Assessing the Antimicrobial Activity of Chitosan Nanoparticles by Fluorescence-Labeling

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Abstract—Chitosan is a natural polysaccharide prepared by the N-deacetylation of chitin. In this study, the physicochemical and antibacterial properties of chitosan nanoparticles, produced by ultrasound irradiation, were evaluated. The physicochemical properties of the nanoparticles were determined by dynamic light scattering and zeta potential analysis. Chitosan nanoparticles inhibited the growth of *E. coli*. The minimum inhibitory concentration (MIC) values were lower than 0.5 mg/mL, and the minimum bactericidal concentration (MBC) values were similar or higher than MIC values. Confocal laser scanning micrographs (CLSM) were used to observe the interaction between *E. coli* suspensions mixed with FITC-labeled chitosan polymers and nanoparticles.

Keywords—Chitosan nanoparticles, dynamic light scattering, zeta potential, confocal microscopy, antibacterial activity.

I. Introduction

DURING the last decades, studies on the use of engineered nanoparticles (NPs) and nanomaterials (NMs) have increased in many research areas, with important applications in water purification, drug delivery, antimicrobial treatments, tissue engineering, biosensors, analyses related to magnetic diagnostics and detection of pathogens, toxins, drug residues and vitamins [1]. Several methods have been developed for NPs production, such as ionotropic gelation, spray drying, emulsification, and coacervation. More recently, ultrasonication [2], a green processing, has been frequently applied in NPs production. Some of the high intensity ultrasound (US) applications consist in effective polymer degradation, breaking up aggregates, and reducing NP size and polydispersity. Ultrasonication leads to the decrease of the mean diameter and polydispersity of particle size, as the time or amplitude of the irradiation process is increased [3]. The applications of nano-based composites and the physical stability of NPs are directly linked to material characteristics, such as chemical composition, size, shape, surface charge density, hydrophobicity [4], polydispersity and the presence or absence of functional groups or other chemicals [5]. These important characteristics define the potential applications of NPs.

Chitosan (CS) has attracted much attention, due to its biocompatibility, biodegradability, and antibacterial activity. Among CS applications, the production of biodegradable films and microcapsule implants for drug and vaccine delivery [6] may be highlighted. In addition, the reported antibacterial properties of CS were shown to depend on its molecular weight (MW), degree acetylation (DA), pH and, of course, the target organism [7]. CS shows several advantages over other types of disinfectants, because it possesses a high antimicrobial activity, a broad activity spectrum, and low toxicity to mammalian cells [8].

Although some studies on nanosized forms of CS concerning to their antimicrobial activities have been published, the small size of CS NPs renders them unique. This is because their physicochemical properties vary as a function of surface area (providing more cationic sites) and reactivity, which potentially determine the charge interactions with microbial surfaces and lead to superior antimicrobial effects [9]. In this context, the aim of the present study was to evaluate the CS NPs produced by US, determining their physicochemical characteristics and testing the antimicrobial activity against *Escherichia coli*.

II. Experimental Details

A. Polymer Degradation

NPs (CS-MMW30 and CS-LMW30) were produced from two commercial CS samples, of medium (CS-MMW) and low molecular (CS-LMW) weights, with an acetylation degree lower than 25% (Sigma-Aldrich® Co, MO, USA). Samples (2%w/v) were solubilized in 0.1 M sodium acetate pH 4.0 and irradiated in an ice bath with an SONIC 750 W model ultrasonic probe (Sonics & Materials, Inc., CT, USA) equipped with a 1/2" microtip, at 4 °C for 30 min. The irradiation was performed at 40% amplitude, under constant duty cycle with 1/1s intervals.

B. Dynamic Light Scattering (DLS) Measurements

The hydrodynamic radius (Rh) and polydispersity index (PdI) measurements of pre (CS-MMW and CS-LMW) and post (CS-MMW30 and CS-LMW30) irradiation NPs were performed using a DynaProNanoStar (Wyatt Technology Corporation, Santa Barbara, CA, USA).

C. Zeta Potential (ζ-Potential)

Zeta potential measurements were performed by applying an electric field across the samples and by measuring the velocity of the electrophoretic mobility of the particles using the laser Doppler anemometry technique. The measurements were performed five times for each sample at 25 °C using a

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NanoBrook 90Plus PALS (Brookhaven Instruments Co,NY, USA).

D. Antimicrobial Activity

The *Escherichia coli* DH5α strain (INCQS-Fiocruz, BRA) was grown in Luria-Bertani (LB) medium (BD, NJ, EUA), in an orbital shaker at 200 rpm and 37 °C for 24 h. 1 mL of the culture was centrifuged at 5.000 x g for 2 min and the supernatant was discarded. The cellular density was adjusted in saline solution (0.85% NaCl) to turbidity equivalent to McFarland 0.5 standard (1.5×10^8 CFU/mL) [10] to be used as an inoculum. The antimicrobial activity of the CS samples (CS-MMW, CS-LMW, CS-MMW30 and CS-LMW30) was evaluated on batch cultures containing CS derivatives -0.5 and 1.0 mg/mL–against *E. coli* in a cell suspension of 10^7 CFU/mL. 100 μL of each mixture was withdrawn and added to 96-well microplates Cellstar (Greiner Bio-One, Kremsmuenster, AUT). The microplates were incubated in the same conditions for 24 h, and the optical densities were determined at 620 nm with a VitorX4 spectrophotometer (PerkinElmer, MA, USA). Inhibition was calculated as described in the literature [11]. The MBC, which allows the initial viable cells to be reduced by at least 99.9%, was evaluated by adding 20 μL aliquots of negative wells (no growth), plated on LB and incubated at 37 °C for 48 h.

E. CS Fluorescent Labeling

A stock solution of fluorescein isothiocyanate (FITC) (1.0 mg/mL in dry MeOH) and CS samples (10 mg/mL in sodium acetate buffer 0.1 M, pH 4.0) were prepared. 10 μL of the FITC solution was added to 5 mL of the CS samples solutions and mixed under stirring for another 72 h at room temperature in the dark. Subsequently, the samples were submitted to dialysis in SnakeSkin® 3.500 MWCO dialysis tubing (Thermo Fisher Scientific, Waltham, MA, USA) against sodium acetate buffer 0.1 M, pH 4.0, for 18h in the dark.

F. Confocal Microscopy

The genetic engineered *E. coli* MG1655mCherry strain, obtained from Nottingham University [12], was incubated overnight in LB medium at 37 °C under stirring. The bacterial suspension was prepared using the overnight inoculum centrifuged at 10.000 g and 4 °C, for 10 min and washed twice with PBS. The cells were finally suspended in a buffer to OD_{600nm} 0.5, and the CS samples were added to the cell solutions at a final concentration of 3.5 mg/mL. A Zeiss LSM 700 confocal microscope (Oberkochen, Germany) was used for fluorescent microscopy studies.

III. RESULTS AND DISCUSSION

A. Characterization of the CS Polymers and NPs

The particle size distributions calculated by DLS and ζ-potential measurements before (CS-MMW and CS-LMW) and after (CS-MMW30 and CS-LMW30) ultrasonication for 30min are displayed in Table I. CS fragmentation by US and its capacity to produce NPs is known. In this study, the

irradiation process increased the percentage of smaller macromolecules obtained from the commercial CS samples. The high range of the polydispersity index (PdI = 2 - 4%) is an indicative of the existence of a group of particles with distinct sizes [13].

The mean radius values estimated at 25 °C are also displayed in Table I. The CS molecule radii ranged from 219 to 3083 nm. The ultrasonic irradiation resulted in the increase of macromolecules with low Rh. After ultrasound processing, the CS-LMW sample showed a major proportion of species with Rh of 3007 nm (72%) and a smaller proportion of Rh = 425 nm macromolecules (18%). The ultrasonic irradiation of the CS-LMW sample for 30 min generated 93 and 26% of CS-LMW-30 macromolecules, with Rh values of 492 nm and 2794 nm, respectively. The CS-MMW sample is composed of 47 and 31% macromolecules displaying Rh values between 403 and 3083nm, respectively. After ultrasound irradiation for 30 min, this sample yielded small CS-MMW-30 species, amounting to 67 and 28%, with Rh ranging from 218 to 482 nm, respectively.

The ζ-potential was high (close to 30 mV) and, consequently, the suspensions are more stable, avoiding particle agglomeration, due to high electrostatic repulsion, when compared to suspensions with lower ζ-potential values [13].

B. Antibacterial Activity of CS Polymers and NPs

The antimicrobial activity of both original and ultrasound-treated NPs was evaluated. The MIC and MBC for the CS samples, before CS-MMW and CS-LMW and after CS-MMW30 and CS-LMW30 ultrasound processing were evaluated for *E. coli*. The influence of the pH of the culture media on the antibacterial activities was also evaluated (Table II).

The MBC values were similar or higher than those found for MIC. The CS concentrations used to kill and inhibit bacteria were very close. CS samples, before and after sonication, showed bactericidal behavior, acting (i) by the lyses of cell wall and/or cell membrane of the target bacteria or (ii) by inhibiting DNA or RNA, injuring the replication nuclear process and/or gene transcription [14]. MIC values ranged from 0.2 to 0.4 mg/mL, depending on the pH of the medium. The CS-LMW30 sample was shown to be the most effective, whereas the least effective were CS-MMW and CS-MM30. The superior activity of CS-LMW and CS-LMW30 corroborates data from literature, in which the antimicrobial activity of CSs was reported to be dependent on MW and DA [15].

CSs show low solubility in weak acid environments, such as sodium acetate buffer, in which the pKa is near 6.0. When the pH is increased over pKa values, solubility is decreased, and precipitation occurs. At pH just below pKa, CS shows polyelectrolyte behavior, due to its charged amine group [16]. CS protonation causes its dispersion, leading to more effective activity against *E. coli*. MIC values against *E. coli* were shown to be pH-dependent.

TABLE I
PHYSICOCHEMICAL ANALYSIS FOR THE CS SAMPLES ESTIMATED BEFORE (SHADED COLUMN) AND AFTER US IRRADIATION (NON-SHADED COLUMN)

Sample	CS-LMW		CS-LMW-30		CS-MMW		CS-MMW-30	
ζ potential (mV)	24.51±1.29		26.12±0.85		26.52±2.4		24.78±2.4	
Rh (nm)	425	3007	492	2794	403	3083	219	482
Intensity (%)	18.8	71.9	93.1	26.4	46.9	31.8	67.0	28.8
PdI	4.49	4.24	1.83	2.88	3.4	1.8	3.3	3.8

Hydrodynamic radius (*Rh*), intensity percentage of light scattering (% intensity), polydispersity index (% PdI) measured by DLS and zeta potential (ζ) of CS samples.

The MIC values estimated at pH 7.0 and pH 5.0 for CS-MMW were 0.47 and 0.40 mg/mL, whereas for CS-MMW30, the values ranged from 0.40 to 0.28 mg/mL. When considering CS-LMW, values of 0.30 and 0.33 mg/mL were obtained, whereas for CS-LMW30, the MIC values were 0.30 and 0.20 mg/mL, in pH 7.0 or 5.0, respectively.

The results of this study demonstrated that NPs production by modification of the polymer chain length, following US irradiation, is a simple and efficient method that dispensed the need for acid addition. The MW modifications allowed for satisfactory changes in the antimicrobial ability of the produced NPs.

A MIC value of 0.2 mg/mL was observed at pH 5.0, as expected for a polyelectrolyte with positive charged amine groups, improving the macromolecule bonds to the negatively charged plasmatic membrane of bacteria and/or interfering with the nuclear replication process and/or gene transcription.

However, this explanation requires further investigation to be confirmed [17].

TABLE II
EFFECT OF MOLECULAR MASS OF CS ON MIC AND MBC AGAINST *E. COLI* CELLS

Sample	MIC (mg/mL)		MBC (mg/mL)	
	pH 7.0	pH 5.0	pH 7.0	pH 5.0
CS-MMW	0.47±0.06[a]	0.4±0.00[a]	0.4	0.4
CS-MMW30	0.4±0.00[a]	0.28±0.03[b,c]	0.4	0.3
CS-LMW	0.3±0.00[b]	0.33±0.06[c]	0.3	0.3
CS-LMW30	0.3±0.00[b]	0.2±0.00[b]	0.4	0.2

MIC and MBC from pre-sonicated, medium molecular mass (CS-MMW), low molecular mass CSs (CS-LMW), post-sonicated medium molecular mass (CS-MMW30) and low molecular mass (CS-LMW30) CS samples measured against *E. coli* (DH5α). Values are expressed as means ± standard deviations. Superscripts in the same column with different letters indicate statistically significant differences (p < 0.05).
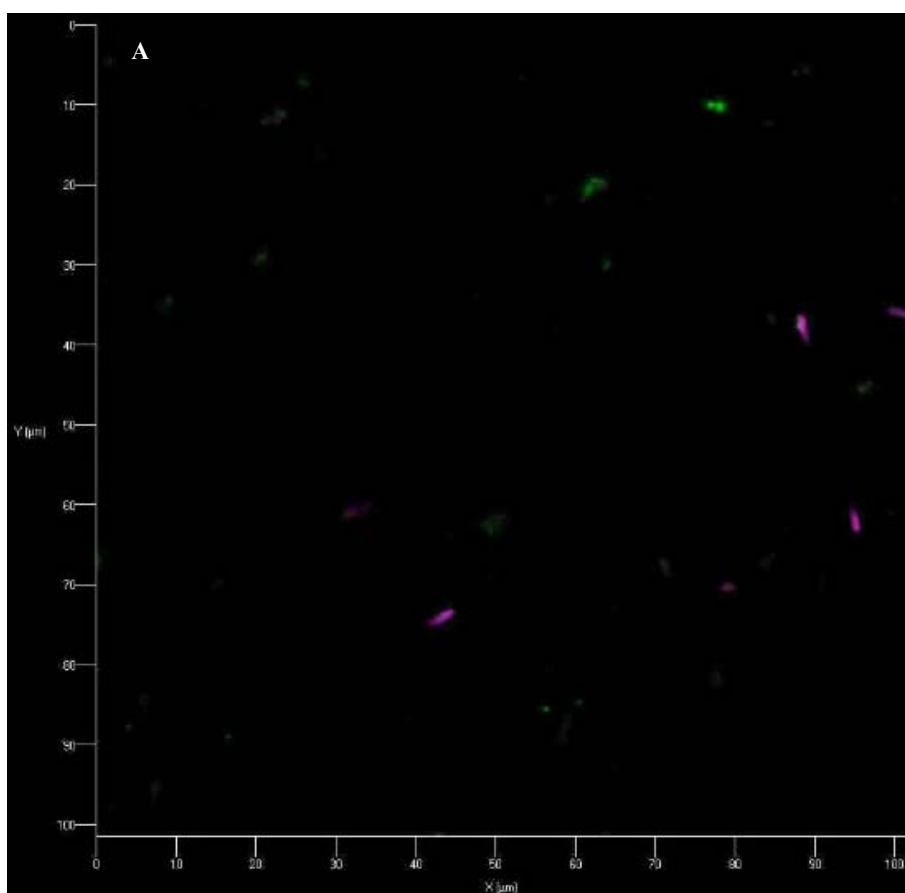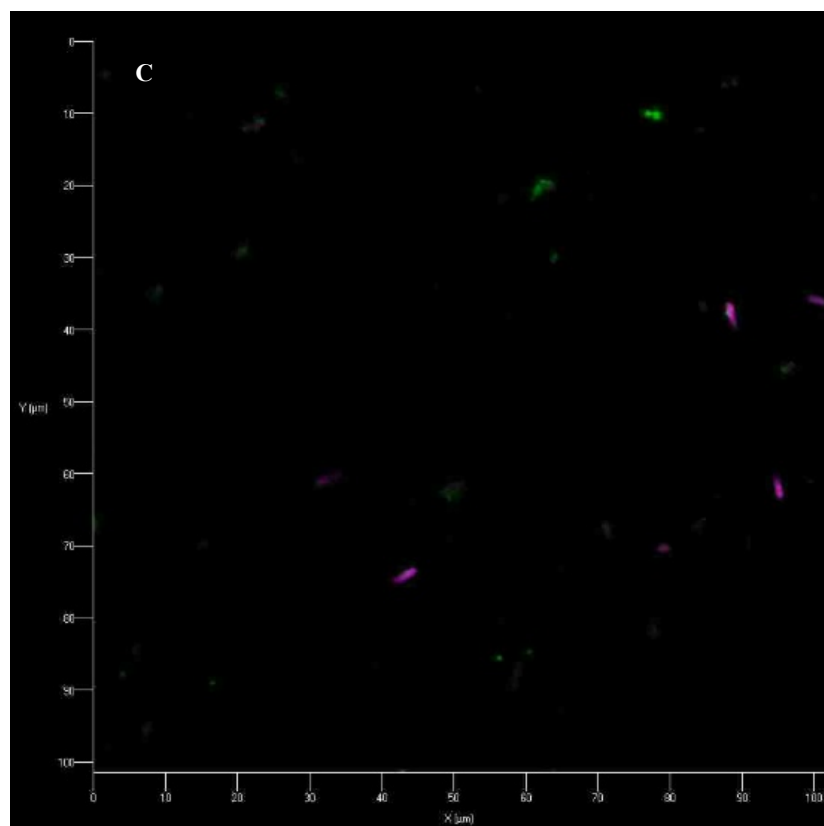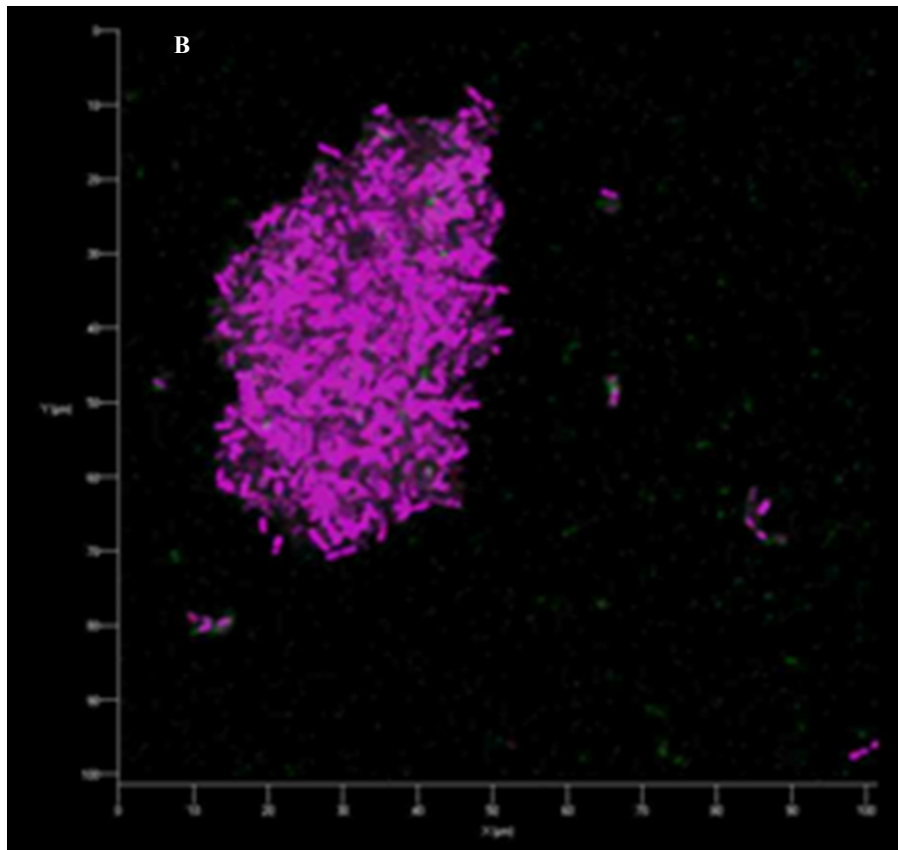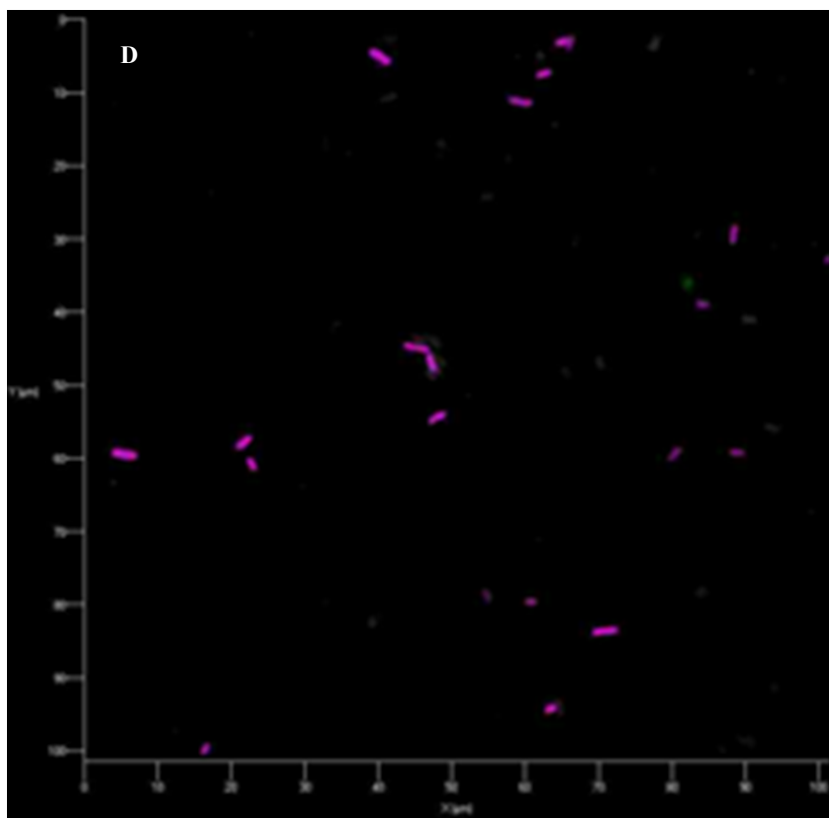
Fig. 1 CLMS of mCherry-*E. coli* (pink) in the presence of FITC labeled CS samples (green). Ortho projections from the overlaid Z-stacks in the red and green channels. (A) CS-LMW, (B) CS-LMW30, (C) CS-MMW and (D) CS-MMW30, transmission micrographs. The red channel has been depicted in pink in all figures

C. Confocal Microscopy Analysis

In order to further probe polymer behavior of on *E. coli* cells, an engineered bacterial strain expressing the fluorescent proteins mCherry (*E. coli*) was mixed with polymers and NPs, labeled with fluorescein isothiocyanate (FITC). CLSM were obtained from *E. coli* (pink fluorescence) suspensions mixed with FITC-labeled polymers and NPs (CS-MMW, CS-LMW, CS-MMW30 and CS-LMW30). The green color of these micrographs clearly indicated, from the analysis of Z-stacks, that the CS-MMW and CS-LMW samples (green fluorescence) were located around individual bacterium (Figs. 2 (A) and (C)). These molecules present higher hydrodynamic radius when compared with the sonicated samples. As already demonstrated, these CS polymers display fibrous characteristics, forming a linear cationic fiber polymer [3], [18].

Fig. 1 (B) shows the cluster formation, and Fig. 2 (D) presents the smallest cluster formation. In addition, the CS NPs (CS-MMW30 and CS-LMW30) showed an easy interaction with bacteria. Because of their large surface areas, the protonated amine groups are nonselective-ligand that bind to *E. coli* by electrostatic attraction. *E. coli* has a natural negative charge membrane, which favors these interactions [19]. According to the literature, electrostatic attraction between negatively charged bacterial cells and positively charged NPs are crucial for NPs activity as bactericidal materials [20], [21]. As displayed in Fig. 1, multiple positive charged-CSs (in green) could strongly attach to the surface of negative charged bacteria (in pink) through attractive electrostatic interactions, resulting in the adhesion of several CSs macromolecules and NPs to the bacterial surface. These results clearly demonstrate the ability of CS, polymers and NPs, to interact with the cell membrane and, particularly, of the NPs to promote the aggregation of *E. coli* cells.

IV. CONCLUSION

The results presented here demonstrated that highly concentrated and nonhazardous nano-sized CS particles can be easily prepared in a cost-effective manner and should be considered as a novel greener bactericidal NM. The physical and chemical characteristics of CS NPs produced by US irradiation were evaluated and compared regarding their *in vitro* inhibition activity towards *E. coli*. The experimental data obtained gave evidence that CS NPs, displaying low and medium molecular masses, can strongly inhibit pathogen growth, and that the inhibitory ability can be controlled by pH and MW. The variation in MW and particle size/zeta potential allowed an easy manipulation of the NPs physicochemical properties. Additional studies would contribute to further improve the understanding of the exact mode of action of CS

NPs and their antibacterial activity.

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