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Preparation of Chitosan Nanoparticles and Chitosan Nanoparticles Loaded with Azithromycin and the Study of Their Characterizations

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Abstract

A noteworthy advancement in recent years has been the identification of nanoparticles, which are made of organic (carbon-based) or inorganic (metal-based) materials and range in size from 1 to 100 nm. This study's objective was to create, standardize, and analyze chitosan nanoparticles as well as chitosan nanoparticles that contained azithromycin. In the experiment, chitosan nanoparticles (CSNPs) and chitosan nanoparticles loaded with azithromycin were biosynthesized and characterized. (AZM-CSNPs) which is performed by spectrophotometry, (FTIR), (XRD), (SEM), (TEM), UV- spectrophotometer analysis and (DLS) Analysis. The results revealed atypical EM micrograph of CSNPs obtained by the reduction of CSNO3 solution. Applying Scherrer's equation, the average grain size of the AZM-CSNPs generated during the bio-reduction process was calculated to be 31.5 nm. At a chosen concentration, CSNPs were 200 nm in size and had a zeta potential of 50 mV, whereas AZM-CSNPs were 51.1 nm in size and had a zeta potential of 50 mV.

Key words: Antibacterial resistances; Azithromycin; Chitosan nanoparticles; Electronic microscope

A good example of an antibiotic that is predominantly used to treat respiratory tract infections is azithromycin, which also treats infections of the ears, sinuses, skin, and throat in addition to pneumonia (infection of the lungs) and bronchitis (infection of the tubes leading to the lungs) (belongs to a class of drugs called macrolide antibiotics). It is also used to treat and manage *Mycobacterium avium* complex, a lung infection that commonly affects people with the human immunodeficiency virus (HIV) (McMullan and Mostaghim). *H. pylori*, a bacteria that causes ulcers, is killed by it when combined with other medications. Antimicrobial resistance is a rising public health concern, and the advent of multi-resistant bacteria has made things much more challenging, such as [*Klebsiellapneumoniaecarbapenemase* and *Haemophilusinfluenzaeblactamase*] (Hernandez-Lauzardo *et al*., 2011). Consequently, there was an urgent need to develop new drugs, but the current rate of this process is very slow. Research on treating infectious diseases has therefore shifted away from looking for new antibiotics and toward discovering alternatives, and nanotechnology has demonstrated that it offers efficient alternatives (Ferreira *et al*., 2019). The discovery of nanoparticles made of organic (i.e., carbon-based) or inorganic (i.e., metal-based) materials with sizes ranging from 1 to 100 nm is one of the key developments in recent years(Fair and Tor, 2014). Since nanoparticles offer significant intracellular penetration and the potential for effective intracellular antibacterial activity over longer time periods, their synthesis or extraction is crucial in this context and has potentially promising uses in the fight against the growing number of antimicrobial resistant pathogenic microbes that pose a constant threat to human health (Yap *et al*., 2014; Mohammed and Attia, 2022). As a result of their high stability, ability to be produced in large quantities, biodegrad-

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ability, non-toxicity, and lack of leakage impurities, polymers are the most common nanoparticles form used in pharmaceutical substances (Uskokovic and Desai, 2014). As an example, Chitosan, a polymer derivative of glucan with repeat units of chitin with the chemical formula (C8H13NO5), is used in the preparation (Zhang *et al*., 2010; Wang *et al*., 2020). Chitosan is used as a wound healer and to lower cholesterol. This substance is employed to deliver the medicine and the gene to the target cells because of its positive charge and its capacity to attach to levels of negative charge (Kammoun *et al*., 2013; Marathe *et al*., 2013). When combined with medications like penicillin G, amoxicillin, and azithromycin, polymer nanoparticles have antibacterial activity against both gram-negative and gram-positive bacteria (Silva, 2004; Han *et al*., 2015; El-Oksh *et al.,* 2022). So, the objectives of this study were to synthesize, standardize, and characterize chitosan nanoparticles as well as chitosan nanoparticles that had azithromycin loaded into them using [spectrophotometry, Fourier transform infrared spectroscopy (FTIR), X-ray spectroscopy (XRD), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and UV-Visible spectrophotometer], chitosan nanoparticles (CSNPs) and chitosan nanoparticles loaded with azithromycin (AZM-CSNPs) were biosynthesized and characterized. As well as comparison evaluation of the antibacterial efficacy of Azithromycin-loaded chitosan NPs, Chitosan NPs and Azithromycin against pathogenic bacteria isolated from individuals with respiratory infectionsusing Minimum Inhibitory Concentrations (MIC) by Well Diffusion Method.

Material and Methods

Synthesis of chitosan nanoparticles:

Chitosan Nanoparticles Preparation

In 2022, chitosan nanoparticles (CS) are synthesized using the ionic gelation process (Sreekumar *et al*., 2018) at the Department of Biotechnology, University of Baghdad. By gelating a CS solution containing sodium tri polyphosphate, CS nanoparticles were produced (TPP). Ionotropic gelation is caused by the interaction of positive charges amino groups with negative charges TPP. Chitosan has been decomposed in 1 percent acetic acid aqueous solutions for 20–24 hours at room temperature with magnetic stirring until a clear solution was produced. Chitosan concentrations ranging from 0.05 to 0.5 percent w/v were synthesized. To prevent particle aggregation, the surfactant span 80 [0.5 percent (v/v)] is added into chitosan solutions, and the pH is altered to 4.6–4.8 using 1N NaOH. A 0.1 % sodium tri poly- phosphate solution is prepared, and 10mg of TPP was dissolved into 10ml of de- ionized water before diluting it to get the following ratios: 0.25, 0.50, 0.75, 1, 1.5, and 2 mg/ml. A 0.22 micron filter was used to filter each solution (Millipore). The TPP solution was then syringed into the chitosan solution while being magnetically stirred at a speed of 800 rpm at room temperature at a concentration of 2.5: 1 (v/v) (chitosan: TPP).The specimens are inspected visually and sorted in three groups: pure solution, opalescent suspension, and aggregates. That opalescent suspension zone is made up of incredibly tiny particles. The resultant chitosan particle dispersion had been centrifuged for another 30 minutes at 12000g. The particle was dissolved again in water. Before using or analyzing this chitosan na- noparticles dispersion, it is freeze-dried.

Characterization of biosynthesized CSNPs:

The following approaches are used to characterize the produced chitosan nanoparticles:

Infrared Spectroscopy Using the Fourier Transform (FTIR)

On the recording, Fourier transform infrared spectroscopy (FTIR, 8000 Series, Shimadzu). Using an FTIR spectrophotometer, the interaction between the biosynthesized CSNPs and biomolecules (which are in charge of reducing, capping, and stabilizing the CSNPs in colloidal solution) was examined. The powder samples from the CSNPs solution were ready for FTIR analysis after 15 minutes of centrifugation at 10,000 rpm. To get rid of any free molecules from the CSNPs surface, deionized water was used to clean the solid pellet three times. The residues were dried at 40 degrees Celsius before the FTIR analysis. The samples were put into discs under high pressure along with the binding agent KBr

(hydraulic pressure). The FTIR spectra were then obtained by scanning the discs (Agarwal *et al*., 2018).

Electron Microscopy studies.(SEM, TEM).

Electron microscopy analysis: After being created, the synthetic chitosan nanoparticles were examined under a stereoscan scanning electron microscope (SEM) (model S360 brand SEM Leica Cambridge, Cambridge, UK). The dimensions and form of the chitosan nanoparticles were also examined using transmission electron microscopy (TEM) (Netherlands, Eindhoven, FEI Company, Philips CM200 EFG). Additionally, the Autoprobe CP Research AFM apparatus was used to take the atomic force microscopy (AFM) images (USA, Thermo microscopes, model AP- 2001) (Agarwal *et al*., 2018).

Tested organisms

All tested bacteria that used in this work obtained from Al-Zahraa Teaching Hospital in Wasit Province (*Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*).

Determination of Minimum Inhibitory Concentration (MIC) by Agar well diffusion method

Following the guidelines provided by CLSI as described by Eloff in, 1998, an Agar well diffusion test was conducted to quantitatively measure the antimicrobial activity of azithromycin and nanoparticles against the test bacterial isolates plate in vitro. In 1.5 ml micro centrifuge tubes (Eppendorff), stock solutions of chitosan nanoparticles and chitosan-azithromycin-loaded nanoparticles were made by melting powdered chitosan nanoparticles and chitosan l-azithromycin-loaded nanoparticles in dimethyl sulfoxide (DMSO) to a final concentration of 2.5 mg/ml and filtering through a 0.22 Millipore filter. Similarly, the antibiotic medication azithromycin was dissolved in an appropriate solvent (DMSO), resulting in an initial concentration of 2.5 mg/ml for all the antibiotics used in the current study. The stock solution was serially diluted by twofold serial dilutionsto obtained concentrations ranging from 1.25 mg/ml to 0.07 mg/ml. A second Eppendorff tube containing 100 µL aliquots of sterile DMSO is aseptically filled with 100 µL of the stock solution (azithromycin, chitosan nanoparticles, and chitosan-azithromycin-loaded nanoparticles), diluting the stock solution by 50% to 1.25 mg/mL. Following the correct mixing of the components in the Eppendorff tube, 100 µL aliquots from the second tube were moved to the third tube, which likewise had 100 L aliquots of DMSO. The antibacterial agent was mixed and then diluted by 50%, or 0.623 mg/ml. The following dilutions were obtained by repeating the same process for every Eppendorff tube: 0.312 mg/ml, 0.15 mg/ml, and finally 0.07 mg/ml. In this investigation, the CLSI-recommended agar well diffusion test was employed. By using a clean swap to distribute the bacterial inoculum over the whole surface of the Muller-Hinton agar medium, the agar plate's surface gets infected, as per the disk diffusion procedure. Next, a sterile cork drill is used to aseptically punch a 6 mm diameter depression

Statistical analysis

Microsoft Excel 2010 and the Statistical Package in the Social Sciences version 19 for Windows software have been utilized for the statistical analysis. Means, SD (standard deviation), plus parametric statistical significance analyses are used to define continuous random variables having appropriately distributed distributions. For descriptive analysis, frequencies as well as estimated percentages were determined (SAS, 2018)

Resultsand Discussion:

Biosynthesis of CSNPs:-

Infrared Spectroscopy using Fourier Transform (FTIR)

The FTIR measurement of the dried and powdered sample of the CSNPs was performed to provide to learn much more about chemical bonds as well as molecular structures of a potential substance that might aid in the CSNPS's CS ion reduction. The biosynthesized CSNPS's FTIR spectra showed numerous distinct peaks at 2920, 1631, 1406, 1048, and 864 cm-1 (Fig 1). The peak at 3273 {cm-1} is attributed to this stretching vibration of something like the [O-H] bond in phenols and alcohol, while the [N-H] stretch vibration in primary amides of

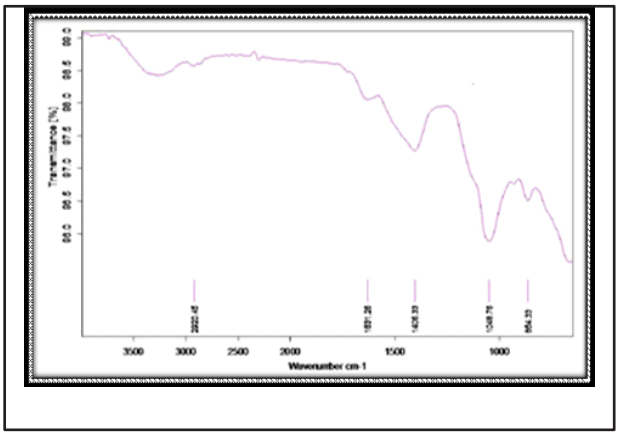

Fig 1: FTIR spectrum of synthesized CSNPs with distinct peaks

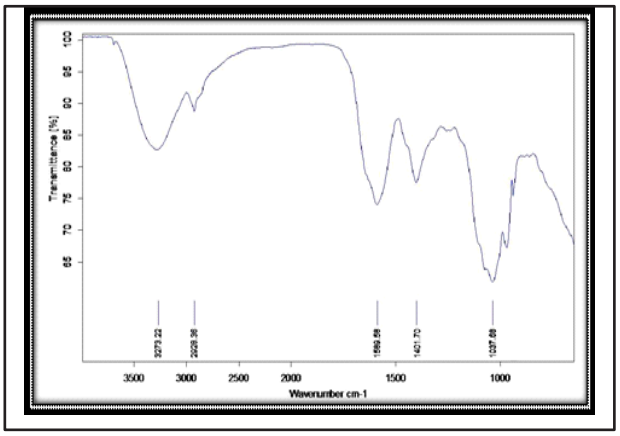

Fig 2: FTIR spectrum of synthesized CSNPs+AZM, with distinct peaks.

protein. The biosynthesized CSNPS + AZM's FTIR spectrum revealed the presence of numerous distinct peaks at 3273, 2982, 1406, 1589, 1401 and 1037 cm-1 (Fig 2). The [C-H] stretch in the protein's methylene groups and the [N-H] stretch for the amine salt might both be used to explain the peaks at 2982 cm-1 and 1406 cm-1, respectively. The absorption peak at 1401 cm-1 may have its roots in this stretching of the C-H symmetry in alkenes. Whereas the band at 970 cm-1 is associated to the connected [C=C] stretching vibration related to an aromatic ring, the band at 1037 cm-1 is related to carbonyl stretches [+N-H] stretches leading to the creation of amide I and amide. The functional biomolecules in AZM were shown to be essential for such biotransformation of CS ions to CSNPs, according to the Fourier transform infrared spectra. This is revealed by distinct peaks located at two IR spectrum regions: functional group region and fingerprint region (Fig 4). The organic compound shows absorbance bands in functional group region, while the metal normally gives absorption bands in fingerprints region resulting from the atomic vibration of molecule.

Electron Microscopy (EM) analysis

The form, size, and morphology of biosynthesized CSNPs were studied using electron microscopy. A representative EM micrograph of CSNPs generated mostly by reduction of CSNO3 solution is shown in (Fig 3). CSNPs had a spherical shape and were distributed evenly (mono-dispersed) without considerable clustering. The size of the particles varied from (10 - 40) nanometers. The biosynthesis of CSNPs was further characterized for its shape, size, morphology and surface chemistry by EM analysis. CSNPs are spherical and evenly scattered (mono dispersed) without considerable aggregation, according to the nanostructural analysis of the current discovery by EM microscopy (Fig 4).

Determination of Minimum Inhibitory Concentrations (MIC) by Well Diffusion Method:

Table (I) provides an explanation of the antibacterial activity in minimum inhibitory concentration (MIC) demonstrated by pure Azithromycin antibiotic and nanoparticles (chitosan nanoparticles and chitosan l-azithromycin loaded nanoparticles) against the tested microorganisms. The study found that the chitosan-azithromycin loaded nanoparticles had Minimal Inhibitory Concentrations (MICs) ranging from 0.312 to 0.07 µg/ml. In contrast, the MICs of the pure azithromycin and the chitosan nanoparticles alone were found to be between 2.5 and 0.07 µg/ml and 2.5 and 0.07 µg/ml, respectively. These results indicate that the chitosan-azithromycin loaded nanoparticles outperformed the pure azithromycin and the chitosan nanoparticles alone in terms of their antimicrobial properties.

Due to the development of antibiotic resistance and the increase in infectious diseases associated with dangerous bacteria, there is an increasing need for the discovery and

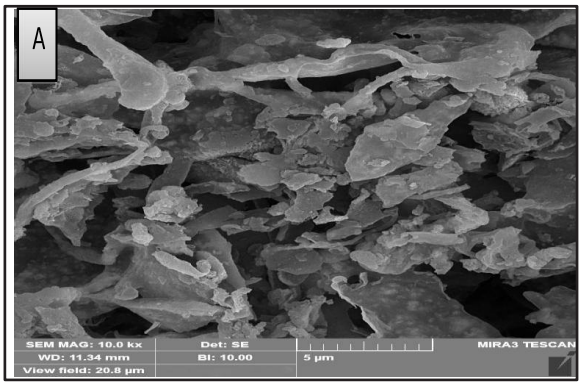

Fig 3: SEM of Biosynthesized CSNPs (A)

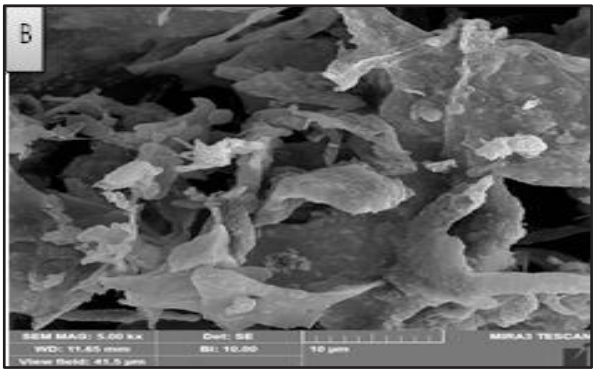

Fig 4: SEM of Biosynthesized CSNPs+AZM (B)

Table I : MIC of bacterial strains for Azithromycin and Nanoparticles.

BacterialIsolates	AZM	CSNPs	CSNPs+AZM
Klebsiellapneumonia	0.07	1.25	0.07
Staphylococcusaureus	2.5	2.5	1.25
Pseudomonasaeruginosa	0.15	1.25	0.07

production of novel antibacterial drugs. After a 24-hour incubation period at 37 °C on Müller-Hinton agar, the minimum inhibitory concentration (MIC) of chitosan-azithromycin-loaded nanoparticles against bacteria is determined. Three different concentrations (0.321, 0.15, and 0.07) of chitosan-loaded azithromycin nanoparticles were tested against K. pneumoniae, S. aureus, and P. aeroginosa. Due to the increasing number of antibiotic-resistant microbes and their apparent ability to address this problem, nanoparticles are currently considered a plausible replacement for antibiotics.

The agar well diffusion method was used to evaluate the antimicrobial activity of CSNPS+AZM against the isolated bacteria. Specifically, the study found that the chitosan-azithromycin-loaded nanoparticles had a range of 0.312 to 0.07 g/mL in their minimum inhibitory concentrations (MICs). In contrast, the MICs of pure azithromycin and chitosan nanoparticles alone were between 2.5–0.07 g/mL and 2.5–0.312 g/mL, respectively. Consequently, it is evident that the combined action of the two active ingredients is more effective as an antimicrobial agent than the action of a single active ingredient alone. This could be related to the ability of NPs to cross the outer membrane and cell envelope.

Scanning electron microscopy [(SEM), energy dispersion x-ray spectroscopy (EDS), and UV-Vis spectroscopy were used to characterize biogenic nanoparticles (UV)]. X-ray diffraction [(XRD) spectroscopy and atomic force microscopy (AFM)].By using the contrast color shift brought on by the surface Plasmon (SPR) phenomena, nanoparticles are produced. The external cell character of the mechanism of NP synthesis is amply demonstrated by the change in external cell medium color (Shaligram *et al*., 2009). A crystal-clear sign that nanoparticles are forming in the reaction mixture is the presence of dark gray color for AZM but also turbid yellow color for CSNPS+AZM in a solution mixture actually contains the biomass. The UV region's absorption peak wavelength is 260 nm. However, in the instance of CSNPS + AZM, a greater intensity level over CSNPs biopolymer had been seen. This is because NPs loaded AZM formed in this situation. As would be demonstrated later, these results were completely consistent with IR as well as X-ray analysis. The peak level at 401.9 nm was visible when CSNPs were characterized using a UV spectrophotometer. This might be because chitosan contains the amido group. Chitosan nanoparticles had a peak at 310 nm in 2014, according to (Krishnaveni and Priya, 2014). Chitosan's peak was measured in 2005 by (Liu *et al*., 2005) at 201 nm. The FTIR measurement of the prepared sample of the CSNPs and CSNPS +AZM as shown in (Fig 1 and 2) were done to offer information about the molecular

structures and chemical bonds of a possible material that could have a role in the reduction of CS ions for CSNPS. Such peak level at {3273 cm-1} for biosynthesized CSNPS + AZM is due to the expanding vibration of the [O-H] bond for alcohol and phenols as well as the [N-H] stretch vibration in basic amides in protein. The peak period at 2982 cm-1 with 1406 cm-1 may be attributed to the [C-H] stretch of methylene groups in proteins or the [N-H] stretch of the amine salt. Alkenes' [C-H] symmetric stretching vibration can be used to explain the optical absorption at 1401 cm-1. The band at 970 cm -1 corresponds to the [C=C] stretch related to an aromatic ring, whereas the peak at 1037 cm -1 indicates the presence of amide I plus amide resulting from either carbonyl stretching with N-H stretching or the C=C stretch associated with an aromatic ring (8). The infrared spectra of the Fourier transform indicated that the AZM included active biomolecules that could be in charge of the bio transformation of CS ions into CSNPs. This is revealed by distinct peaks located at two IR spectrum regions: functional group region and fingerprint region (Fig 2). The organic compound shows absorbance bands in functional group region, while the metal normally gives absorption bands in fingerprints region resulting from the atomic vibration of molecule. The biosynthesis of CSNPs was further characterized for its shape, size, morphology and surface chemistry by EM analysis. The nanostructural analysis of the current discovery using EM micrographs revealed that CSNPs are spherical and evenly scattered (mono dispersed), with little to no aggregation (Fig 4). The CSNPs' SEM pictures clearly depict a spherical-like shape, aggregated particles, and a range of particle sizes between 10 and 40 nm this result almost in line with (Ali and Yasssein, 2021).While the size of CS-AZM nanoparticle was ranged from 30-50 nm.The obtained results confirmed that the antibiotic was successfully loaded in the polymer matrix. Additionally, the consistency of the nanoparticles' sizes can be seen. These findings are entirely consistent with earlier research (OH *et al*., 2019). The data presented above clearly reveal that the CNPs have a very porous surface as a result of agglomeration characteristics. The CNPs are beneficial as a crucial chitosan-based bio-nanopesticide due to their porosity nature and aggregation abilities (Saharan *et al*., 2015). For the synthesis of new CSNPs in biomedical applications and nanomedicine, agglomeration has been identified as the fundamental phenomena (Ghadi *et al*., 2014).

Conclusion

Ionic gelation was used to successfully create chitosan nanoparticles. These nanoparticles produced nanoparticles very efficiently. The presence of azithromycin in the nanocomposite was confirmed by UV-Vis spectroscopy, SEM, and TEM examination. At the selected concentration, CSNPs had a size of [(200 nm) and a zeta potential of {50 mV}], whereas CSNPs+AZM had a size of {51.1 nm} and a zeta potential of (50 mV). The technology used in the current research is both economical and environmentally friendly for creating azithromycin nanoparticles. Hence, in upcoming biomedical applications, the produced CSNPs and AZM-chitosan nanoparticles can be used as an effective antibacterial material.

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Conflict of interest:

There was no conflict of interest.

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