

# EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF NANOPARTICLES BASED ON CHITOSAN CROSSLINKED WITH PHYTIC ACID AND LOADED WITH DAPTOMYCIN AGAINST SENSITIVE *STAPHYLOCOCCUS AUREUS* STRAINS

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## Abstract

This study focused on determining whether the antimicrobial activity of the lipopeptide antibiotic daptomycin improves when it is loaded into chitosan-based nanoparticles (NPs). The nanoparticulate systems were developed through the ionic gelation method employing phytic acid as a crosslinking agent. Physicochemical characterisation of the nanoparticles (NPs) was carried out by measurements of particle size, polydispersity index (PDI), and zeta potential. Antimicrobial evaluation was performed using a susceptible *S. aureus* strain using the broth microdilution method. The results showed a particle size of  $453.9 \pm 4.7$  nm, a polydispersity index of  $0.245 \pm 0.04$ , and a zeta potential of  $+29.4 \pm 0.3$  mV. Notably, the antimicrobial evaluation revealed that daptomycin reduces the minimum inhibitory concentration (MIC) from 64 to 4  $\mu\text{g/mL}$  upon loading into the nanoparticle, demonstrating an interesting potential as a pharmaceutical nanoformulation.

**Keywords:** chitosan, daptomycin, antibiotic-loaded nanoparticles, ionic gelation, antimicrobial activity

**Received:** 31.03.2025

**Accepted:** 25.05.2025

## 1. Introduction

Over the last century, antibiotics have saved millions of lives and revolutionised medicine. However, a growing concern is compromising their effectiveness; one of the most significant challenges is the difficulty drugs face in reaching their site of action. As a result, they often must be administered in high doses and at higher administration frequencies, which commonly leads to prolonged treatments and an increase in side effects [1]. In recent years, chitosan has become a viable alternative for the development of nanoformulations in various fields, such as pharmaceuticals, cosmetics and food. This biopolymer is a renewable polysaccharide derived from naturally abundant chitin that acts as a supporting material for the exoskeleton of crustaceans, insects and arthropods, as well as in the cell walls of lower plants (fungi and green algae) [2]. Based on the physicochemical properties described by this biopolymer, such as its ability to self-organise in a micro- and nanostructured manner when present with polyanionic systems such as phytic acid, it has become a fundamental raw material for the formation of encapsulation and modified-release systems for various active ingredients [3–14]. Chitosan also exhibits biodegradability, biocompatibility and non-toxicity [15]. It is a water-soluble biopolymer, which allows it to form films, hydrogels, fibres and nanoparticles in a mild acidic aqueous medium; it has better solubility at a pH < 6.5 [16]. The synthesis of chitosan nanoparticles has emerged as an innovative therapeutic strategy within the development of controlled drug release systems. This approach motivated the present study, which aimed to synthesise, characterise and evaluate the biological potential of chitosan nanoparticles containing daptomycin, an antibiotic approved by the FDA in 2003 [17]. Daptomycin is a lipopeptide drug used as a last resort for the treatment of severe infections caused by *S. aureus* strains. However, it raises significant concern due to its nephrotoxicity when administered in high doses, particularly in patients with renal impairment [18]. Therefore, dosing frequency must be carefully considered in the clinical setting. Consequently, it is essential to explore strategies that can reduce the dose of daptomycin, minimise its side effects, and simultaneously improve its efficacy in treating infections caused by *S. aureus*, a Gram-positive bacterium common in both hospital-acquired and community-acquired infections, ranging from mild to severe [19]. In this study, the ionic gelation method was carried out to obtain a nanoparticle system loaded with daptomycin, where the phytic acid was used as a cross-linking agent, which allowed obtaining nanosystems with the potential to reduce the minimum inhibitory concentration of daptomycin against *S. aureus* and demonstrated that nanostructured systems based on chitosan and phytic acid can significantly influence this result. In this manner, the obtained nanoparticles will be physicochemically characterised by means of dynamic and static light scattering studies, while their antimicrobial evaluation will be studied on a sensitive strain of *S. aureus* ATCC 25923 by means of the broth microdilution method.

## 2. Materials and Methods

### 2.1. Deacetylation of Chitosan

Deacetylation (hydrolysis) of chitosan was performed with conventional microwave heating equipment. Specifically, 3 g of chitosan lot (BCCF8502, Sigma-Aldrich Co., St. Louis, MO, USA) with a deacetylation degree of 75% was weighed and simultaneously, a 10 N NaOH solution was prepared. They were added to a 500 mL round-bottom flask coupled with a spiral reflux condenser through an opening at the microwave's top. The reflux heating assembly was performed. Its objective was to produce the heating of the reaction through 2-minute cycles for 90 minutes with 6-minute rest periods. The mixture was then neutralised with 6 N HCl until pH 6 was obtained, then left under

constant stirring, rinsing with deionised water until a stable conductivity was obtained. The obtained suspension was vacuum filtered and lyophilised. The degree of deacetylation of chitosan was determined by infrared spectroscopy. The characteristic functional groups of the chitosan sample were evaluated in an infrared spectrophotometer using cells with potassium bromide (KBr), working within the 400 - 4000  $\text{cm}^{-1}$  frequency range. The interpretation of the spectrum was carried out through Shimadzu FT-IR computer software (IR Affinity-1S). Baseline correction and automatic noise removal were performed throughout the IR in addition to the quantification of bands belonging to the amide group and hydroxyl group. Degree of deacetylation (DD) was calculated using the following Baxter equation [20]:

$$DD = \frac{A_{1650}}{A_{3450}} \cdot 115\% , \quad (1)$$

where:

DD – degree of deacetylation [%];

$A_{1650}$  – absorbance of the FTIR band at 1650  $\text{cm}^{-1}$ , amide group;

$A_{3450}$  – absorbance of the FTIR band at 3450  $\text{cm}^{-1}$ , hydroxyl group;

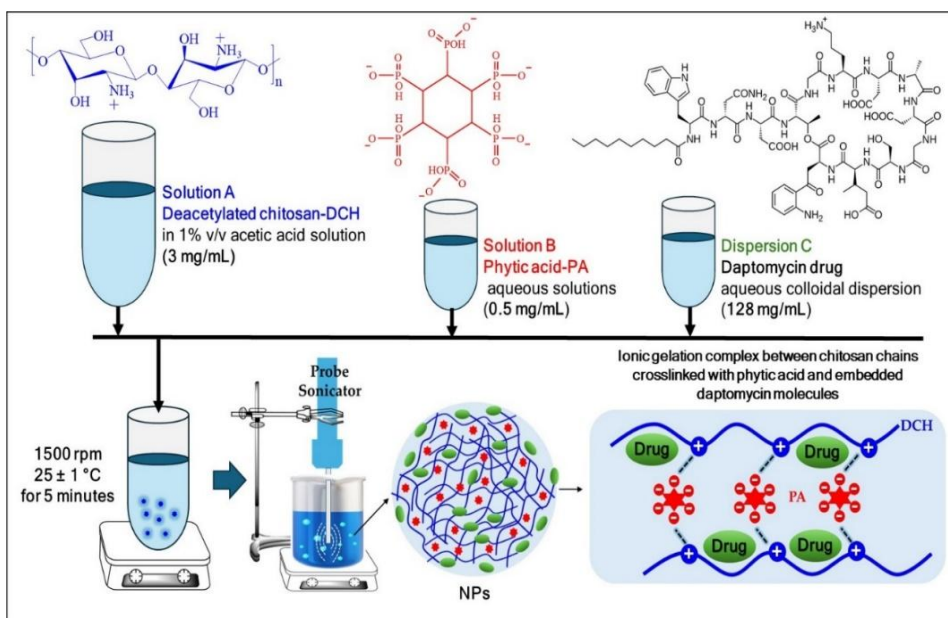
115 – the ratio of molecular weights of the *N*-acetyl-glucosamine and *N*-glucosamine subunits.

## 2.2. Preparation of Nanoparticles

A 3 mg/mL chitosan solution (in 1% acetic acid, v/v) of pH 3.5 was prepared and marked as solution A. At the same time and independently, an aqueous 0.5 mg/mL phytic acid solution (solution B) and a colloidal dispersion of daptomycin (2 mg/mL) (solution C) were prepared. Then, 0.872 mL of solution A, 1.0 mL of solution B and 0.128 mL of dispersion C were mixed in a Flacon tube, left under constant magnetic stirring at 1500 rpm for 5 minutes at  $25 \pm 1^\circ\text{C}$  until the formation of ionic complexes was achieved (Figure 1). Once the ionic association complexes were developed, the particle size was reduced employing a probe sonicator. For this, 2.0 mL of the final blend was taken and subjected to ultrasonic treatment using an ultrasonic probe (CL-18, tip 4422, diameter of 3 mm), where pulses of 15 s each followed by a 15 s resting time and an energy of 919 W were employed for a total treatment of 1 minute [21].

## 2.3. Physicochemical characterisation of Nanoparticles

These analyses were conducted using a Zetasizer nano ZSP (Malvern Instrument, Worcestershire, United Kingdom) equipped with a red He/Ne laser (633 nm). Particle size was measured using a dynamic light scattering (DLS) with a scattered angle of  $173^\circ$  at  $25^\circ\text{C}$ , and a quartz flow cell (ZEN0023). The zeta potential was determined using a disposable folded capillary cell (DTS1070). This instrument reports the particle size as the z-average diameter, and PDI ranging from 0 to 1, corresponding to a monodisperse and very broad distributions, respectively. All the nanoparticles (NPs) were dispersed in ultra-pure water employing a  $\sim 1:100$ , v/v dilution factor. All measurements were performed in triplicate and reported as the mean  $\pm$  standard deviation [21, 22].



**Figure 1.** Representative scheme of the development of chitosan nanoparticles crosslinked with phytic acid and loaded with daptomycin.

#### 2.4. Evaluation of the Antimicrobial Activity of Nanoparticles

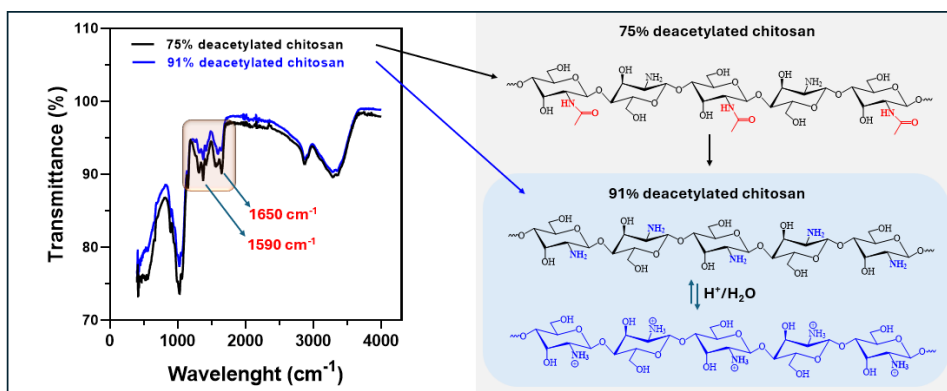
The antimicrobial activity of daptomycin-loaded NPs was determined by the broth microdilution method according to the guidelines of Clinical and Laboratory Standards [11]. At the first step, the bacterial strain (*S. aureus* ATCC 25923) was isolated in nutrient agar at 37°C between 24 and 48 h. Three to five isolated colonies were selected from the nutrient agar plate and suspended in a test tube with 0.9% saline solution. Subsequently, the turbidity was adjusted with a Vitek Densichek to a density corresponding to 0.5 of the McFarland standards. This pattern corresponds to a homogeneous suspension of approximately  $1.5 \cdot 10^8$  CFU/mL. A 1:100 solution was continuously prepared by transferring 10  $\mu$ L of the 0.5 McFarland solution to a test tube containing 9.99 mL of Mueller-Hinton Broth and vortexing thoroughly. A 1/1000 dilution factor ( $\sim 1 \cdot 10^5$  CFU/mL) was subsequently used for the tests. 50  $\mu$ L of this bacterial culture was inoculated for 18 - 20 h in 96-well plates at 37°C together with 50  $\mu$ L of each sample of the encapsulated and antibiotic systems with concentrations of 128, 64, 32, 16, 8, 4, 2, 1, and 0.5  $\mu$ g/mL. This procedure aims to determine the minimum inhibitory concentration (MIC), that is, the lowest concentration that prevents the growth of the bacteria [23].

### 3. Results and Discussion

#### 3.1. Deacetylation of Chitosan

The deacetylation process of commercial chitosan purchased from Sigma-Aldrich with a deacetylation degree of 75% was performed to remove the residual acetyl group and generate new amino groups that compose the new chitosan structure [24]. Chitosan is soluble in acidic aqueous solutions because the acidic medium protonates the free amino groups of the polysaccharide; as a result, chitosan becomes a cationic polyelectrolyte [25]. These positive charges increase its affinity for water, thus facilitating its solubility in the

acidic medium and influencing its use in multiple applications. By achieving a higher degree of deacetylation, chitosan of better solubility is formed [7]. Hence, the degree of acetylation is one of the most essential parameters, since it determines the polymer's chemical, physical and biological properties, making it suitable for application in fields such as biotechnology, biomedicine, food and pharmaceuticals [26]. The IR results, i.e., the decrease in the band corresponding to the amide group ( $-\text{CONH}_2$ ), which appears around  $1650\text{ cm}^{-1}$ , and the increase in the band associated with the amino group ( $-\text{NH}_2$ ), which is found around  $1590\text{ cm}^{-1}$ , allowed to establish the change in the degree of deacetylation of chitosan, which increased from 75 to 91% (Figure 2). IR results confirm that the deacetylation reaction occurred when the biopolymer was exposed to a highly concentrated alkaline medium at temperatures above  $60^\circ\text{C}$  [27]. As the deacetylation reaction proceeds, the number of amide groups decreases, and the number of amino groups increases [28]. The degree of deacetylation identifies the relative ratio of amino groups in the polymer; in addition, its crystalline form and protonatable amino groups ( $\text{NH}_3^+$ ) allow it to be soluble in acidic aqueous solutions, since the polarity and electrostatic repulsions increase [29].



**Figure 2.** Chitosan deacetylation process: (left) chitosan FTIR spectra, (right) scheme of the deacetylation process and formation of the cationic polyelectrolyte system in an acidic aqueous medium.

### 3.2. Physicochemical Characterisation of Nanoparticles

The results of particle size, as well as the polydispersity index (PDI) and the zeta potential for the NPs system, are summarised in Table 1.

**Table 1.** Results of the physicochemical characterisation of NPs in aqueous media.

Nanoparticle system	Particle size [nm]	PDI	Zeta potential [mV]
Unloaded NPs	$446.9 \pm 9.6$	$0.244 \pm 0.007$	$+10.7 \pm 1.3$
Daptomycin-loaded NPs	$453.9 \pm 4.7$	$0.245 \pm 0.037$	$+29.7 \pm 1.3$

The results show that the particle size remains unchanged when daptomycin is loaded into the NPs, describing values of  $446.9 \pm 9.6\text{ nm}$  and  $453.9 \pm 4.7\text{ nm}$  for unloaded and daptomycin-loaded NPs, respectively. The only slight increase in particle size can be attributed to the entrapment of the drug within the cross-linked system formed between chitosan and phytic acid. On the other hand, the size polydispersity values were found to

remain practically constant at  $\sim 0.245$ , indicating that the systems tend to be monodisperse and that the nanoparticle production process was appropriate. In contrast, the zeta potential results showed a moderate increase when the drug daptomycin was embedded inside the NPs. This result suggests a rearrangement of the chitosan polymer chains, with the protonated groups orientated toward the nanoparticle surface, which was reported previously in the literature [21, 30–34].

### 3.3. Minimum Inhibitory Concentration

Antibacterial activity was studied in terms of minimum inhibitory concentration (MIC), which is the lowest concentration of the antibiotic that inhibits bacterial growth in cell culture [35]. According to the Clinical and Laboratory Standards Institute (CLSI), *S. aureus* strains with a daptomycin MIC of  $\leq 4 \mu\text{g/mL}$  are considered susceptible [36]. The MIC value for encapsulated daptomycin was compared with the activity of the free antibiotic. Free daptomycin exhibited a minimum inhibitory concentration of  $64 \mu\text{g/mL}$  (Figure 3). Although this MIC result appears to be a high concentration, it is important to note that this antibiotic has extreme chemical instability in aqueous media. Therefore, using this antibiotic always requires administration under additional stabilisation, or, if used directly, the dose and frequency must be increased [37, 38]. Moreover, the antibacterial activity of daptomycin is calcium-dependent, as calcium ions induce conformational changes essential for its mechanism of action. These changes also facilitate daptomycin oligomerisation and membrane insertion, highlighting the importance of using a suitable culture medium for its analysis. As previously mentioned, this higher-than-expected value of MIC for sensitive *S. aureus* was obtained, which could be due to the physicochemical instability of daptomycin under storage. In a study, daptomycin concentrations decreased by more than 70% when stored at  $4^\circ\text{C}$  for 6 months [39]. Fernandez *et al.* [40] reconstituted vials of daptomycin ( $50 \text{ mg/mL}$ ) in aqueous solution and reported that the vial's content remained physicochemically stable for one week when refrigerated between  $2^\circ\text{C}$  and  $8^\circ\text{C}$ . Although the MIC value of daptomycin considerably exceeded the standard value for sensitive *S. aureus*, the ampicillin control was  $0.5 \mu\text{g/mL}$  [36].

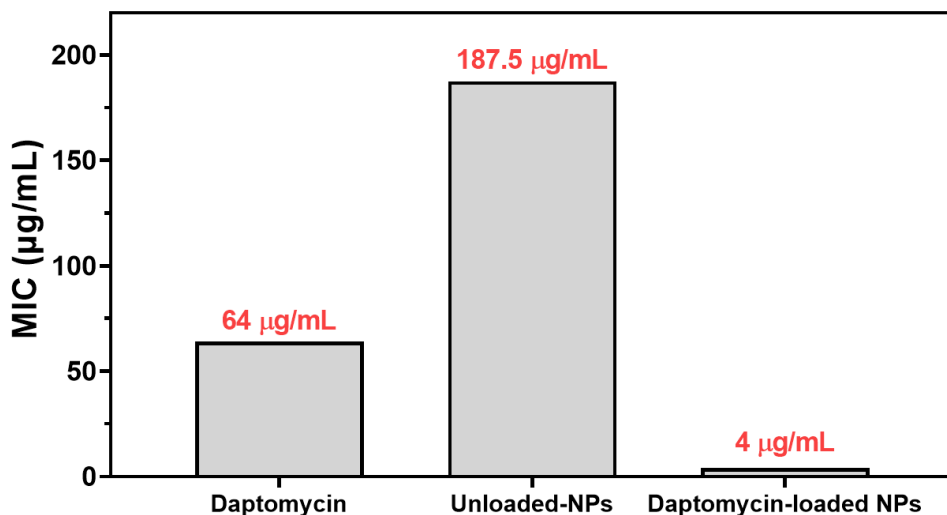


Figure 3. Minimum inhibitory concentration (MIC).

Daptomycin-loaded NPs allowed to reach a minimum inhibitory concentration of 4 µg/mL (Figure 3). This result allows to establish that chitosan nanoparticles contributed to increasing the effectiveness of the antibiotic against sensitive strains of *S. aureus*, reflecting a synergistic effect between daptomycin and chitosan. Daptomycin has been reported to require the presence of ionic species such as Ca<sup>2+</sup> to interact with undecaprenyl-coupled cell envelope precursors in the presence of the anionic phospholipid phosphatidylglycerol and form a tripartite complex that disrupts cell wall biosynthesis [41]. Besides, the dimerisation of anionic lipopeptides is dependent on the presence of Ca<sup>2+</sup>, which results in a structure exhibiting hydrophobic groups concentrated on one side of the dimer, while the flat surface that is formed on the opposite side is markedly hydrophilic [42]. Thus, several protonated amines of chitosan could contribute to the role of the surrounding Ca<sup>2+</sup>, favouring the construction of the daptomycin complex, which results in a structure suitable for the accommodation of a target molecule. However, to confirm this phenomenon, more detailed studies are necessary. Therefore, nanoparticles exhibit a potential for effective drug delivery, controlled drug release and improved therapeutic efficacy [43]. It was possible to evaluate that neat chitosan nanoparticles have MIC at a concentration of 187.5 µg/mL, confirming that this biopolymer can interact with *S. aureus* because its cell wall comprises networks with pores that allow the nanoparticles to easily enter the cell. Chitosan alters its structure through interactions with the cell membrane, and as a result, cellular inclusions are leaked (enzymes, nucleotides, proteins and ions) [44].

#### 4. Conclusions

It was established that the methodology used to develop nanoparticulate systems was adequate for obtaining high monodispersity (PDI < 0.3). Likewise, it was found that the antibiotic loading does not considerably affect NPs' size, while their zeta potential did increase moderately. This zeta potential change is interesting as its increase is usually related to the decrease in NPs aggregation and causes improvement of NPs' physical stability. Likewise, the increase in the zeta potential also improves bio-adhesion processes and consequently, the NPs could interact better with the microbial system. Finally, it was established that the unloaded-NPs do not have a relevant antibiotic effect against *S. aureus*. Moreover, when the antibiotic daptomycin is embedded in these NPs, there is a notable decrease in the MIC, from 64 to 4 µg/mL, indicating that this NP system improves antibiotic activity. Nevertheless, future studies are needed to determine the encapsulation yield of the antibiotic within the NPs and the mechanism of its release. It is also necessary to establish whether NPs improve the chemical stability of this drug, which is widely known for its high instability in aqueous media.

#### 5. Acknowledgements

*The authors thank the Universidad Santiago de Cali for the funding granted under the project, the investigative No. 939-621122-071.*

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