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Efficacy of ferulic acid encapsulated chitosan nanoparticles against *Candida albicans* biofilm

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ABSTRACT

Candida albicans, an opportunistic fungal pathogen is a major causative agent of superficial to systemic life-threating biofilm infections on indwelling medical devices. These biofilms acts as double edge swords owing to their resistance towards antibiotics and immunological barriers. To overcome this threat ferulic acid encapsulated chitosan nanoparticles (FA-CSNPs) were formulated to assess its efficacy as an antibiofilm agent against *C. albicans*. These FA-CSNPs were synthesized using ionotropic gelation method and observed through field emission scanning electron microscopy (FESEM) and fluorescent microscopy. Assessment of successful encapsulation and stability of ferulic acid into chitosan nanoparticles was made using Fourier transform infrared spectrum (FTIR), ^{1}H NMR and thermal analyses. Synthesized FA-CSNPs, were found to be cytocompatible, when tested using Human Embryonic Kidney (HEK-293) cell lines. XTT assay revealed that FA-CSNPs reduced the cell metabolic activity of *C. albicans* upto 22.5% as compared to native ferulic acid (63%) and unloaded CSNPs (88%) after 24 h incubation. Disruption of *C. albicans* biofilm architecture was visualized by FESEM. Results highlighted the potential of FA-CSNPs to be used as an effective alternative to the conventional antifungal therapeutics.

1. Introduction

Candida species present one of the leading causes of hospital acquired systemic infections, displaying striking ability to form drug-resistant biofilms on surgical implants such as an intravascular or urinary catheter, prosthetic heart valves, cardiac pacemakers and joint replacements [1,2]. Biofilms are microbial communities that are often embedded in a matrix of slimy extracellular polymers and are significantly less susceptible to antifungal drugs, becoming the foremost cause of virulence associated with *Candida* species. Within entire *Candida* genus, *Candida albicans* is the major species causing life-threatening infections among immunocompromised individuals like HIV, patients relying on various kinds of implants and cytotoxic chemotherapies [3]. *C. albicans* represents second highest colonization-to-infection rate and the overall highest crude mortality [4]. *C. albicans* biofilms are reported to be 4000 times more resistant to antifungal drug fluconazole when compared to planktonic or free-floating counterparts [5]. As a result, biofilm community will survive, disseminating candidemia and leading to device removal in some cases [1,6]. Hence, there is an urgent need to search alternatives to the conventional drugs so as to combat *C. albicans* biofilm. Natural biological molecules are currently being evaluated for their antibiofilm activity in order to develop alternative preventive or therapeutic rationale. One such alternative is provided by plant derived polyphenols which interacts with nucleophilic groups present in *Candida* cell membrane and causes its disruption [7]. An important plant phenolic compound, ferulic acid (FA) and its derivatives have been screened recently for their antibiofilm potential against *C. albicans* [8,9]. As compared to conventional synthetic antifungal drugs that have become unyielding towards the biofilm matrix [10], there is a less likelihood of the development of resistance by biofilm cells against naturally occurring FA. However, applicability of FA is limited owing to its low permeability and instability [11]. This can be circumvented by FA nano-encapsulation into chitosan which is a biocompatible, biodegradable and selectively non-toxic polymer [12,13]. Earlier, studies have reported the antifungal activity of chitosan-pentasodium tripolyphosphte (CS-TPP) nanoparticle against *C. albicans* [14]. Chitosan based zinc oxide NPs also have been synthesized and evaluated for antimicrobial and antibiofilm potential against various microbial strains including *C. albicans* [15].

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In the present study, FA encapsulated CSNPs were prepared by ionic gelation method, which is a low cost and environment friendly technique. We hypothesized that FA-CSNPs could easily penetrate the biofilm cells and matrix owing to their high surface area to volume ratio and disrupt or alter the permeability of fungal cell plasma membrane, as well as reduce the cell population. Synthesized FA-CSNPs were characterized using various biophysical techniques such as FTIR, thermogravimetric (TG) and differential scanning calorimetric (DSC) analyses and their stability was assessed using ^{1}H NMR technique. The biocompatibility of FA-CSNPs with HEK-293 cell lines was confirmed by MTT assay, FESEM and fluorescent microscopy.

2. Experimental

2.1. Materials

Medium molecular weight chitosan (190–310 kDa) with 75–85% deacetylation degree, heat inactivated fetal calf serum (FBS), XTT (2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium salt), RPMI 1640 medium, glutamine, TPP, penicillin–streptomycin, EDTA and trypsin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Cell culture-grade dimethyl sulfoxide (DMSO), ferulic acid, acridineorange (AO), ethidium bromide (EtBr), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), gluteraldehyde and yeast peptone dextrose (YPD) were purchased from Himedia (India). HEK-293 cells lines were procured from National Centre for Cell Science (NCCS) Pune, India. All reagents were used without further purification.

2.2. Preparation of solutions

Chitosan solution (1% w/v, 40 ml) was prepared by dissolving chitosan in 1% acetic acid using a magnetic stirring at ambient temperature. Varying concentrations of ferulic acid viz. 0.0264 (FA_1), 0.0528 (FA_2), 0.1056 (FA_3), 0.2112 (FA_4), 0.4224 (FA_5) mg/ml were used to prepare different nanoparticles' formulation as well as native ferulic acid solution in 2% DMSO. For XTT assay, stock solution of menadione (10 mM) was prepared by dissolving 0.017 g of menadione in 10 ml acetone.

2.3. Synthesis of nanoparticles

Ferulic acid encapsulated chitosan nanoparticles were prepared by modifying an earlier described protocol [16]. Briefly, 10 μl each of varying FA conc. as mentioned above was added to 1 ml chitosan solution (1% w/v) with intermittent vortexing (MX-F, Dragon Lab instruments, China) to obtain different nanoparticles' formulations (NF_1, NF_2, NF_3, NF_4, NF_5). To this mixture, 0.25 ml TPP (1 mg/ml) was added drop-wise to yield FA encapsulated chitosan nanoparticles. Unloaded (without ferulic acid encapsulation) CSNPs were prepared under similar conditions by adding TPP to the chitosan solution. Nanoparticles thus obtained were centrifuged at 40,000 g for 30 min at 4 °C, pellet rinsed with distilled water and freeze dried. To determine the encapsulation efficiency (EE) of nanoparticles, supernatant was collected and non-encapsulated ferulic acid was quantified spectrophotometriclly at 319 nm using FA standard curve (Lasany double beam LI-2800). The EE (%) was calculated by using the equation:

$$\text{EE}\ (\%) = (\text{Total FA loaded} - \text{non-encapsulated FA/Total FA loaded}) \times 100$$

FA extinction coefficient (ε) was calculated using Beer–Lambert equation:

$$\varepsilon_{319} = A/(c \cdot b)$$

where, ε_{319} is extinction coefficient at 319 nm; A is absorbance; c is concentration of native FA (mol/L); b is thickness of cuvette (1 cm).

After freeze drying, a small fraction of FA-CSNPs was desiccated under vacuum for 180 days in order to assess their stability in terms of particle size, zeta potential and polydisperity index. Autoflorescence property of FA was utilized in order to validate its encapsulation into chitosan nanoparticles using fluorescent microscope equipped with GFP light cube at 470–510 nm (EVOS-FL,AMG,USA).

2.4. Physicochemical characterization of nanoparticles

In order to measure the mean particle size and zeta potentials, 500 μg/ml nanoparticles (de-ionized distilled water) were analyzed at 25 °C with scattering angle of 90° (Malvern zetasizer, Malvern Instruments Ltd., U.K.). To determine nanoparticles' morphology by scanning electron microscopy, (FESEM; Quanta 200F Model, FEI, Netherland) samples were immobilized on the surface of glass slides followed by 1 min gold coating (sputter coater: Biotech SC005, Switzerland). FTIR spectra were obtained using a spectrometer (FTIR; Thermo Nicolet Nexus 6700, US). An average of 32 scans over a wavenumber range of 400–4000 cm^{-1} with 4 cm^{-1} resolution were recorded. Thermal behaviors of native ferulic acid and nanoformulations (CSNPs, FA-CSNPs) were analyzed using thermogravimetric analyzer and differential scanning calorimeter (EXSTAR, TG/DTA 6300 with STAR^{e} software). For TGA, samples were heated from 20 °C to 550 °C at 10 °C min^{-1} heating rate while for DSC, samples were packed in an aluminum pan and heated at the same rate. Stability of ferulic acid upon encapsulation was evaluated by performing ^{1}H NMR spectra analysis with 500-MHz NMR spectrophotometer (Bruker, Germany) at 25 °C with deuterated DMSO as solvent.

2.5. Cytocompatibility evaluation

2.5.1. Cell culture

HEK-293 cell lines were propagated in augmented DMEM medium (100 μg mL^{-1} streptomycin, 100 U mL^{-1} penicillin and 10% FBS) and used for the cytocompatibilty evaluation of native FA, CSNPs and FA loaded CSNPs.

2.5.2. MTT assay and FESEM analysis

Cell metabolic activity study using MTT assay and evaluation of morphological alterations (FESEM analysis) in HEK-293 cells in presence of different formulations were carried as described earlier [17]. Concisely, nanoformulations (NF_1–NF_5) along with native FA (FA_1-FA_5) and unloaded CSNPs were separately placed in 24 well microtiter plate, subsequently HEK cells were added to the wells while keeping the untreated cells as control. The plate was incubated in 5% CO_2 atmosphere at 37 °C for 24 h, followed by MTT assay and absorbance was recorded at 540 nm with the help of a microplate reader (Fluostar optima, BMG labtech, Germany). The cell viability (%) was calculated using equation given below:

$$\text{Cell viability}(\%) = (\text{OD}_{540\text{nm}}\text{test samples}/\text{OD}_{540\text{nm}}(\text{control})) \times 100$$

For FESEM analysis, the above 24 h incubated nanoformulations were fixed onto a glass slide by immersing it for 4 h into 2.5% glutaraldehyde PBS solution. The samples were than subjected to