Effect of Lipopeptide-Loaded Chitosan Nanoparticles on *Candida Albicans* Adhesion and on the Growth of *Leishmania Major*

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Abstract

A strain B84 producing surfactin and bacillomycin D lipopeptides was identified as *Bacillus amyloliquefaciens*. B84 lipopeptides were extracted by acid precipitation, identified by mass spectrometry and loaded onto chitosan nanoparticles by ionotropic gelation process. The lipopeptide / chitosan nanoparticles have an average size of 569 nm, a zeta potential range of 38.8 mV and encapsulation efficiency (EE) of 85.58 %. The treatment of *C. albicans* cells with encapsulated lipopeptides showed an anti-adhesive activity of 81.17 % and a decreased effect of cell surface hydrophobicity « CSH » by 25.53%. An anti-leishmanial activity was also recorded with IC₅₀ of 14.37 µg mL⁻¹ and 22.45 µg mL⁻¹ against *L. major* promastigote and amastigote forms, respectively. Cytotoxicity studies on human cells showed low cytotoxicity effect with HC₅₀ value of 770 µg mL⁻¹ towards human red blood cells and LC₅₀ value of 234.56 µg mL⁻¹ towards macrophage Raw 264.7 cell line. These lipopeptide / chitosan nanoparticles could be used as a potential delivery system of lipopeptides to improve both their anti-adhesive effect against *C. albicans* cells colonizing medical devices and their anti-infectious activity against *leishmania*.

Introduction

The pathogenic yeast *Candida albicans* has the ability to colonize surfaces including biomedical devices such as catheters and prostheses [1]. This pathogen develops a biofilm which consists of a well-structured surface cell community that is responsible for pathogenesis and plays an important role in the protection of cells against host defense and conventional antifungal drugs [2]. In addition, leishmaniasis is one of the major infectious zoonotic parasitic diseases. About 12 million people are affected by the disease each year, and cutaneous leishmaniasis is the most common. Given the inadequacy of current treatments, their toxicity and parasitic resistance, the exploration of new drugs could be an alternative solution to conventional treatments.

Cyclic lipopeptides produced by *Bacillus* species with biosurfactant properties have shown a wide biological activity spectrum such as antimicrobial [3], anti-adhesive [4] and anti-parasitic activities [5]. Lipopeptides share a similar form as they are composed of a cyclic peptide linked to a β-amino or β-hydroxy fatty acid of different length [6]. Lipopeptides target the cytoplasmic membrane and form ion-conducting pores in the lipid membrane [7]. In addition, lipopeptides affect the adhesion of microorganisms by modifying cell surface properties [8]. They have the capacity to adsorb to microbial surfaces, resulting in a change in the hydrophobicity of the cell surface and a decrease in the adhesion process [9].

However, in most cases, these lipopeptides have shown cytotoxicity towards human cells which limits their biomedical application [10]. Drug delivery system (DDS) is an attractive technology and an interesting strategy to reduce the toxicity of drugs by controlled release of charged compounds [11].

Ionotropic gelation methods, liposomes, niosomes and emulsions have rapidly developed in the medical field as an effective DDS for the treatment of infectious diseases. These formulations have been shown to be more effective than free active compounds [12].

Chitosan nanoparticles widely used as nano transporters are an amino-polysaccharide naturally derived from chitin which is the second most abundant natural polymer after cellulose [13]. It is a constituent of the cell wall of most fungi, algae, insects and crustaceans. Chitosan is obtained by deacetylation of chitin. It has shown several advantages as it is biocompatible, biodegradable, muco-adhesive and non-toxic [14]. Its muco-adhesive properties improve the lifetime and the penetration of drugs into infected areas [14].

In this regard, the current study aimed to prepare and characterize chitosan nanoparticle loading *Bacillus* lipopeptides. The anti-adhesive property of lipopeptide-loaded chitosan nanoparticles on *Candida albicans* cells, their antileishmanial and toxicity properties have also been assessed. To our knowledge, this is the first report that highlighted the potential role of chitosan as nanocarrier of *Bacillus* lipopeptides that could reduce the toxicity of lipopeptides toward mammalian cells and could maintain their biological activities.

Materials And Methods

Microorganisms and culture conditions

A strain B84 producing lipopeptides was isolated from olive leaves of a Tunisian cultivar and was cultivated in Luria-Bertani "LB" medium at 30 °C for 24 h. The reference yeast strain *C. albicans* ATCC 10231 was cultured in Sabouraud-dextrose broth (Pronadisa, Hispanlab. S.A., Spain) at 30 °C for 24 h. Microbial strains were stored at -80 °C in culture media supplemented with 25% glycerol.

Molecular Identification of lipopeptide producing strain

The genomic DNA was extracted from the bacterial culture in LB medium. DNA extraction was performed by the DNeasy blood and tissue Kit (QIAGEN, Hilden, Germany). The 16S rRNA gene of the selected strain B84 was amplified using primers: rd1 AAGGAGGTGATCCAGCCGCA and fd2 AGAGTTTGATCATGGCTCAG [15]. PCR products were purified using the Promega PCR purification kit (Wizard SV gel and PCR clean-up 95 systems). Almost full length 16S rRNA genes were sequenced and submitted to the GenBank. The alignment of the nucleotide sequences was carried out using the "Blast Search Algorithm".

Production and extraction of lipopeptides

The lipopeptide producing strain B84 was cultured in LB broth at 30 °C for 24 h at 150 rpm as previously described [16]. Lipopeptides were extracted according to Kim et al with minor modifications [17]. Briefly, the culture medium of the producer strain was centrifuged at (10,000 ×g) for 15 min at 4 °C. The cell-free supernatant was subjected to acid precipitation at pH 2.0 with 3 N HCl and held overnight at 4 °C. After

centrifugation (10,000 ×g) for 20 min at 4 °C, the precipitate was collected and was dissolved in chloroform / methanol (2:1, v:v). The solvent was removed and the resulting pellet was dissolved in methanol to the desired concentration.

Mass spectroscopy analysis of B84 lipopeptides

The active fraction was injected into QTRAP mass spectrometer (Applied Biosystem) equipped with an electrospray ionization interface, at a flow rate of 10 µl min⁻¹. The parameters of the ion source were as follows: ion spray voltage: 5500 V; curtain gas (nitrogen 99.9 %):10 psi, nebulizer gas (gas 1): 20 psi. The mass spectroscopy analysis was performed in positive ion mode and the mass spectra were analyzed by Analyst 1.5.

Encapsulation of B84 lipopeptides in chitosan nanoparticles

The chitosan nanoparticles were prepared by the ionic gelation method with slight modifications [11]. Chitosan was dissolved in 0.1% aqueous acetic acid solution. The lipopeptide extract was then added to a chitosan solution with moderate magnetic stirring at room temperature in order to obtain a lipopeptide / chitosan mass ratio of 2 : 1. Chitosan nanoparticles loaded with lipopeptides (LP/Cs-NPs) are formed spontaneously upon dropwise addition of TPP solution (1 mg mL⁻¹) to a pH of 5.5. LP/Cs-NPs were collected by centrifugation at 4,000 x g for 10 min at 4 °C.

Determination of encapsulation efficiency (EE)

The encapsulation efficiency of LP/Cs-NPs was determined as described previously [14]. Residual lipopeptides of surfactin and bacillomycin D homologues in the supernatant after centrifugation and the total amount of lipopeptides used for encapsulation were determined using QTRAP mass spectrometer. Encapsulation efficiency was calculated as the difference between the total amount of lipopeptides used for encapsulation and the amount of residual lipopeptides present in the supernatant after centrifugation of the encapsulated suspension using the following formula:

Encapsulation efficiency = [(Total amount of lipopeptides - residual amount of lipopeptides) / Total amount of lipopeptides] x 100

***In vitro* lipopeptide release**

In order to measure the release of lipopeptides in *vitro*, LP/Cs-NPs were suspended in a phosphate buffered saline solution (PBS) pH 7.4 and were incubated at 37 °C. The mixture was centrifuged at 10,000 x g for 5 min at different time intervals 0, 30, 45, 60, 120, 180 min and 24 h and the supernatant was removed and replaced with the same volume of PBS buffer [14]. The amount of released lipopeptides of surfactin and bacillomycin D homologues was determined by QTRAP mass spectrometer.

Characterization of lipopeptide/chitosan nanoparticles

Chitosan (Cs), sodium tripolyphosphate (TPP), chitosan nanoparticles (Cs-NPs), free lipopeptides (LP) and lipopeptide-loaded chitosan nanoparticles (LP/Cs-NPs) were analyzed by FTIR spectrophotometer (Frontier, Perkin Elmer) with spectrum software and with an attenuated total reflectance (ATR) accessory. Spectra were scanned over the wavenumber range 400-4000 cm^{-1} .

The particle size, the polydispersity Index (PDI) and the Z-potential of Cs-NPs and LP/Cs-NPs were determined on aqueous dispersion (0.1 % w/v) using a Malvern Zetasizer nano ZS instrument (malvern Instruments, UK) at 25°C and Zetasizer software.

The morphological structure of Cs-NPs and LP/Cs-NPs was observed with a scanning electron microscope (SEM, JSM-5400 JEOL) operating at an applied voltage of 10 KV.

Anti-*Candida* activity

Anti-*Candida* activity was assessed against *Candida albicans* ATCC 10231. The minimal inhibitory concentration (MIC) was established using the standard microbroth dilution method in sterile 96-well plates as described previously [18]. Two-fold serial dilutions of samples ranging from 62.5 to 1000 $\mu\text{g/mL}$ have been checked. The MIC value was determined to be the lowest concentration of the active compound which inhibited the growth of *C. albicans* ATCC 10231. Amphotericin B (AMB) was used as positive control.

Anti-adhesive activity

Cs-NPs and LP/Cs-NPs were tested for their ability to inhibit *C. albicans* cell adhesion in 96-well flat-bottom polystyrene microplates. *C. albicans* cell suspension was diluted to an OD_{590} of 0.6 as described previously [1]. Two-fold serial dilutions of the tested samples were added to *Candida albicans* cells and were incubated for 3 h at 37 °C. Adherent cells were revealed with 0.05% crystal violet solution [19]. The excess stain was removed by rinsing the wells with sterile deionized water. After addition of an acetic acid solution (33%), the absorbance was measured at 595 nm using a microplate reader (Bioteck, ELx 800). The percentage of adherent cells was calculated relative to the control (cells without samples tested) according to the following formula:

$$\text{Adhesion (\%)} = [1 - (\text{Abs}_t / \text{Abs}_c)] \times 100$$

Where Abs_t is the absorbance of treated *C. albicans*, Abs_c is the absorbance of untreated *C. albicans* cells (control).

Cell surface hydrophobicity

The effect of free LP and LP/Cs-NPs on cell surface hydrophobicity (CSH) of *C. albicans* was established as described previously [8]. Briefly, *C. albicans* cell suspension was washed with 0.9% (w/v) physiological

water and diluted to an OD₆₀₀ of 0.5. Samples were added to the cell suspension at two-fold serial dilutions ranging from 125 to 2000 µg mL⁻¹. Incubation was carried out at 37 °C for 2 h with constant stirring at 150 rpm. Microbial adhesion to hydrocarbon (MATH) was determined to assess CSH of *Candida* cells treated with Hexadecane. After vortexing for 3 min and phase separation for 1 h, the OD₆₀₀ of the aqueous phase was measured and the CSH was calculated as follows:

$$\text{CSH (\%)} = [1 - (\text{Abs}_a/0.5)] \times 100$$

Where Abs_a is the optical density of aqueous phase at 600 nm

Anti-promastigote assay

The anti-promastigote activity of free LP and LP/Cs-NPs was evaluated on *Leishmania major* (LC04) as described previously [20]. The pathogenic strain was cultured in RPMI 1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U mL⁻¹) and streptomycin (100 µg mL⁻¹) and was incubated at 24 °C in a humidified atmosphere with 5% CO₂. In the stationary phase, promastigotes were seeded at 2 × 10⁵ / well in 100 µL of growth medium containing serial dilutions of tested samples (7.81-1000 µg mL⁻¹). The 96-well plate was incubated at 24 °C for 72 h. The viability of *Leishmania* was measured by a colorimetric test with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma–Aldrich, USA). After 4 h of incubation, the formazan crystals were solubilized with pure DMSO and the absorbance was measured spectrophotometrically at 570 nm (OD_{570 nm}) using a microplate reader (Bioteck, ELx 800). Antileishmanial activity was expressed as IC₅₀ which is the concentration of the sample required to reduce the growth of the parasite by 50%. AMB was used as positive a control. All assays were performed in triplicate.

Anti-amastigote assay

Raw 264.7 macrophages (5×10⁴ cells) were infected with *L. major* promastigotes at *their* stationary growth phase in a ratio of 1:10 in chamber slides and were incubated for 24 h at 37°C in 5% CO₂. Non-adherent cells were eliminated by washing with serum-free RPMI-1640 medium. LP, chitosan and LP/CS-NPs were then added with increasing concentrations (6.25 to 200 µg mL⁻¹) and were further incubated for 48 h at 37°C in 5% CO₂ [21]. Plates were stained with 10% Giemsa and examined under light microscopy (X100). The number of intracellular amastigotes was determined by counting amastigotes in at least 100 macrophages. The percentage of reduction of the infection rate (% IR) was determined:

$$\% \text{IR} = 100 - [(\text{infection rate of the treated culture} / \text{infection rate of the untreated culture}) \times 100].$$

Cytotoxicity assay and selectivity index

The cytotoxicity of samples was evaluated using Raw 264.7 macrophage cell line cultured in RPMI 1640 medium supplemented with 10% FBS and with an antibacterial and an antifungal solution (Gibco, USA).

Incubation was carried out at 37 °C in 5% CO₂ in an humidified atmosphere. The viability of macrophage cells was examined under a microscope after staining with 0.1% trypan blue solution. Macrophage cells were inoculated into a 96-well tissue culture plate at 10⁵ cells/well and allowed to adhere overnight. The adherent cells were treated with two-fold serial dilutions of samples and were incubated for 72 h at 37 °C. Cell viability was estimated by MTT test as described above. Cytotoxicity was expressed as CC₅₀ corresponding to the drug concentration causing 50% of cell death. The selectivity index (SI) was determined as the ratio of macrophage CC₅₀ to the parasite IC₅₀ [20].

The hemolytic activity was determined on erythrocyte cells as described previously [22]. Briefly, washed human blood cells were incubated with two-fold serial dilutions of samples at 37 °C for 30 min. After centrifugation at 3000 rpm for 15 min, the absorbance of the supernatant was measured at 450 nm. The percentage of hemolysis was calculated relative to the positive control suspended in distilled water. The hemolytic concentration HC₅₀ was determined as the concentration required to lyse 50% of erythrocyte cells.

Statistical analysis

All assays were collected from three independent experiments and were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using student *t* test with GraphPad Prism. *P* value of < 0.05 was considered significant.

Results

Molecular identification of the strain B84

The strain B84 producing lipopeptides was isolated from olive leaves of a Tunisian cultivar. Blast analysis of the partial sequence of the 16S rRNA gene revealed a 99.86% similarity with *Bacillus amyloliquefaciens* strain BCRC 11601 (NR116022). The sequence has been deposited in the NCBI Gene Bank nucleotide sequence database under the accession number MT883340.

Mass spectrometry analysis of B84 lipopeptides

Lipopeptides of strain B84 were analyzed by mass spectrometry as illustrated in Table. 1. Signals at the *m* / *z* range 1000-1102 were obtained. The ions at *m* / *z* 1022.6, 1036.8, 1050.8 and 1064.8 have been assigned to protonated surfactin homologues with C14, C15, C16 and C17 fatty acid chains , at *m* / *z* 1072.7 and 1086.8 attributed to sodium surfactin homologues with C16 and C17 fatty acyl chains and at *m* / *z* 1102.8 to potassium surfactin homologue with C17 fatty acyl chain. The second set of ions at *m* / *z* 1045.7, 1059.7 and 1073.7 corresponded to the protonated bacillomycin D homologues with C15, C16 and C17 acyl chains and at *m* / *z* 1081.7 was assigned to the sodium bacillomycin D homologue with C16 acyl fatty acid. Data showed that strain B84 produced 68.83% surfactin homologues and 31.16% bacillomycin D homologues. The most prominent signal ion belongs to the C 17 surfactin homologue.

Fourier transform infrared analysis (FT-IR)

The FTIR analysis of Cs, TPP and Cs-NPs is reported in Table 2. The spectrum of Cs showed a broad band at 3350 cm^{-1} assigned to the -N-H stretching vibration mode superimposed on the -O-H stretching vibration mode. The peak at 2870 cm^{-1} is attributed to the -C-H stretching vibration band. A characteristic peak at 1650 cm^{-1} is associated with the vibration of -C=O bonds of amide group, while the -N-H bending vibration is observed at 1585 cm^{-1} . The -C-H bending vibrations at 1420 cm^{-1} and 1375 cm^{-1} are due respectively to the $-\text{CH}_2$ and $-\text{CH}_3$ groups. The peaks observed from 1150 cm^{-1} to 1030 cm^{-1} are related to the vibrations of the -C=O groups.

The TPP spectrum showed a characteristic peak at 1260 cm^{-1} corresponding to the stretching vibration of P=O. The symmetric and anti-symmetric stretching vibrations in PO_2 are sustained by a peak at 1120 cm^{-1} while the symmetric and anti-symmetric stretching vibrations of PO_3 groups are confirmed by the presence of a peak at 1060 cm^{-1} . The peak at 860 cm^{-1} could be assigned to the asymmetric stretching vibration of the P-O-P bridge. Examination of FTIR spectrum of Cs-NPs showed a broad band at 3350 cm^{-1} resulting from the stretching vibration of the $-\text{NH}_2$ and -OH groups. Two peaks at 1650 cm^{-1} and 1585 cm^{-1} corresponding to -C=O and -N-H vibrations of the amide groups in Cs are respectively shifted to 1640 cm^{-1} and 1540 cm^{-1} with increased intensity. This change could be due to the bond between the protonated amino groups $-\text{NH}_3^+$ of Cs and the P-O- group of TPP. In addition, the Cs-NPs spectrum revealed the presence of typical peaks of P=O and P-O-P bridge vibrations at 1260 cm^{-1} and 860 cm^{-1} respectively. Furthermore, the FTIR spectrum of LP reported in Fig. 1 showed the presence of a characteristic peptide band at 3290 cm^{-1} indicating the N-H stretching mode. Absorption bands at 1644 and 1518 cm^{-1} correspond to stretching vibrations of C=O and C-N in the amide bond of the peptide residues. The presence of an aliphatic chain was recognized by absorption bands at 2953 - 2927 cm^{-1} , 2851 cm^{-1} and 1460 cm^{-1} ($-\text{CH}_3$, $-\text{CH}_2-$). The 1736 cm^{-1} band reflects the absorption of lactone carbonyl. The FTIR results confirmed the lipopeptide nature of compounds produced by B84. The LP/Cs-NPs spectrum revealed appreciable changes from that of Cs-NPs. As shown in Fig. 1, the intensity of the C=O vibration band in the chitosan amide group ($\sim 1650\text{ cm}^{-1}$) was increased after LP loading while, bands between 1030 and 1150 cm^{-1} were faded away. Bands at 1440 cm^{-1} , 1644 cm^{-1} , 1736 cm^{-1} , 2857 cm^{-1} , 2927 cm^{-1} and 2953 cm^{-1} assigned to LP clearly indicated incorporation of LP into Cs-NPs.

Particle size and zeta potential determination

The zeta potential of Cs-NPs and LP/Cs-NPs was determined (Table 3). Both displayed positive zeta potential values corresponding to $+46.7$ and $+38.8\text{ mV}$ respectively, which could be related to the cationic nature of Cs-NPs. Furthermore, the encapsulation of LP in Cs-NPs led to a decrease of the zeta potential value compared to that of free Cs-NPs, suggesting a phenomenon of interaction between LP and Cs-NPs. The mean size of Cs-NPs and LP/Cs-NPs was also measured (Table 3). Results showed that the size of LP/Cs-NPs increased to reach 569.2 nm compared to that of Cs-NPs which was 270.7 nm . These results

could confirm the incorporation of LP into Cs-NPs. Furthermore, Cs-NPs and LP/Cs-NPs showed a polydispersity index (PDI) of less than 0.5 (Table 3), indicating good homogeneity of nanoparticle size distribution. Scanning electron microscopy images showed that Cs-NPs and LP/Cs-NPs were spherical shaped particles (Fig 2). The particle size of Cs-NPs ranged from 230 to 330 nm with an average size of 276.66 nm. An increase in the particle size of LP/Cs-NPs was recorded in a range of 500-660 nm with an average size of 576.66 nm corresponding to the dual size range of Cs-NPs. These results are comparable to those obtained by dynamic light scattering (data not shown).

Encapsulation efficiency and *in-vitro* release kinetics of B84 lipopeptides

The lipopeptide encapsulation efficiency reported in Table 3 was approximately 85.58%. Furthermore, surfactin homologues showed higher encapsulation efficiency (93.5%) than those of bacillomycin D (68.07%). The release profile of LP from LP/Cs-NPs revealed a continuous release of LP after 3 h of incubation reaching 34.5% release. After 24 h, only 47% of LP had been released. The bacillomycin D homologues were released in a high and continuous manner reaching 48.5% after 3 h and 70% after 24 h while surfactin homologues were rather weakly released with 28% after 3 h and 37% after 24 h (Fig. 3). Total release of LP was achieved within 72 h (Fig. 3).

Anti-*Candida albicans* and anti-adhesive activities

Anti-*Candida* activity was determined for both LP and LP/Cs-NPs (Table. 4). The results showed that the LP was more active than the LP/Cs-NPs when tested against *Candida* cells with MICs of 156 and 312 $\mu\text{g mL}^{-1}$ respectively. While, LP/Cs-NPs displayed potent anti-adhesive activity ($\text{IC}_{50} = 360 \mu\text{g mL}^{-1}$) being more effective than free lipopeptides ($\text{IC}_{50} = 410 \mu\text{g mL}^{-1}$) (Table 4). Cs-NPs showed less anti-adhesive activity and exhibited anti-adhesive activity with IC_{50} value of 900 $\mu\text{g mL}^{-1}$. Furthermore, the anti-adhesive activity for tested samples was concentration dependent and was reduced at lower concentrations (Fig. 4). The best anti-adhesive activity was recorded at 2 mg mL^{-1} for LP/Cs-NPs, LP and Cs-NPs. The important effect was determined for LP/Cs-NPs which inhibited 81.17% adhesion of *Candida albicans* cells and was even more active than LP with 79.25% inhibitory effect ($P < 0.05$). However, Cs-NPs showed only 63.93% inhibition of *C. albicans* adhesion.

Cell surface hydrophobicity

The adhesion of cells to surfaces is related to cell surface hydrophobicity (CSH). In this way, the influence of LP, LP/Cs-NPs and Cs-NPs on *C. albicans* CSH was studied (Fig. 5). The effect of LP/Cs-NPs on CSH was concentration dependent. At 2 mg mL^{-1} , LP/Cs-NPs significantly decreased CSH by 4.25-fold compared to untreated cells. At lower concentrations, the effect on CSH was reduced (Fig. 5). On the other hand, LP was less active than LP/Cs-NPs ($P < 0.01$) and reduced CSH by 3.21 times at 2 mg mL^{-1} . Note that a small decrease in CSH of 1.89 times was recorded for Cs-NPs.

Antileishmanial activity

The antileishmanial activity of LP, LP/Cs-NPs as well as Cs-NPs was assessed against promastigote (Table 5) and intracellular amastigote forms of *L. major* (Table 6). Results showed significant antileishmanial activity of LP/Cs-NPs with IC₅₀ values of 14.37 µg mL⁻¹ and 22.45 µg mL⁻¹ against promastigote and amastigote form of *L. major*, respectively. Interestingly, the antileishmanial activity of LP/Cs-NPs was 3.73 and 3.57 times greater than that of LP with IC₅₀ of 53.27 µg mL⁻¹ and 80.33 µg mL⁻¹ against promastigote and amastigote forms of *L. major*, respectively. It should be noted that Cs-NPs exhibited low antileishmanial activity against promastigotes (IC₅₀ = 1 mg mL⁻¹) and no inhibitory effect was recorded against amastigotes. AMB, used as a positive control, showed significant antileishmanial activity against promastigote and amastigote cells with IC₅₀ of 0.97 ± 0.08 µg/mL and 0.5 µg mL⁻¹, respectively.

Cytotoxicity effect on human erythrocyte and Raw 264.7 cells

The hemolytic activity was tested and the concentration leading to 50% lysis of human erythrocytes (HC₅₀) was determined (Table 4). Results indicated that LP's HC₅₀ was approximately 420 µg mL⁻¹ and was more cytotoxic than LP/Cs-NPs with an HC₅₀ value of 770 µg mL⁻¹. The cytotoxic effect was also tested on macrophage Raw 264.7. The LP was highly cytotoxic with LC₅₀ value of 124.17 µg mL⁻¹ and a selectivity index of 1.54 and 2.33 (Table 5). In contrast, LP/Cs-NPs showed a weak cytotoxic effect with LC₅₀ = 234.56 µg mL⁻¹ and a high selectivity index of 10.44 and 16.32. Hence, LP/Cs-NPs could be considered as safe for mammalian cells and effective against parasites. The reference compound AMB was less cytotoxic with a selectivity index of 10.94 and 21.24.

Discussion

Cyclic lipopeptides produced by *Bacillus* species showed promising therapeutic potential. However, their clinical use remains limited due to their low stability, their undesirable interactions with host macromolecules and their potential toxicity to mammalian cells [18; 23]. Encapsulation could protect them against external aggressions, increase their structural stability and reduce their toxicity [24; 25]. Different formulation techniques have been developed. The most common is encapsulation in chitosan nanoparticles [26]. The incorporation of LP into Cs-NPs was obtained by ionotropic gelation method based on the spontaneous formation of complexes between Cs and TPP. This method allowed the formation of positively charged nanoparticles with spherical shape and with 200 to 500 nm diameter [14].

The FTIR spectrum of Cs-NPs showed a spectrum similar to that of Cs with slight differences due to the interaction of the phosphate group of TPP with the NH₂ group of Cs. These results are consistent with previous reports for Cs and Cs-NPs [27, 28]. The spectrum of LP/Cs-NPs showed peaks characteristic of LP which confirmed the stability of these lipopeptides after encapsulation in Cs-NPs. Furthermore, shifted peaks appearing in the spectrum of LP/Cs-NPs at 1030-1150 cm⁻¹ indicated the electrostatic interaction between Cs-NPs and LP. No new peak was observed suggesting that no new bond formed during the encapsulation process and that only electrostatic interaction could be involved [29].

The zeta potential value of LP/Cs-NPs was reduced from +46.7 to +38.8 mV compared to Cs-NPs. A similar reduction in zeta potential has been reported in previous studies [11, 30]. In fact, the neutralization of some positive surface charge of Cs-NPs could have taken place and could be related to the adsorption of LP on the surface of nanoparticles [30]. The ionotropic gelation method allowed the formation of Cs-NPs with an average particle size of 270.7 nm. Encapsulation of LP in Cs-NPs increases the mean size of LP / Cs-NPs to 569.2 nm compared to that of Cs-NPs. Similar results suggest that the increase in the size of the charged nanoparticles is linked to the adsorption of bioactive compounds to the surface of the nanoparticles or to their incorporation inside the nanocapsules [31, 32].

SEM analysis of LP/Cs-NPs showed a spherical shape and a smooth surface. Spherical particles have been shown to be able to provide maximum drug incorporation [33].

LP compounds were efficiently loaded into Cs-NPs with an encapsulation efficiency of about 85.58%. It has been previously reported that daptamycin, a cyclic lipopeptide, has shown significant encapsulation efficiency ranging from 80.82% to 97.93% [14]. Our results appear to be in agreement with other compounds encapsulated in Cs-NPs prepared by ionotropic gelation. For example, hydrophobic cyclosporin A has shown an encapsulation efficiency of 73.4% when loaded into Cs-NPs [34]. It has been shown that an increase in the efficiency of proteins encapsulation in Cs-NPs was observed with increasing protein concentrations [35]. For example, other studies have reported a decrease in encapsulation efficiency with increasing insulin concentration [36].

Likewise, the encapsulation efficiency was related to the concentration of LP. In fact, the 2:1 LP; Cs-NPs ratio showed the best encapsulation efficiency. By increasing the LP : Cs-NPs ratio to 3:1 a 2.39-fold decrease in encapsulation efficiency was observed (data not shown). In addition, surfactin homologues have shown high encapsulation efficiency compared to bacillomycin D homologues. This fact could be related to the charge of encapsulated compounds [37]. It has been shown that negatively charged proteins can be attracted to positively charged Cs allowing high encapsulation efficiency. In contrast, positively charged proteins were less attracted which reduced encapsulation efficiency [37]. In our case, surfactin homologues containing two negatively charged amino acid residues (1 Glu and 1 Asp) should be attracted to Cs-NPs, resulting in high encapsulation efficiency. However, bacillomycin D homologues with a single negatively charged amino acid (1 Glu) should be less attracted to Cs-NPs, leading to less encapsulation process.

Cumulative LP release studies showed moderate release (47%) during 24 h of incubation. The release of bacillomycin D homologues from Cs-NPs was greater than surfactin homologues. These results could be explained by the higher binding of surfactin homologues to Cs-NPs compared to bacillomycin D homologues. It has been previously shown that proteins with negatively charged surfaces are loaded more efficiently in Cs-NPs and are released more slowly [38].

Anti-*Candida* activity was determined for both LP and LP/CS-NPs. It was observed that the MIC value of LP/CS-NPs was 2 times higher than that of LP. Likewise, previous studies have shown that CS-NPs loaded with daptamycin have a higher MIC value than free daptamycin [14]. It has been suggested that

the decrease in the susceptibility of daptamycin/CS-NPs may be related to the interaction of CS with negatively charged cells which blocked the access of active compounds to the binding site. In contrast, the conventional antifungal drug amphotericin B loaded lecithin / chitosan nanoparticles exhibited comparable anti-Candida activity as the standard amphotericin B [39].

Candida albicans has the ability to adhere to surface-forming biofilms [40]. Microbial adhesion results from a specific interaction between the cell surface structure and the substrate [8]. This is because hydrophobic cells adhere more to epithelial cells and abiotic surfaces than hydrophilic cells. The adhesion of *C. albicans* to the polystyrene microplate was reduced after incubation with LP. The anti-adhesive effect was more pronounced with LP/Cs-NPs than with Cs-NPs. CS-NPs have been reported to reduce *C. albicans* biofilm on resin [41]. In addition, de Carvalho et al reported that Cs-NPs reduced the initial adhesion of *C. albicans* and showed a 25-50% inhibition of *C. albicans* adhesion [42]. This phenomenon could be linked to the efficiency of Cs-NPs in reducing CSH of *C. albicans* cells to 44% at 0.25% (w/v) [43]. Similar results were observed in the present study showing a 39.8% reduction in cell surface hydrophobicity by Cs-NPs at 2 mg mL⁻¹. Furthermore, surfactin homologues have been reported to significantly reduce the adhesion of *C. albicans* by 67–69 % at 2 mg mL⁻¹ [9]. This effect is partly linked to the reduction in the CSH of hydrophobic strains [8]. In fact, biosurfactants can decrease CSH due to their adsorption to the surface of yeast cells in order to inhibit cell adhesion to surfaces [8,44].

LP and LP/Cs-NPs were also tested against *L. major*. LP/Cs-NPs exerted strong antileishmanial activity against promastigote and amastigote forms with an IC₅₀ of 14.37 µg mL⁻¹ and 22.45 µg mL⁻¹, respectively. They were more active than free LP with an IC₅₀ of 53.7 µg mL⁻¹ and 80.33 µg mL⁻¹, respectively. The reduced antileishmanial activity of free compounds could be linked to their partial inactivation or to their sequestration on components of the medium or on dead cells [11]. The increase in the antileishmanial activity of the LP/Cs-NPs could be explained by the progressive release of LPs from chitosan delivery system (Fig. 3) allowing a continuous reduction of the viability of parasites and a prevention of their regrowth during 72 h of incubation and ensuring a long-lasting antileishmanial effect. Moreover, Cs-NPs showed antileishmanial potential with direct intercalation into the parasitophorous vacuole of the parasite [21]. Therefore, Cs-NPs could enhance the antileishmanial activity of LP. Similar results were observed for amphotericin B incorporated into chitosan nanoparticles which were more potent than free amphotericin B in inhibiting leishmania major promastigotes and amastigotes [45]. LP/Cs-NPs showed less toxicity than LP to human red blood cells and Raw 264.7 cells. These results were in agreement with previous studies showing that encapsulated drugs in Cs-NPs were less cytotoxic than free compounds. Amphotericin B in nanoparticles also showed less hemolysis than free amphotericin B [46]. It was also observed that the toxicity of the peptide RBRBR was significantly reduced by incorporation into Cs-NPs [47]. This fact could be linked to the slow and continuous linear release of drugs from the nanocarrier system [46, 48]. The decrease in the hemolytic activity of the encapsulated compounds could also be related to the reduced cytotoxic effect of Cs-NPs showing no hemolysis activity even at higher concentrations superior to 1mg mL⁻¹ [48, 49].

Conclusion

The current study showed that LP produced by the strain B84 was successfully encapsulated in a chitosan nanocarrier system. To our knowledge, no study has been reported on the properties of chitosan nanoparticles loaded with B84 lipopeptides. Such a powerful system represents a promising and innovative strategy to avoid the degradation of LP and reduce their toxicity. Further studies should be conducted to investigate the ability of the developed nanocarrier system to treat candidiasis and leishmaniasis *in vivo* and to study the delivery of chitosan loaded lipopeptides in infected sites.

Declarations

Ethical Approval This paper does not involve any human participants and animals performed by any of the authors.

Consent to participate All authors have agreed to participate in the publication of this paper.

Consent to publish All authors have agreed to publish this paper in Journal of Applied Biochemistry and Biotechnology.

Author contribution Siwar Soussi, Rym Essid and Ines Karkouch and Sarra Bachkouel performed the experiments; Houda Saad and Ferid Limam analysed the experimental data; Olfa Tabbene wrote the manuscript. All authors read and approved the final version of the manuscript.

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Competing interests All Authors have no conflict of interest to declare.

Availability of data and materials The authors confirm that all data of this finding are fully available without restriction. All relevant data are within the paper

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Tables

Table 1. Mass spectrometry analysis of lipopeptide fraction of B84 strain

Pics (m/z)	Assignment	Intensity (%)
1022.6	C14 Surfactin [M + H] ⁺	1.81
1036.8	C15 Surfactin [M + H] ⁺	5.02
1050.8	C16 Surfactin [M + H] ⁺	11.72
1064.8	C17 Surfactin [M + H] ⁺	21.54
1072.7	C16 Surfactin [M + Na] ⁺	8.58
1086.8	C17 Surfactin [M + Na] ⁺	18.69
1102.8	C17 Surfactin [M + K] ⁺	1.48
1045.7	C15 Bacillomycin D [M + H] ⁺	4.27
1059.7	C16 Bacillomycin D [M + H] ⁺	13.05
1073.7	C17 Bacillomycin D [M + H] ⁺	8.65
1081.7	C16 Bacillomycin D [M + Na] ⁺	5.19

Table 2. FTIR analysis of the original chitosan (Cs), sodium tripolyphosphate (TPP), chitosan nanoparticles (Cs-NP), lipopeptides (LP) and lipopeptide - loaded chitosan nanoparticles (LP/Cs-NP) produced by *Bacillus* B84 strain

Samples	Wave number (cm ⁻¹)	Assignment
Cs	3350	-N-H stretching vibration overlapped with –O-H stretching vibration
	2870	-C-H stretching vibration
	1650	-C=O of –CONH group
	1585	Bending vibration of –N-H
	1420	Bending vibration of –C-H of – CH ₂ group
	1375	Bending vibration of –C-H of – CH ₃ group
	1150	-C=O vibration
	1060 and 1030	-C=O vibration
TPP	1260	Stretching vibration of P=O
	1120	Symmetric and anti-symmetric stretching vibration in PO ₂
	1060	Symmetric and anti-symmetric stretching vibration in PO ₃
	860	Anti-symmetric vibration of P-O-P
Cs-NP	3350	-N-H stretching vibration overlapped with –O-H stretching
	1640	Bending vibration of-C=O
	1540	-N-O-P stretching vibration
	1260	Stretching vibration of P=O
	860	Anti-symmetric vibration of P-O-P
LP	3290	-N-H stretching mode
	1644	Stretching vibration of –C=O
	1518	Stretching vibration of –C-N
	2953 ; 2927 ; 2857 and 1460	Aliphatic chains –CH ₃ ; -CH ₂
	1736	Lactone carbonyl absorption
LP/CS-NP	2953 ; 2927 ; 2857 and 1460	Aliphatic chains –CH ₃ ; -CH ₂
	1736	Lactone carbonyl absorption
	1644	Stretching vibration of –C=O
	1540	-N-O-P stretching vibration

Table 3. Characterisation of Cs-NPs and LP/Cs-NPs: average size, surface charge and encapsulation efficiency

Samples	Particle size (nm±SD)	Polydispersity	Zeta Potentiel (mV±SD)	Encapsulation efficiency (%)
Cs-NPs	270.7±11.43	0.373	46.7±0.87	ND
LP/Cs-NPs	569.2±13.48	0.225	38.8±1.37	85.58%

Cs-NPs: chitosan nanoparticles, LP/Cs-NPs: lipopeptide-loaded chitosan nanoparticles

Table 4. Anti-*Candida*, anti-adhesive and hemolytic activities of Cs-NPs, LP and LP/Cs-NP

Samples	Anti- <i>Candida</i> activity (µg/mL)	Anti-adhesive activity IC ₅₀ (µg/mL)	Hemolytic activity HC ₅₀ (µg/mL)
Cs-NPs	>1000	900±10.73	>1000
LP	156±6.87	410±8.52	420±2.43
LP/Cs-NP	312±4.28	360±3.13	770±5.32
AMB	2±0.03	8±0.58	16±0.24

Table 5. Antileishmanial activity of CS-NP, LP and LP/CS-NP against *L. major* promastigotes and cytotoxicity effect

Samples	Anti-promastigote activity CI ₅₀ ±SD (µg/mL)	Cytotoxicity test CL ₅₀ ±SD(µg/mL)	Selectivity index (SI)
CS-NP	1000	>1000	ND
LP	53.27±1.45	124.17±1.23	2.33
LP/CS-NP	14.37±0.66	234.56±2.65	16.32
AMB	0.97±0.08	10.62±0.58	10.94

Leishmanicidal activity tested against *L. major*, cytotoxicity tested on Raw 264.7 cells

CS-NP: chitosan nanoparticles, LP: lipopeptides, LP/CS-NP: lipopeptides-loaded chitosan nanoparticles

Table 6. Antileishmanial activity of CS-NP, LP and LP/CS-NP against *L. major amastigotes*

Samples	Anti-amastigote activity $CI_{50} \pm SD$ ($\mu g/mL$)	Specificity (SP)	Selectivity index (SI)
CS-NP	ND	ND	ND
LP	80.33 ± 1.22	0.66	1.54
LP/CS-NP	22.45 ± 0.87	0.64	10.44
AMB	0.5 ± 0.15	1.94	21.24

ND: not determined

Specificity: is the ratio between promastigote IC50 and intracellular amastigote IC50.

Figures

Figure 1

FTIR spectra of LP/Cs-NPs (A), LP (B) and Cs-NP (C) Cs-NP: chitosan nanoparticles, LP: Lipopeptides from B84 strain, LP/Cs-NPs: lipopeptide -loaded chitosan nanoparticles

Figure 2

Scanning electron microscopy of Cs-NPs (A) and LP/Cs-NPs (B)

Figure 3

Release kinetics of lipopeptides, bacillomycin D and surfactin isoforms from chitosan nanoparticles at phosphate buffered saline, pH 7.4

Figure 4

Anti-adhesive activity of LP, Cs-NPs and LP/Cs-NPs. Statistical analysis was performed with t test: * P < 0.05, ** P < 0.01, *** P < 0.001

Figure 5

Cell surface hydrophobicity of *C. albicans* cells after treatment with LP, Cs-NPs and LP/Cs-NPs. Statistical analysis was performed with student test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. UC: untreated cells, LP: lipopeptides, Cs-NPs: chitosan nanoparticles, LP/Cs-NPs: lipopeptide - loaded chitosan nanoparticles