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Can the Antimicrobial Peptide Ctx(Ile^{21})-Ha-Ahx-Cys Grafted onto Nanochitosan Sensitize Extensively Drug-resistant *Mycobacterium tuberculosis*? †

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Abstract: The infectious agent Mycobacterium tuberculosis (MTB) has several defense and resistance mechanisms to be eliminated. The treatment is prolonged, which in many cases generates susceptibility to generate microbial resistance. This research aimed to study whether the antimicrobial peptide Ctx(Ile^{21})-Ha-Ahx-Cys (Ctx-SH) functionalized in nanochitosan matrices could eliminate resistant MTB. For this, a nanosystem was developed with chitosan matrices previously modified with N-acetylcysteine functionalized to Ctx-SH. Modified chitosan nanoparticles (NPQ) were obtained by ionic gelation using sodium tripolyphosphate and loaded with rifampicin. Both chitosan and NPQ modifications were analyzed for physicochemical parameters by Fourier/Raman transform infrared spectroscopy and Zeta potential. Antimicrobial activity was performed in a level 3 biosafety laboratory with strains H37Rv (standard) and CF169 (extensively drug-resistant, XDR) incubated in 7H9 broth supplemented with oleic acid, albumin, dextrose, and catalase at 37°C and 5% CO_2, and read using fluorescence with 0.01% resazurin after 7 days. Insertion and mapping of NPQ into macrophages were assessed using a confocal microscope after 24 h with NPQ conjugated to fluorescein isothiocyanate. Preliminary results show that the spectroscopies corroborate the hypothesis of the functionalization of the Ctx-SH peptide to the chitosan-N-acetylcysteine system because, when comparing the three spectroscopies, a gradual increase in the intensity of several bands and the formation of captive disulfide are observed; and the Zeta potential (+30mV) confirmed high application stability. Bacterial inhibition studies revealed that rifampicin-loaded antimicrobial peptide-conjugated chitosan nanoparticles have better activity than rifampicin alone against CF169 with a minimum inhibitory concentration of <0.977 μg/mL similar to the standard strain. In addition, it was shown that NPQ would be able to enter the macrophage without causing toxicity and thus take better advantage of the activity of rifampicin. Finally, it is possible to verify that the nanobioconjugation of the Ctx-SH-N-acetylcysteine-chitosan compound is capable of enhancing the activity of obsolete drugs and/or sensitizing XDR bacteria.

Keywords: *Mycobacterium tuberculosis*; nanobioconjugation; antimicrobial peptides

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1. Introduction

The tuberculosis (TB) has as etiologic agent the bacillus alcohol- acid resistant *Mycobacterium tuberculosis* (MTB), and it is reported by the World Health Organization

(WHO) as one of the world's leading infectious killers [1,2]. The pathology can be briefly described as: an infection by air pathway, a phagocytosis of the bacteria by lung´s resident macrophages, an adaptation of the mycobacteria to the phagolysosome environment, and an inhibition of MTB death[1,3]. The disease can be characterized by a chronic inflammation of the air pathway and pulmonary cavitation[4]. The current world situation is critical, especially with the increase of cases of drug-resistant TB, that hinders the use of first-line anti-MTB drugs (as isoniazid, rifampicin and ethambutol) [5]. Antimicrobial peptides (AMPs) are macromolecules that have fast bactericidal effect, low minimal inhibitory concentration (MIC) and low possibility of bacterial resistance[6,7]. They are also compounds of the innate immune system, and as so, they can modulate the immune respond [8]. The AMP Ctx(Ile^{21})-Ha is a peptide with twenty one residues of amino acids, cationic feature, and with action against bacteria Gram-negative and Gram- positive [9–11]. The mechanism of action of Ctx(Ile^{21}) is by barril-stave model, which means, it forms pores in the bacterial membrane [9]. This AMP has a potentially biologic application when insert in polymeric superficies, as chitosan [12]. Chitosan is a polysaccharide with a great biocompatibility, a low toxicity, and antibacterial activity[13,14]. Its mechanism of action is encompassing the bacterial cell and does a cationic multi-attack[15]. Because TB is an intracellular facultative pathogen of macrophages, it is necessary to develop strategies of drug- delivery to these cells [7,12]. One of these strategies is using nanotechnology as target delivery, in a way to improve pharmacology proprieties of molecules and to enable controlled released of drugs [11,16]. Ctx(Ile^{21})-Ha AMP grafted onto nanochitosan, by bisulfate bond, demonstrated lowers MICs to *S.aureus* and *P.aeruginosa* [12]. It can also add the strategy of modify chitosan with *N*-acetylcysteine (Figure 1), to improve pharmacology proprieties and half-time [17]. It was demonstrated that N-acetylcysteine has anti-biofilms action and an important inhibition of air pathway infection [18]. The aim of this work was to analyzes the application of Ctx (Ile^{21})-Ha-Ahx-Cys grafted onto nanochitosan to sensitize extremely resistance MTB strains.

2. Material and Methods

2.1. Chemical Reagents

Chitosan; *N*-acetylcysteine; sodium tripolyphosphate, Resin, Fmoc-aminoacids and other reagent for peptide synthesis was purchased from Sigma-Aldrich, dimethylformamide (DMF) was purchased from Neon Comercial (São Paulo, Brazil); dichloromethane (DCM) was purchased from Anidrol Laboratory (São Paulo, Brazil).

2.2. Synthesis of Antimicrobial Peptide

The synthesis of AMP Ctx(Ile^{21}) was assembled by standard solid phase peptide syntheses methodologies by Fmoc/tBu. It was used Rick amide MBHA resin as the solid support. It started with 1:1 v/v DMF/DCM to wash the resin and then it was add a solution of 20% 4-methylpiperidine in DMF in order to remove the Fmoc group of it. Then it was add the amino acid Fmoc- Ile (1.2 eq, molar equivalents) with 1.2/1.2 eq of hydroxybenzotriazole/*N*,*N*′-diisopropylcarbodiimide and 1:1 v/v DMF/DCM for 2 h at 40 °C. To confirm the couplings, it was used the Kaiser test, with blue color for negative results. With positive result, it was add the solution of 20% 4-methylpiperidine in DMF and the next amino- acid of the sequence. With the primary sequence complete coupling, the resin was removed using a solution of trifluoroacetic acid, triisopropylsilane and water 95:2.5:2.5 v/v/v for 2 h at room temperature. After that, the peptide was precipitated with cold ethyl ether and centrifuged three times (at 6500×*g* for 5 min). The supernatant was dry and then add aqueous solvent and acetonitrile solvent. After the centrifuge and freeze-dried [7].

Purification and characterization of Ctx(Ile^{21})-Ha peptide, was carry out used liquid chromatography/mass spectroscopy and the HPLC (Shimadzu, with DGU-20A5R membrane degasser, CTO-20A column oven, sampler automatic SIL-10AF, fraction collector

FRC-10A, UV detector SPD-20A and LC-20AT of double pump), respectively. The mobile phase was aqueous solution with TFA and acetonitrile solution with TFA. The method had a 1 mL min^{-1} flow and a dual mode with wavelengths at 220nm and 280 nm. The products of the separation were collected by an automatic fraction collector and were analyzed comparing the chromatographic profile [7].

2.3. Purification of Commercial Chitosan

Accordingly the method of purification of Costa et al. [19], the chitosan was solubilized with acetic acid 1% and it was precipitated with sodium hydroxide 1M. After that, it was washed with distillate water, centrifuged and filtrated with 0.2 nm membrane filter. Latter it was lyophilized.

2.4. Modification of Chitosan with N-acetylcysteine

The modification of chitosan with *N*-acetylcysteine was by the method of carbodiimide [20]. The chitosan previously purificated was solubilized with acetic acid 1% and add to a conjugation solution (50 mM of *N*-ethylcarbodiimide hydrochloride and 50mM of *N*-hydroxysuccinimide). To this solution it was add *N*-acetylcysteine 1:1:1 v/v/v in room temperature, with agitation for 24 h.

2.5. Functionalization Ctx(Ile21)-Ha onto N-acetylcysteine-chitosan

Disulfide bonds were spontaneously formed to graft the Ctx(Ile^{21})-Ha onto the surface of N-acetylcysteine-chitosan. *N*-acetylcysteine-chitosan compound was solubilized with 1% acetic acid and after 12 h of stirring it was precipitated with 0.1 M sodium hydroxide. Finally, it was filtered and lyophilized [21].

3.6. Nanoparticles Formation

In order to obtain the nanoparticles of N-acetylcystein-chitosan with Ctx(Ile21), it was used the method of ionic gelation [7]. The compound N-acetylcystein-chitosan with Ctx(Ile21) was solubilized with acetic acid 1% for 2 h, and then it was add rifampicin and Tween 80. It was homogenized and add 5mg/mL of sodium tripolyphosphate in a controlled agitation.

2.7. Physicalchemical Characterization

It was used Infrared spectroscopy (Perkin-Elmer, Frontier model,USA) with attenuated total reflectance fixture (Bruker Vertex 70 FTIR) and a resolution of 4 cm-. It was also used Raman spectroscopy (Burker Ram II Raman spectrophotometer). Both spectras were analyzed by Origin Pro 2019 software. It was also determinate the Zeta potencial by a Zetasizer.

2.8. Activity Against Mycobacterium Tuberculosis

Accordingly to Palomino et al. [22], antimicrobial activity against MTB was carried out using Resazurin Microtiter Assay (REMA) method. Briefly, nanoparticles solution was added in Middlebrook 7H9 Broth (Difco®) supplied with oleic acid, bovine albumin, sodium chloride, dextrose, and catalase. Rifampicin and isoniazid (25 μg/mL) was used as a controls. Nanoparticles loaded and controls was teste against MTB strains H37Rv (standard) and the CF169 (clinical isolated extremely resistant). The samples was incubated for seven days in 37 °C and 5% of CO_2, and read with 30 μL of resazurin 0.001% by fluorescence.

2.9. Confocal Microscopy

The polymers before their encapsulation were conjugated with fluorescein isothiocyanate, precipitated with NaOH and washed with distilled water until pH 7 was obtained.

Nanoparticles were then formed and brought to a confocal microscope 24 h after incubation in murine macrophages at a concentration of 1 mg/mL.

3. Results and Discussion

The spectroscopies confirm the modification and functionalization of the nanocomposites with Ctx(Ile^{21}) Ha-Ahx-Cys. The infrared spectroscopy (FTIR) was used in three samples of nanoparticles: chitosan purification, chitosan modified with *N*-acetylcysteine, chitosan modified with *N*-acetylcysteine and functionalized with Ctx(Ile^{21})Ha-Ahx-Cys. The intensity of the band 2364 cm^{-1}, corresponding to nitrile groups, were higher in the nanoparticles with modifications, and the intensity of the bands 1029cm^{-1} and 1074 cm^{-1}, corresponding to free amine groups, were lower in the same samples [23]. That confirmed that the modification of chitosan with *N*-acetylcysteine was effective. It was also possible to observe in the spectroscopy with modification and functionalized a band in 1457cm^{-1} (Figure 2.), corresponding to CH_2 and CH_3 of aromatics structures of the secondary structure of Ctx(Ile^{21}) -Ha-Ahx-Cys, which shows that the functionalization of the peptide were successful [7]

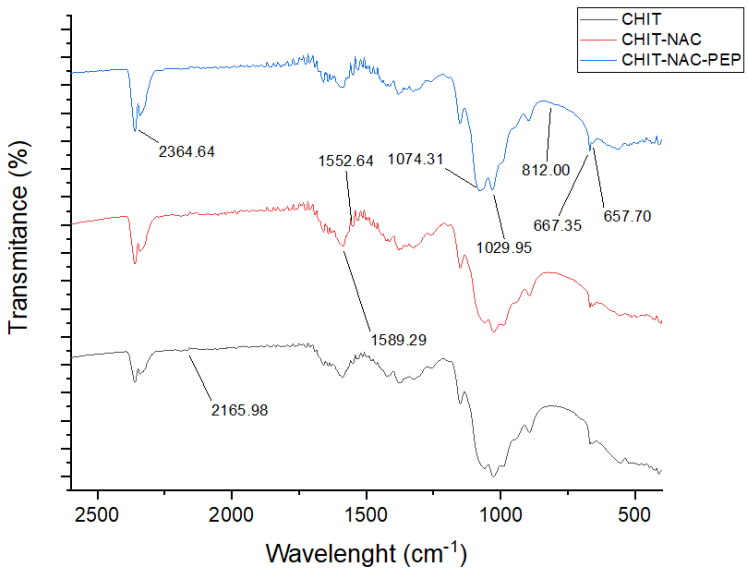

Figure 2. Infrared spectroscopy of the samples.

The method of spectroscopy RAMAN was used with the same samples. It confirm the functionalization of Ctx(Ile^{21})-Ha-Ahx-Cys onto modified polymer, because It has shown bands as 885cm-, 1004cm- and 1333cm-, corresponding to aromatics amino acids in the peptide (Figure 3.); It was also observed a band in 1230 cm-, characteristic of the secondary structure of Ctx(Ile^{21})-Ha-Ahx-Cys [7].

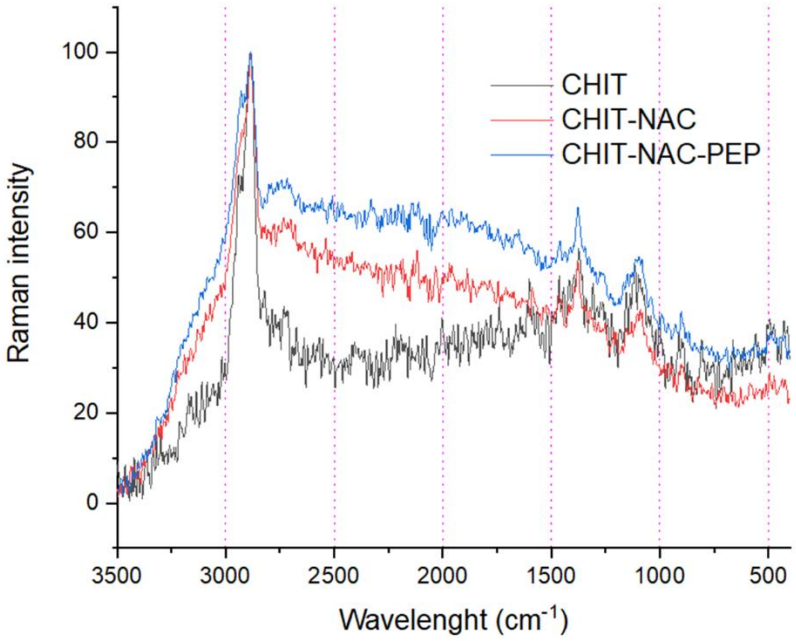

Figure 3. Spectroscopy RAMAN of the samples.

The anti-*Mycobacterium tuberculosis* activity was evaluated with the Minimal Inhibitory Concentration (MIC) of the nanoparticles of chitosan- *N*- acetylcysteine functionalized with Ctx(Ile[21])-Ha-Ahx-Cys and loaded with rifampicin. The MIC of the compound against CF169, clinically isolated multi resistant of tuberculosis, was 0,977 ug/mL. The value of MIC of this compound against H37Rv MTB strain was lower than 0,977 ug/mL. Considering that the MIC to rifampicin, the control compound, against CF169 is 25 ug/mL [24] and the similarity of the values of MIC (Table 1.), it can be concluded that the nanoparticles were able to sensiblizate this clinical isolated multi- resistant to drugs.

Table 1. This table shows the results of minimal inhibitory concentration values from chitosan modified nanoparticles loaded with rifampicin and the control compound (rifampicin) against the strain H37Rv and CF169.

Compound	Minimal Inhibitory Concentration (MIC) against CF169 (ug/mL)	Minimal Inhibitory Concentration (MIC) against H37Rv (ug/mL)
Nanoparticles loaded with rifampicin	0,977	<0,977
Rifampicin	25	0,977

The images obtained with confocal microscopy (Figure 2) showed that the nanoparticles of chitosan-*N*-acetylcysteine functionalized with Ctx(Ile[21])-Ha-Ahx-Cys and loaded with rifampicin was able to go through the plasmatic membrane of macrophages. It can be observed that the fluorescence intensity in the image of the compound is higher than in the image of the control- rifampicin isolated. Considering that macrophages are the most common cells infected with MTB [25], the ability of the compound to enter in the intracellular environment can indicate a select transport of drug propriety. It is also possible to conclude that the compound is not cytotoxic to this cell.

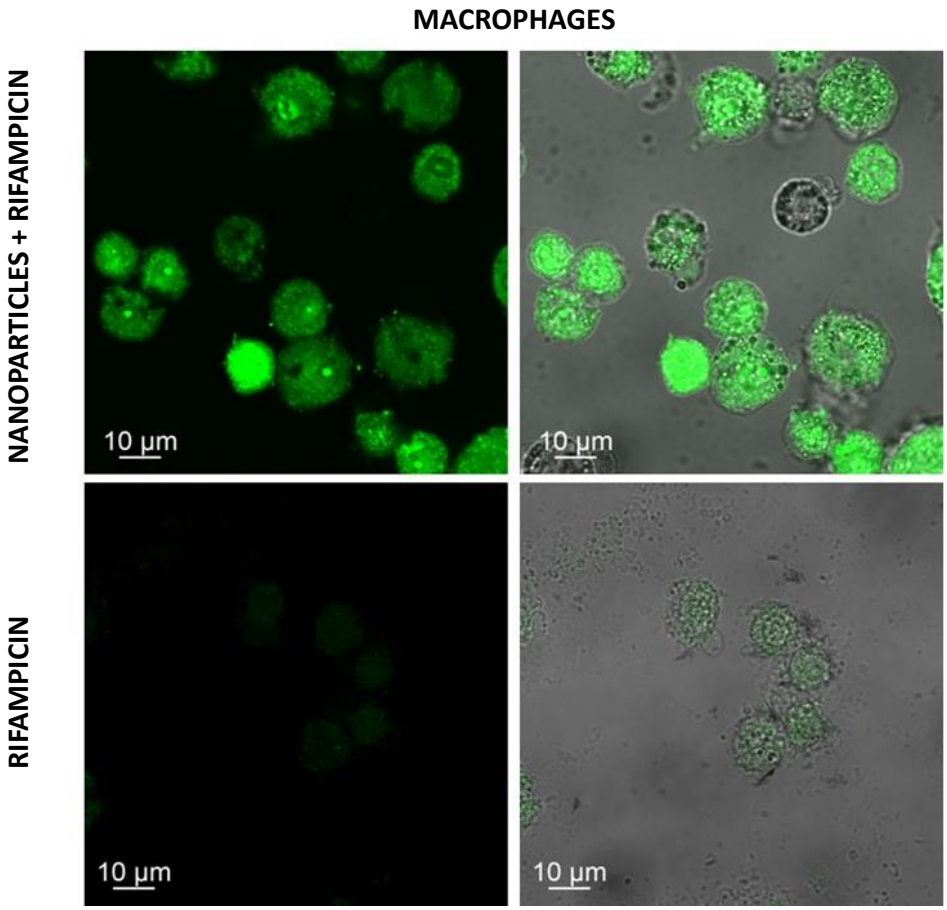

Figure 4. Images of the confocal microscopy of the nanoparticles of chitosan-*N*-acetylcysteine functionalized with Ctx(Ile^{21})-Ha-Ahx-Cys loaded with rifampicin and the control, rifampicin isolated. The first and second were corresponding to the nanoparticles and the third and fourth are corresponding to rifampicin.

4. Conclusion

It can be conclude that the antimicrobial peptide Ctx(Ile^{21})-Ha-Ahx-Cys grafted onto nanochitosan was able to sensiblizate an strain extremely resistance of *Mycobacterium tuberculosis* and intensity the effect of rifampicin, drug obsolete against CF169.

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