA ilaç taşıma sisteminin *C. albicans*'ın neden olduğu enfeksiyonlarda kullanılabilecek yeni bir tedavi yaklaşımı olduğunu göstermektedir.

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*Sustained release*
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*Biofilm*

## Introduction

*Candida* species are among the most commonly isolated human fungal pathogens. The main colonization site of *Candida* members is the gastrointestinal tract from the mouth to the rectum, but they can also be found commensally in the vagina, urethra, skin, fingers and toes. In addition to humans, *Candida* can survive in the digestive system of animals and in air, water and soil. Candidiasis are endogenous or exogenous infections that occur when the normal flora of the host is under conditions suitable for infection. The incidence of invasive *Candida* infections has increased significantly in recent years. Although there are more than 200 *Candida* species described, the most commonly isolated agents from invasive fungal infections are *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* and *C. krusei* (Hazen & Howell 2003). Despite the increase in the prevalence of non-albicans *Candida* species in recent years, *C. albicans* is still the major pathogen of the genus due to its high adaptability. The current treatment approaches in fight against candidal diseases include the use of natural compounds, antifungal agents and their combinations, and photodynamic therapy. The success of

antifungal therapy depends not only on the drugs used, but also on host-specific factors such as pH of the environment, cellular barriers, and degrading enzymes that hinder drugs from reaching their maximum effectiveness. The emergence of fungal resistance to traditional antifungals has led to difficulties in the treatment of candidiasis and candidal biofilms. Candidal biofilms are complex microbial communities attached to a surface in an extracellular matrix (ECM) and have become a threat to human health. Therefore, it is important to develop new therapeutic approaches against candidal biofilms (de Barros *et al*. 2020).

Nanobiotechnology-based drug delivery systems have become attractive research targets in the last few decades with their advantages such as maximizing bioavailability, targeting molecules and reducing side effects. Nanoparticles are seen as potential sources for drug delivery systems due to their small particle size, ability to easily cross biological barriers and openness to modifications. It has been proven that the delivery of antifungal compounds through nanoparticles and that metallic nanoparticles have direct antibiofilm/antifungal effects against *Candida* (Ahmed *et al.* 2019, Vangijzegem *et al.* 2019, Wani & Ahmad 2013, Yenice Gürsu 2020). Polymeric drug delivery system was chosen in our study because polymers are biodegradable, compatible with biological systems, not toxic, and their physicochemical properties can be controlled by production variables. Researchers have proven that the use of PLGA (poly lactic-co-glycolic acid) nanoparticles is ideal for both antibiotic-derived drugs and proteins (Cheow & Hadinoto 2012, Water *et al.* 2015). PLGA is a safe, clinically approved (FDA), biodegradable and biocompatible polymer for drug delivery systems (Lü *et al*. 2009). It is a polyester composed of one or more different hydroxy acid monomers, D-lactic, L-lactic and/or glycolic acids. PLGA nanoparticles can be synthesized with different physicochemical properties (size, morphology, size distribution, zeta potential) by keeping the preparation method under control.

The use and selection of a stabilizer, that is, a surfactant, plays a critical role in the formulation process of the nano-suspension. The stabilizer provides a stable formulation by providing steric or ionic barriers to prevent aggregation of nanoparticles. Povidones, cellulosics, polysorbates, poloxamers and lecithin are the most studied stabilizers (Nakarani *et al.* 2010). The choice of stabilizer to be used in the drug delivery system and the optimum concentration have important effects in terms of particle size, size distribution, zeta potential and encapsulation efficiency (Türk *et al*. 2009, Menon *et al.* 2012, Sharma *et al*. 2016). Researchers have focused on the existence of new surfactants and increasing the activity of this system in the formulation process of the nano-suspension. For this purpose, rhamnolipid, a biosurfactant, will be used in addition to PVA in our study. It has been known that rhamnolipid is used as a drug encapsulation agent, surfactant and as a release triggering molecule in the drug delivery system (Cheow & Hadinoto 2012, Yi *et al.* 2019, Lee *et al.* 2021). However, there is no study on the use of rhamnolipid as a cosurfactant in the drug delivery system against Candidal biofilm.

Here, we describe a new formulation to prepare PLGA-NPs using rhamnolipid as cosurfactant. NP formulations were evaluated in terms of surface charge, size, appropriate release profile, antibiofilm efficacy and NP formulation with rhamnolipid surfactant was found to be an attractive candidate for inhibit Candidal biofilm.

## Materials and Methods

*Materials and microorganisms*

FLZ (98%), PLGA (MW=76000-115000) and PVA (MW=30000-70000) were purchased from Sigma Aldrich. Ethyl acetate(EtAc) and dimethyl sulfoxide (DMSO) (HPLC grade) were obtained from Fisher Scientific Co. *Candida albicans* ATCC 90028 was used as the reference pathogen and was maintained in potato dextrose agar (PDA) at 30°C. XTT kit was purchased in biological industries (BI).

*Preparation of NPs (PLGA-NPs)*

NP formulations were prepared with two different surfactant systems, PVA and PVA-rhamnolipid (1:1). The formulations were prepared by the solvent-evaporation-method with some modifications. Briefly, 5 mg FLZ 0.5 mL of 5 mM phosphate buffer (pH =7.4) and DMSO (5:1) was emulsified in 0.9 mL of EtAc containing 10 mg PLGA (75:25) using a homogenizer-disperser (IKA Ultra-Turrax T-18), at 20.000 rpm for 30 s. The mixture was added dropwise to 1 mL aqueous solution containing 1% (w/v) PVA/PVA-R at 20.000 rpm. EtAc was removed by evaporation at 500 mbar for 2 min using a rotary evaporator. To eliminate the free FLZ from NP preparation, NPs containing FLZ (FLZ-NP) were centrifuged at 14.000 rpm for 60 min at +4°C. The pellet was washed three times with distilled water (Gómez-Sequeda *et al.* 2017). The prepared formulations were used freshly in future experiments.

*Particle morphology by SEM*

Morphology of NPs was observed by SEM (SEM-Quanta FEG 250). The zone magnification for the image was kept around 8000×. Measurements were performed under 1 and 3 kV (Kalam *et al.* 2017).

*Particle size, polydispersity and zeta-potential measurement*

The mean particle size (d.nm), polydispersity index (PDI) and zeta-potential of the NP formulations were determined by a Zetasizer Nano ZSP (Malvern Instruments, UK). Dynamic light scattering (DLS) function was used to determine the particle size and size distribution (PDI) of NPs at 20 °C after appropriate dilution. Laser Doppler Velocimetry (LDV) function of the same device was used for measure of zeta potential (mV) of the NPs after an appropriate dilution.

*Encapsulation efficiency (EE%) and drug loading capacity (DL%)*

EE% and DL% of NPs were measured by centrifuged suspension of NPs at 14.000 rpm for 60 min at +4°C. Then, the supernatant was analyzed spectrophotometrically at 260 nm and the amount of FLZ was measured using a calibration curve of FLZ. The EE% and DL% were measured by using the following equations (Shafique *et al.* 2017).

$$EE\% = \frac{(\text{amount of FLZ loaded in NP(mg)})}{(\text{total amount of FLZ used(mg)})} * 100$$

$$DL\% = \frac{(\text{amount of FLZ loaded in NP(mg)})}{(\text{total amount of formulation(mg)})} * 100$$

*In vitro drug release*

The in vitro release assay of Gómez-Sequeda *et al.* (2017) was performed with some modifications in the protocol. Briefly, 2 mL of FLZ-NP was dispersed in the dialysis membrane (MWCO=12kDa). Then, the NPs were released into 20 mL of PBS (pH=7.4) and were stirred in a shaker at 37°C, 100 rpm. NPs were analyzed spectrophotometrically at 260 nm at specified time intervals (0, 6, 12, 24, 48, 72 hours.).

*Antibiofilm activity*

Antibiofilm activity test was determined with the XTT (2,3-bis (2-methoxy-4-nitro-5- sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction test. XTT test is used to test cell proliferation in respect to different growth factors. This test depends on the reduction of the tetrazolium salt XTT to orange colored formazan compounds by metabolically active cells. Antibiofilm test was performed with some modifications. *Candida* cells were suspended at $10^7$ cells/mL in YPG (yeast-pepton-glucose) medium. Then, 100 µL of the suspension was added into each well of polystyrene 96-well plates and incubated at 37°C, 75 rpm for 1.5 hour to prevent cell adherence. At the end of the incubation period, the medium was removed and 100 µL medium and NP formulations (loaded FLZ-NP, free-FLZ, free-NP) were added to the wells. Antibiofilm activities of PVA and PVA-R-NP were determined after 48 h of incubation. To stain, wells were washed with 200 µL of PBS to eliminate the planktonic cells, then 100 µL of PBS and 10 µL XTT solution were added to each well. Plate was incubated at 37°C for 3 h in the dark and the colour change was measured at 490 nm by a microtiter plate reader. Untreated *Candida* cells are used as control. The biofilm %inhibition was measured by using the following equations (Alteriis *et al.* 2018).

$$\%\text{inhibition} = \left(\frac{(\text{untreated candida cells} - \text{treated candida cells})}{(\text{untreated candida cells})}\right) 490\text{nm} * 100$$

*Statistics*

The results were analyzed as mean ± SD and tested for statistical significance ($p < 0.05$) by two-way ANOVA (GraphPad Software Inc. San Digeo, USA).

## Results

*Physicochemical characterization of NPs*

NP formulations were characterized by SEM and zetasizer to determine mean size, zeta potential, PDI and morphology of NPs (Fig. 1). PLGA NPs were observed in spherical morphology.

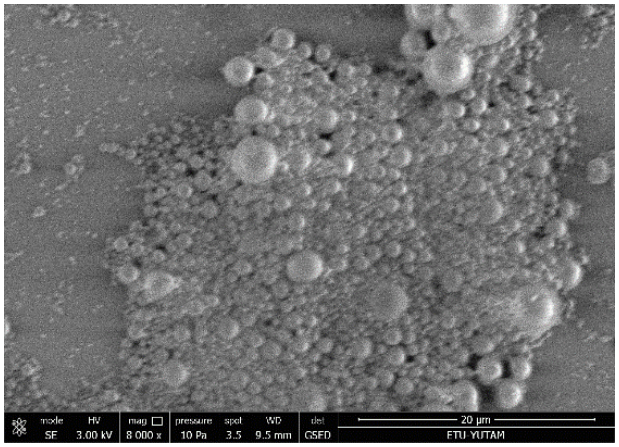


**Fig. 1.** SEM image showing the morphology of PLGA NP

ZP, PDI, d.nm values of the NP formulations are shown in Table 1. The formulations prepared with PVA and PVA-R surfactant were compared with flucanozole loaded and unloaded.

**Table 1.** Zeta potential, polydispersity index, and % mean particle size of NP formulations (n = 3).

| Formulations | ZP ± SD | PDI ± SD | d.nm ± SD |
|---|---|---|---|
| **PVA-NP** | -23.2±0.7 | 0.409±0.011 | 959.2±55.2 |
| **PVA-R-NP** | -40.9±7.39 | 0.497±0.042 | 374.2±95.68 |
| **FLZ-PVA-NP** | -25.9±1.99 | 0.291±0.032 | 602.3±56.65 |
| **FLZ-PVA-R-NP** | -48.1±2.46 | 0.192±0.037 | 265.4±7.324 |

**Note:** Results were represented as mean ± SD, n = 3
**Abbreviations:** ZP, zeta potential; PDI, polydispersity index; d.nm, mean size.

*Encapsulation efficiency and drug loading capacity of FLZ*

The addition of rhamnolipid to the nanosuspension prepared with PVA surfactant increased the EE% and DD% amount by 35%, 11%, respectively (Table 2).

**Table 2.** EE% and DL% value of FLZ-PVA-NP and FLZ-PVA-R-NP.

| | EE% ± SD | DL% ± SD |
|---|---|---|
| **FLZ-PVA-NP** | **50±3.82** | **17±1.21** |
| **FLZ-PVA-R-NP** | **85±2.43** | **28±2.22** |

**Note:** Results were represented as mean ± SD, n = 3
**Abbreviations:** EE, Encapsulation efficiency; DL, drug loading capacity.

*In vitro drug release*

Drug release profiles of FLZ loaded nanoparticles prepared with PVA and PVA-R surfactants were determined with an in vitro release test (Fig. 2). It was found that PVA and PVA-R PLGA-NPs exhibited biphasic release in PBS, with an initial burst release of about 45% and 49% after 6 h, respectively. The second phase was a stable release profile with about 62% and 59% of drug released for 72 h, respectively.

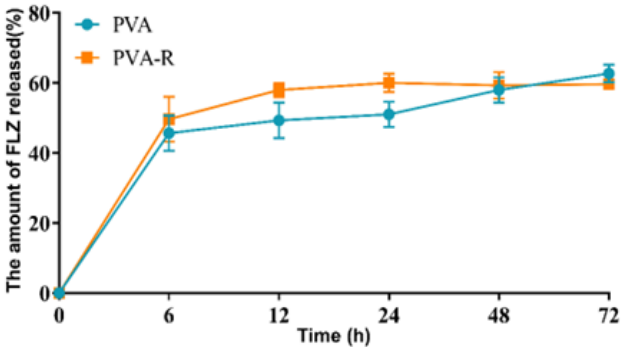


**Fig. 2.** In vitro drug release profiles of FLZ loaded-NPs. Results were represented as mean ± SD, n = 3.

***Abbreviations:** PVA, PLGA-NP prepared with PVA surfactant; PVA-R, PLGA-NP prepared with PVA-R surfactant.

*Antibiofilm activity of PLGA NPs*

The results of XTT assay revealed the high potential of the FLZ loaded PVA and PVA-R-NPs for inhibition and treatment of *C. albicans* biofilms compared to free FLZ and NPs. In our experiments, free NPs show a lower activity compared to free-FLZ on *Candida* biofilm formation; these results suggest that PVA/PVA-R PLGA NPs enhance FLZ activity rather than inhibiting biofilm formation directly (Fig. 3).

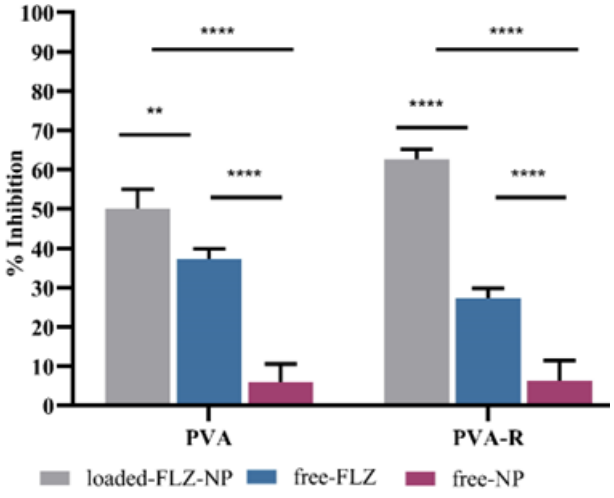


**Fig. 3.** Efficiency of biofilm inhibition of PVA and PVA-R NPs.

***Abbreviations:** PVA, PLGA-NP prepared with PVA surfactant; PVA-R, PLGA-NP prepared with PVA-R surfactant

Results were represented as mean ± SD, n = 3. Statistically significant differences between groups: $^{**}P<0.0067$, $^{****}P<0.0001$ (two-way ANOVA).

## Discussion

The zeta potential is defined as the electrical potential of molecules at the slipping plane. The zeta potential of a nanosuspension provides information about stabilization. The zeta potential of a nanosuspension is governed by the stabilizer, encapsulator, and drug. The zeta potential value suitable for nanoemulsions and drug delivery is ±30 mV (Müller & Jacobs 2002). It was observed that drug-free and drug-loaded formulations prepared with rhamnolipid surfactant of nanoparticle formulations gave better ZP values than formulations prepared without the cosurfactant. The results confirm that rhamnolipid cosurfactant is a good choice for stability. The difference between the drug-loaded and non-drug-loaded groups clearly showed that the presence of the FLZ on the surface did not significantly modify the charge of the NPs. PDI is a parameter to evaluate the physical stability of nanosuspensions and should be as low as possible for long-term stability. The value of PDI should be between 0 and 1 for homogeneous dispersion of nanoparticles, and values of 0.2 and below are considered the most commonly suitable for polymer-based nanoparticle materials in practice (Patravale *et al.* 2004). Our results showed that the FLZ-loaded formulations had PDI values of 0.2 and below. Our experiment results demonstrated the suitability of FLZ-loaded PLGA nanoparticles for polymeric drug delivery.The average nanoparticle sizes should be in the range of 10-100 nm, but this value can reach up to 1000 nm for drug delivery studies. From this perspective, it was determined that nanoparticle formulations have suitable size properties. It was also measured that the formulations prepared with rhamnolipid surfactant were approximately 337 nm and 585 nm smaller size in drug-free and drug-loaded formulations, respectively. The lower size of PVA-R-NPs compared to other formulations can be explained by the addition of rhamnolipid surfactant, the ability of this surfactant to form droplets between the water and organic phase and emulsify with its cohesive effect. In the study of Lee *et al.* (2021), rhamnolipid-coated PLGA nanoparticles were prepared by double emulsion method to be delivered to tumor tissues. The formulation using rhamnolipid reduced the nanoparticle size by approximately 128 nm. This result highlights the suitability of rhamnolipid for preparing suitable nanocarrier systems. For nanobiotechnological drug delivery, nanoparticles with appropriate characterization should have uniform size distribution, spherical morphology and high zeta-potential value. Our results provided clear support for the appropriate nanobiotechnology drug delivery system. In the study of Shi et al. (2016), the anticancer properties of tetrandrine loaded PLGA nanoparticles prepared without the use of surfactants were investigated. It has been shown that they do not show much different effects from PLGA nanoparticles prepared using PVA surfactant as size, efficacy, drug release, but have much more negative values in zeta potential (Shi *et al.* 2016). This result supports that the surfactant to be used in the drug delivery system, as mentioned above, significantly affects the

stabilization. Menon *et al.* (2012) evaluated the efficacy of doxorubicin (DOX) loaded PLGA nanoparticles in the presence of PVA and pluronic surfactants and found that dox-pluronic-PLGA nanoparticles kill more cancer cells. In these studies, it was determined that the use and selection of surfactants for stabilization of the nanocarrier system increased the therapeutic efficacy by increasing the percentage of drug encapsulation. In another study, particle size, encapsulation efficiency and in vitro drug release kinetics were evaluated by including a co-surfactant in the drug delivery system to reduce particle size. The insertion of vitamin E TPGS, Poloxamer 188 or Tween 80 in addition to the PVA surfactant has been observed to reduce the particle size from 438 nm to 230, 244 or 301 nm, respectively. As the encapsulation efficiency increased to 0.010% and 0.25% concentrations, respectively, for both Vitamin E TPGS and Poloxamer 188 (Sharma *et al.* 2016). In another study, it was determined that the percentage of drug encapsulation increased by 10% when the surfactant concentration was increased by 2 times (Pezeshki *et al*. 2014). The concentrations of surfactants are determined by their critical micelle concentration in the nanoemulsion. Since these surfactants used in our study were used according to this value, optimization study was not performed. FLZ release from PLGA NPs showed biphasic release, burst release and second phase (Fig. 2). Burst effect is commonly known as the release of the drug present near the nanoparticle surface. After the burst release, it was observed that the stable release profile for both nanoparticle formulations continued with nanoparticle degradation and drug diffusion. Based on these results, it is conceivable that after 6 hours of burst release, PVA-R-FLZ-NPs can deliver a steady dose of drug for 3 days. In drug delivery studies, the second phase of release is used to increase the biological half-life and decrease the dosing frequency. The second phase release profiles of PVA-FLZ-NPs were found to be less stable compared to the formulation prepared with PVA-R. In the study of Gómez-Sequeda et al. (2017), the release profile of FLZ-PLGA-NPs prepared according to the double emulsion solvent-diffusion method with poloxamer 407 surfactant was investigated. A burst release was observed in the first 24 minutes (81.5%) and after 3 hours an almost complete FLZ release (≥90%) was observed, indicating no controlled release. These results showed that the addition of rhamnolipid to the drug-loaded nanoparticle formulation increased drug release and resulted in a more appropriate release profile in our study. The drug release profiles of other antifungal drugs prepared by similar methods also consist of two phases as burst and steady release, and their release times vary according to the drug-polymer interaction (Alhowyan *et al.* 2019, Sadozai *et al.* 2020).

Free FLZ can be degraded between cells by the action of various molecules. However, the encapsulation of FLZ with PLGA nanoparticles gives it stability. Antibiofilm inhibition percentages of PVA and PVA-R PLGA NPs were 55% and 63%, respectively (Fig. 3). The significant difference ($P$<0.0001) between free NP and loaded-FLZ-NP shows that this effect is not directly caused by rhamnolipid, but by the formulation used as a cosurfactant. In our previous experiment, PVA and PVA-R-FLZ-NPs showed a sustained release of 62% and 59%, respectively, after 6 hours of burst release according to the in vitro release test, indicating that not all of the encapsulated amount was released. Considering that the amount of PVA/PVA-R-FLZ-NPs showing antibiofilm activity is approximately 60% FLZ, it has been determined that the efficiency will increase more by making modifications that increase the release. According to these results, PVA-FLZ-NPs showed 18% and PVA-R-FLZ-NPs 36% more antibiofilm activity compared to free FLZ at the same concentration. Both antifungal molecules and NPs of different structure (organic, inorganic, or hybrid) are potential sources for combating candidal biofilm. On the other hand, increasing cell penetration, targeting and controlled release of NPs increase the antibiofilm activity of antifungal molecules (Han *et al.* 2017). Alteriis *et al.* (2018) investigated a new nanosystem as indolicidin-loaded gold nanoparticles (AuNPs-indolicidin) against pathogenic *Candida albicans* biofilms. According to the results, AuNPs-indolicidin showed the effect of inhibiting early biofilm formation and eradicating mature biofilms. AuNPs-indolicidin showed 60% greater antibiofilm inhibition properties against a *Candida* strain versus indolicidin alone. Another study evaluating the antibiofilm activity of farnesol-loaded poly(DL-lactide-co-glycolide) (PLGA) nanoparticles against *Candida albicans* showed that F-PLGA NPs could reduce biofilm formation by 57% at lower concentrations than farnesol alone (Yenice Gürsu 2020). Although our test results are similar to the examples in the literature, the antibiofilm activity varies according to the drug-encapsulating substance interaction and concentration.

## Conclusion

Surfactants used in drug delivery systems are very important in terms of stabilization and efficiency of the system. In this study, the effects of rhamnolipid surfactant with PVA on FLZ-loaded-PLGA-NP as zeta potential, PDI, size, in vitro release, drug encapsulation efficiency, and antibiofilm activity were evaluated. As a result, PVA-R-FLZ-NPs with appropriate properties compared to PVA-FLZ-NPs inhibited candidal biofilms as a new drug delivery formulation. Our findings support that PVA-R-FLZ-NPs have attractive source for nanocarrier system. In summary, a new formulation is presented for the first time using rhamnolipid cosurfactant for the treatment of candidal biofilms.

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**Author Contributions:** Concept: A.Ü., S.Ö., Desing: A.Ü., S.Ö., Execution: A.Ü., S.Ö., Material supplying: S.Ö., Data acquisition: A.Ü., S.Ö., Data analysis/interpretation: A.Ü., S.Ö., Writing: A.Ü., S.Ö., Critical revision: A.Ü., S.Ö.

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