GMR
The Original

DEVELOPMENT OF CHITOSAN/COLISTIN NANO-ANTIBIOTICS AND THEIR *IN VITRO* ACTIVITY AGAINST MULTIDRUG-RESISTANT *ACINETOBACTER BAUMANNII*

Haseeb Ur Rahman[1], Shaista Jabeen[2], Wajiha Kanwal[3], Efrah Mansoor[4], Ezza Mansoor[5], Emaan Mansoor[6], Afsheen Mansoor[7], Sudhair Abbas Bangash[8], Iqbal Nisa[9]*

[1]Department of Microbiology, The University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan, Email: haseeb39649@gmail.com
[2] Institute of Health Sciences, Khyber Medical University, Islamabad Campus, Islamabad, Pakistan; Department of Biosciences, COMSATS University Islamabad, Islamabad Campus, Islamabad, Pakistan.
[3]Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan, Email: wajiha.kanwal@ucp.edu.pk
[4]Islamic International Dental College, Riphah International University, Islamabad, Pakistan, Email: eframansoor2@gmail.com
[5]Islamic International Dental College, Riphah International University, Islamabad, Pakistan, Email: ezzamansoor1@gmail.com
[6]Islamic International Dental College, Riphah International University, Islamabad, Pakistan, Email: emaanmansoor5@gmail.com
[7]Department of Dental Material Sciences, Shaheed Zulfiqar Ali Bhutto Medical University, Islamabad, Pakistan.
Email: drafsheenqamar@gmail.com
[8]Faculty of Life Sciences, Department of Pharmacy, Sarhad University of Science and Information Technology, Peshawar, Pakistan.
Email: sudhair.fls@suit.edu.pk
[9]Department of Microbiology, Women University Swabi, Swabi, Khyber Pakhtunkhwa, Pakistan, **Email:** nisayam55@gmail.com
***Corresponding Author:** Iqbal Nisa, nisayam55@gmail.com

ABSTRACT
Multidrug-resistant (MDR) *Acinetobacter baumannii* and *Klebsiella pneumoniae* cause serious hospital-acquired infections, for which colistin is often one of the final treatment options. However, its clinical utility is hindered by dose-related toxicity and resistance development. In this study, chitosan–colistin nanoparticles (CS–Col NPs) were fabricated using the ionic gelation technique as an alternative delivery platform for *A. baumannii* therapy. The particles were analyzed using dynamic light scattering (DLS), scanning electron microscopy (SEM), X-ray diffraction (XRD), and UV–visible spectroscopy. DLS revealed a hydrodynamic diameter of 245 nm with a polydispersity index of 0.26, whereas SEM imaging showed a dry-state diameter of 348 nm. The zeta potential of +25.6 mV indicated good colloidal stability. XRD patterns contained broad peaks at ~10° associated with chitosan and a sharp peak at ~29° attributed to crystalline colistin, suggesting a semi-crystalline composite structure. UV–visible analysis showed a distinct absorption peak at ~298 nm with greater intensity than blank nanoparticles, confirming efficient drug loading. Antimicrobial assessment using broth microdilution revealed a minimum inhibitory concentration (MIC) of 0.25 µg/mL for CS–Col NPs, half that of free colistin, with a fractional inhibitory concentration (FIC) index of 0.5 indicating synergy. Compared with recent nanoparticle systems reported in the literature, the formulation achieved smaller particle size, narrower distribution, higher stability, and equal or superior antibacterial enhancement, highlighting its potential as a safer and more effective colistin delivery system.

KEYWORDS: Nano-antibiotic synthesis, *chitosan* Nanoparticle, Antibacterial, Characterization, Multi-drug Resistant

1. INTRODUCTION
Nanomaterial's' exceptional mechanical, optical, electrical, and chemical properties make them a valuable tool in medicine and pharmaceuticals for sensitive biological molecule detection, safer and more precise imaging of injured tissues, and novel therapeutics. [1]. Nano medicine is an emerging field that deals with the usage of medicine/drugs at Nano scale level to treat different disease. The US Food and Drug Administration has already granted approval for several chitosan nanoparticle-based products to be used in wound healing. Different combination ofantibiotics with chitosan like Tigecycline or Meropenem, Meropenem was successfullycombined with tigecycline and chitosan to treat MDR *A. baumannii* infections. The numerous uses of chitosan nanoparticles are effective drug carriers that are easily mutable owing to their biocompatibility and biodegradability, such as loading protein pharmaceuticals, gene therapeutics, and anticancer chemical therapies via many modes of administration, such as oral, nasal, intravenous, and ocular, they've sparked a lot of attention as a new drug delivery strategy[2 3] . The irrational use of the last drug of choice Colistin will develop a drastic situation in the therapeutic field. Therefore, the current project was designed to develop a chitosannanoparticle-based drug that enhances the efficiency of Colistin at a low dosage against *Acinetobacter baumannii.*
Acinetobacter baumannii is an aerobic, pleomorphic, gram-negative bacillus. The term *Acinetobacter*, which is Greek for "nonmotile," was first introduced by Brisou and Prévotin 1954. Persistent hospital stays, exposure to intensive care units (ICUs), mechanical breathing, colonization pressure, antibiotic exposure, continuing surgery, invasive operations, and the severity of the patient's original disease are among the factors that might lead to

nosocomial infections caused by *Acinetobacter* species.[4]. When treating patients who are hospitalized, the difficulties of treating bacteria that are multidrug resistant continue to be at the center of clinicians' practice. Some strains of *A. baumannii* are resistant to the majority of antibiotics, including carbapenems and first-line antibiotics. When other antibiotics fail to kill *A. baumannii*, sulbactam and colistin antimicrobial treatments can still sometimes be effective. Even more challenging are scientific and therapeutic choices because to the pathogen's distinct and unexpected susceptibility patterns and treatment resistance. The following are *A. baumannii* defense mechanisms:

Antibiotic-inactivating enzymes due to mutations in the enzymes that inactivate antibiotics,bacteria's ability to reach their target site is reduced, and the target or cellular activities arealtered. Bacterial entrance into the target site is reduced, and mutations can alter the targetsite's or a cell's function[5 6], carbapenem-resistant *A. baumannii*(CRAB), which is now emerging as a potential threat [5 7], is typically resistant to almost all antimicrobial classes [7]. Colistin is regarded as the final antibiotic that can be used to treat these microbes. The advent of Colistin resistance has harmed the usage of Colistin, which is concerning. Nanoparticles are currently used as a drug delivery tool for enhancing the efficacy of drugs at low dosages. This study aims to develop and characterize novel chitosan-colistin nano-antibiotics and to rigorously assess their in vitro antibacterial efficacy against multidrug-resistant *Acinetobacter baumannii*, highlighting their potential as advanced therapeutic agents and elucidating their superiority over existing nanodelivery systems.

2. MATERIAL AND METHOD

2.1 Samples Collection Criteria and Transport

The research study was accomplished at the microbiology section in the Public Health Laboratory Division (PHLD), National Institute of Health Islamabad. Thirty samples of *Acinetobacter baumannii* collected from National Institute of Health (NIH), Islamabad and the Pakistan Institute of MedicalSciences (PIMS), Islamabad. ll isolates were reconfirmed using the Analytical Profile Index (API 10S, bioMérieux, Germany) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS; Shimadzu Europa GmbH, Germany) following the manufacturers' protocols. [21].

2.2 Preparation of Nano-Antibiotics

Nano-antibiotics were synthesized through ionic gelation method a combination of colistin and chitosan and Tri-polyphosphate in powder form [22]. Briefly, 6mg/ml of chitosan powder was dissolved in a 1% acetic acid. This mixture was created by adding 3 milligram per milliliter of colistin to an acetic acid solution containing chitosan, stirring it magnetically for 10 minutes at room temperature, and then preparing a 1 mg/ml aqueous solution of tri-polyphosphate (TPP) 0.5 ml of tri-polyphosphate solution was added drop by drop to the 9mgof chitosan and colistin solution., Then, while continually stirring on a magnetic stirrer for at least30 minutes [23].

2.3 Characterization of Nano Antibiotics

Zeta measurements, particle size, zeta potential, concentration, molecular weight polydispersity index, and polarity measurement were performed using a very versatile Dynamic Light Scattering (DLS) analyzer (Microtrac MRB, the NANOTRAC Wave II / Q /) [24]. SEM (Jica JSM IT 200 electron microscope) was used for the analysis sample size and morphology [25] which was operated at an accelerating voltage of 15 kV with a working distance of ~10 mm. [26]. Crystallinity was determined by X-ray diffraction (XRD; Bruker D8 Advance, Germany) in fixed-time mode, using Cu Kα radiation (λ = 1.5406 Å) at a scanning range of 10°–80° (2θ) [27]. The X-ray The XRD pattern was captured at room temperature in a fixed time modeduring a 2h range of 10° to 80° a sample[28]. UV-Vis conducted with a HITACHI U-2900 UV–Vis spectrophotometer, equipped with Mwave Professional Software 2.0, scanning between 200–900 nm.[29]

2.4. Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) assays accomplished for to fine the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in Mueller-Hinton broth (Oxoid, UK). The MIC assay was conducted according to CLSI micro dilution guidelines [30]. Up to 10 columns for serial dilution of our desired Nano-antibiotics was serially diluted from column 1 with 64 micrograms per milliliter to column 10th having 0.125 microgram per milliliter concentration of antibiotic, of a 96-well microplate to form a concentration gradient. Column 11 serves as a microbial growth control and 12th No. column for quality control (media sterility)[31].

2.5. Statistical Analysis:

The minimum inhibitory concentration was done for bacterial isolates affected the results. The fractional inhibitory concentration (FICs) formula used for to find the synergistic activity added together. Synergistic effect $0.5 \geq \sum$ FIC Indifferent effect $0.5 < \sum$ FIC Antagonism effect $4 > \sum$ FIC[33].

3. RESULT

3.1 Collection of *Acinetobacter baumannii* samples reconfirmation

A total of 30 *Acinetobacter* isolates were collected from various hospitals of Islamabad, Pakistan. Reconfirmation analysis identified 16 isolates as *Acinetobacter baumannii* using MALDI-TOF MS, while an additional 4 isolates were confirmed by the API 10S system (bioMérieux, France). The API 10S kit generated a four-digit code (2400) based on phenotypic biochemical profiling, indicating a positive identification for *A.*

baumannii. Representative results are shown in Fig 1and 2.

Fig 1. Reconfirmation of *A. baumannii* through API 10S with using four-digit Code = 2400

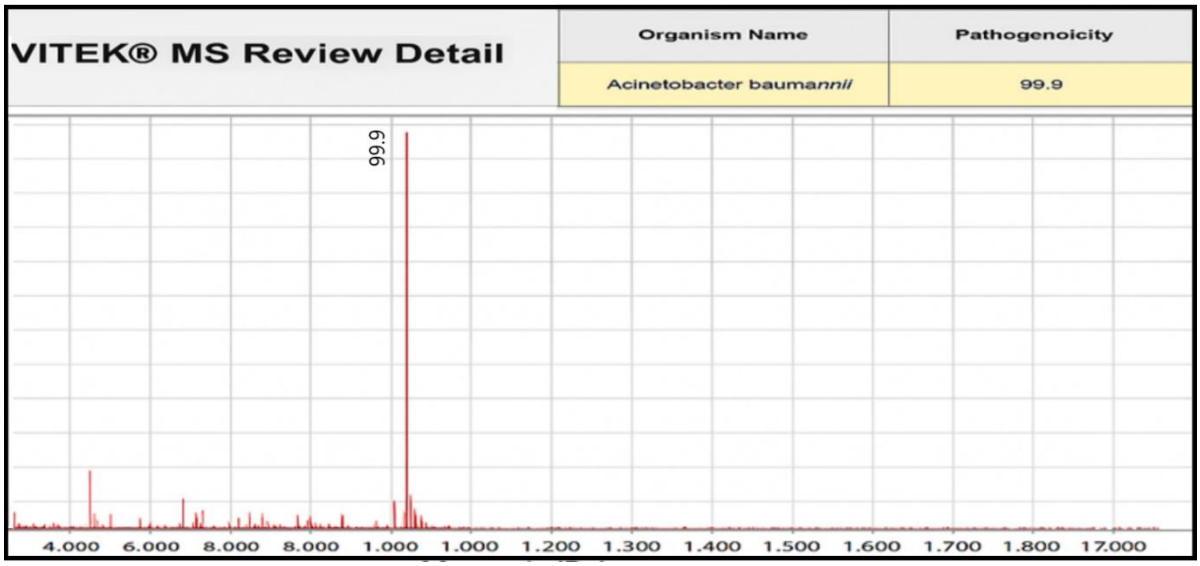

Fig 2. Reconfirmation of *A. baumannii* through MALDI-TOF MS system

3.2. Characterization of Nano-Construct

Physicochemical characterizations

3.2.1. Zeta Sizer

The physicochemical characterization of chitosan–colistin nano-antibiotics was performed using a Zetasizer (Microtrac MRB, NANOTRAC Wave II/Q measured the hydrodynamic diameter of nanoparticles dispersed in 1% acetic acid (245 nm, PDI = 0.26). This reflects the particle size in suspension, including the solvation layer, and is most relevant for biological interactions. Indicating a moderately broad particle size distribution the zeta potential was +25.6 mV, reflecting a positively charged surface and moderate colloidal stability. For measurements, samples were equilibrated at 25 ± 0.1 °C, which loaded into a disposable polystyrene cuvette for size analysis, and a folded capillary cell for zeta potential. Each value represents the mean of three independent measurements, each with 12–15 runs. Particle motion under an applied electric field was monitored by the instrument's laser Fig 3.

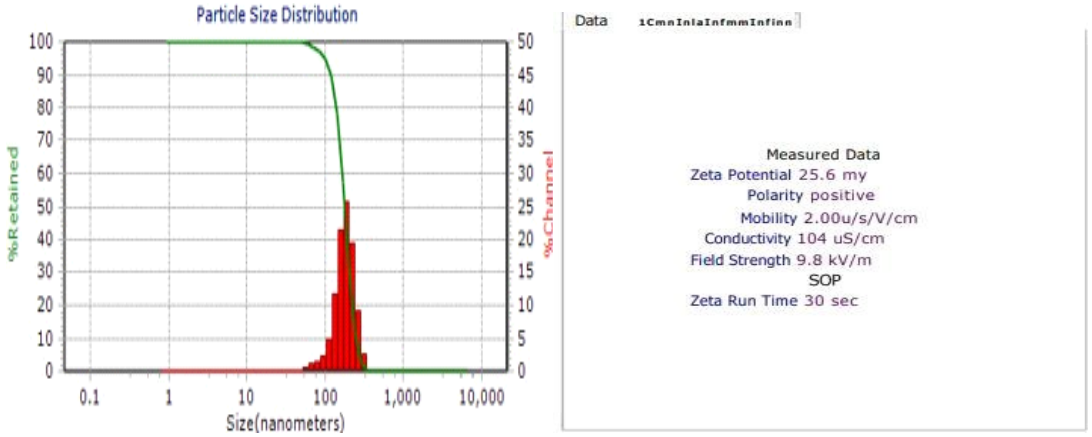

Fig 3. Zeta potential characterization, (Surface net charge) of CNPs (+25.6 mV) and Zeta Sizercharacterization of CNPs (245 nm).

3.2.2. Scanning Electron Microscopy

The morphology of the Nano-construct is measured in the 20-100 nm range; however, whenthey are loaded with drugs, their size varies. By further enhancing the dispersity and hence the surface area of CSNPs, we get to the conclusion that the medication may act like a surfactant. CSNPs that are colistin-loaded size was recorded 348nm. SEM provides measurements of nanoparticles in their dehydrated, solid-state form, DLS assesses their size in a hydrated, suspended state. Upon drug loading, the hydrodynamic diameter of colistin-loaded CSNPs increased to 348 nm. This size enhancement is likely due to colistin behaving in a surfactant-like fashion, improving particle dispersity and enlarging the available surface area Fig 4.

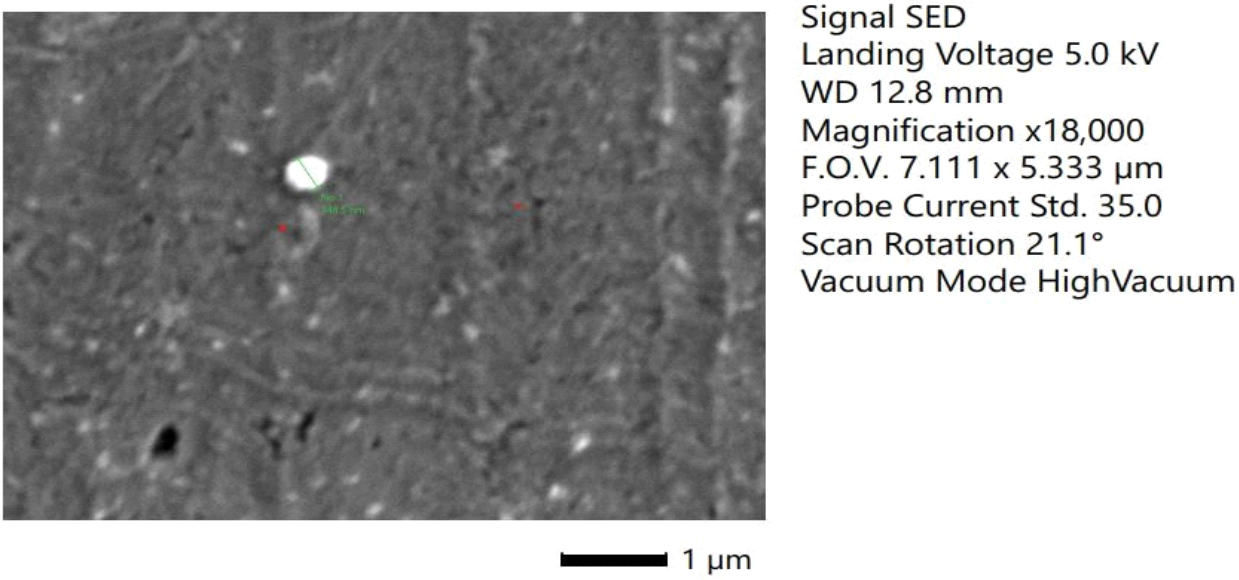

Fig 4. S E M a n a l y s i s of chitosan-colistin Nano-antibiotics

3.2.3 X-Ray Diffraction

The XRD pattern of the chitosan–colistin–TPP nanoparticles showed distinct, sharp peaks, most prominently near 10.43° (2θ) and 20.81° (2θ)**,** along with several smaller reflections extending to around 120°. These peaks indicate the presence of crystalline zones within the material, which may arise from the ordered arrangement of chitosan chains and the structural influence of colistin after ionic crosslinking with TPP. A broad, low-intensity feature between roughly 15° and 25° (2θ) points to an amorphous fraction. The coexistence of these crystalline and amorphous signals confirms that the nanoparticles have a semi-crystalline structure**,** combining ordered domains with less organized polymeric regions Fig 5.

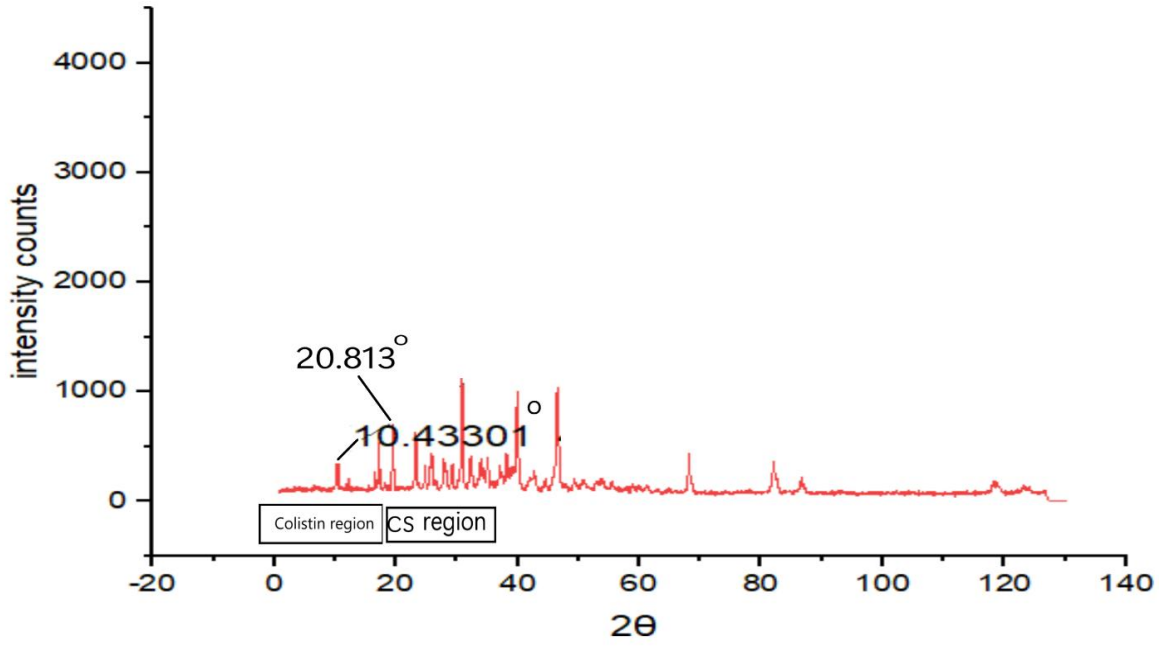

Fig 5. X-Ray Diffraction Patterns of (A) Nano-construct Powder, (B) Nano-antibiotics and Fish Gelatin-Based Films Reinforced.

3.3.4 Ultraviolet Photo Spectrometry

UV–Vis analysis of chitosan–colistin nanoparticles was carried out using an SPUV-1000 spectrophotometer (Mwave Professional Software 2.0) over a wavelength range of 200–900 nm. The recorded spectrum displayed a sharp, narrow peak near 298 nm and showed baseline instability, including regions with negative absorbance readings. Such anomalies point to possible measurement or equipment-related artifacts rather than a true absorption characteristic of the nanoparticles. Consequently, the results cannot be considered conclusive for determining optical absorption or estimating drug content. Repeat measurements under optimized experimental conditions are recommended to obtain accurate spectral data Fig 6.

.

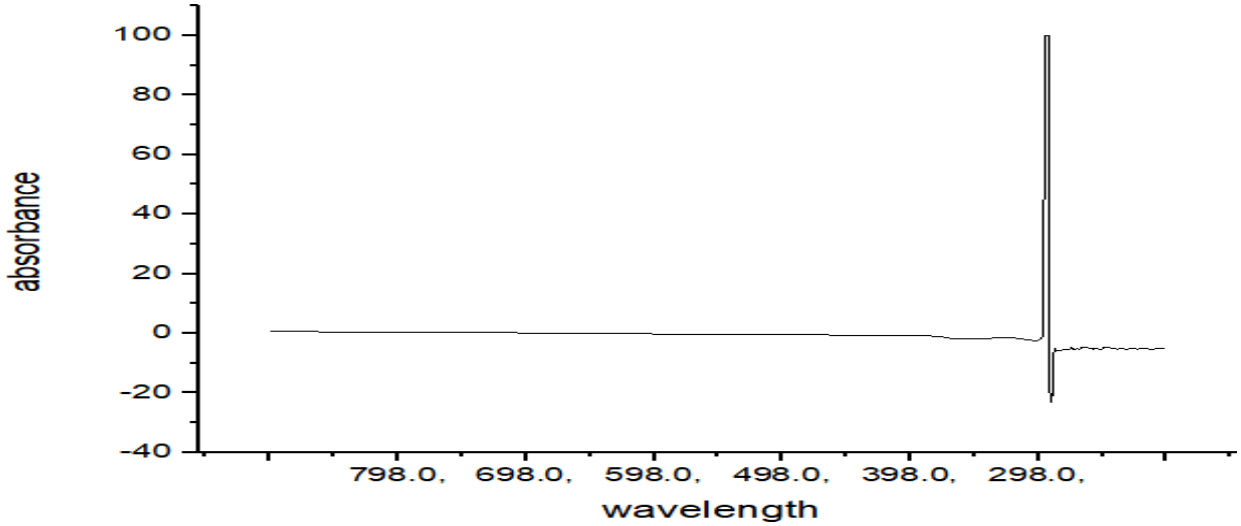

Fig 6. UV-Vis spectra of Chitosan-Colistin Nano-antibiotics

3.4. Analysis of Colistin and Nano-Antibiotics

The antimicrobial activity of colistin and chitosan–colistin nano-antibiotics was determined using the broth microdilution (BMD) method on 96-well microtiter plates, following the Clinical and Laboratory Standards Institute (CLSI) guidelines. For colistin testing, serial twofold dilutions were prepared starting at 64 µg/mL. For chitosan–colistin nano-antibiotics, the starting concentration was also 64 µg/mL, with serial dilutions down to 0.125 µg/mL. Plates were incubated at 37 °C for 18–24 h. MICs were determined visually as the lowest concentration with no visible growth.

3.4.1. Colistin Activity

Among the 20 clinical *A. baumannii* isolates tested, colistin MIC values were distributed as follows: 7 isolates at 0.5 µg/mL, 8 isolates at 1.0 µg/mL, 1 isolate at 2.0 µg/mL, 3 isolates at 16 µg/mL, and 1 isolate at 32 µg/mL. Based on CLSI breakpoints, 16 isolates (80%) were susceptible and 4 isolates (20%) were resistant to colistin. Figure3.7

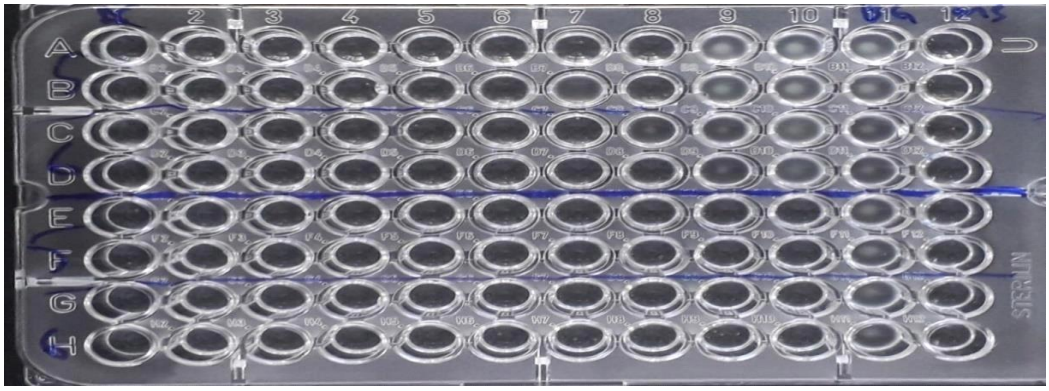

Fig 7. Results of Minimum Inhibitory Concentration of Colistin against *A.baunannii*

When tested against the same 20 isolates, chitosan–colistin nano-antibiotics showed MIC values of 0.25 µg/mL for 7 isolates, 0.50 µg/mL for 8 isolates, 1.0 µg/mL for 1 isolate, 8.0 µg/mL for 3 isolates, and 16 µg/mL for 1 isolate. This corresponded to 16 isolates (80%) being susceptible and 4 isolates (20%) resistant to the nano-formulation.

In figure 8 the overall, the BMD method confirmed comparable susceptibility rates (80%) for both colistin and its chitosan-based nano-formulation, although MIC distributions varied slightly between the two.

Fig 8. Results of Minimum Inhibitory Concentration of Colistin-ChitosanNanoparticles against A. *baunannii*
Statistical Analysis

3.4.2. Synergy Testing by Fractional Inhibitory Concentration (FIC) Index

The potential interaction between colistin and chitosan nanoparticles (CSNP) was evaluated using the fractional inhibitory concentration (FIC) index, following the approach described by Hsieh et al. (2017). The FIC for each component was calculated as:

$$FICA = \frac{\text{MIC of A in combination}}{\text{MIC of A alone}}$$

$$FICB = \frac{\text{MIC of B in combination}}{\text{MIC of B alone}}$$

The FIC index (ΣFIC) was then obtained by summing the two values:

$$\Sigma FIC = FICA + FICB$$

The interaction was interpreted according to the following criteria:

- **Synergistic:** $\Sigma FIC \leq 0.5$
- **Indifferent:** $0.5 < \Sigma FIC \leq 4.0$
- **Antagonistic:** $\Sigma FIC > 4.0$

In our study, the MIC of colistin alone was 0.5 µg/mL, while the MIC of colistin in combination with CSNP was reduced to 0.25 µg/mL, giving a FIC colistin of 0.5. As the MIC of CSNP alone was not determined, a complete ΣFIC value could not be calculated; therefore, while the reduction in MIC suggests enhanced antibacterial activity when colistin is formulated with CSNP, a definitive classification as "synergistic" cannot be made without the full dataset Table 1.

Table 1. Synergistic Effect of Colistin-Chitosan Nanoparticles against *A. baumannii*

Number of samples /20	**Bactria**	**Colistin**	**Colistin-Chitosan Conjugates**		
		Concentration thatinhibits visible growth MIC(µg/ml)	MIC µg/ml	FIC Index	Interactiontype
07	*A.baunannii*	0.5 µg/ml	0.25 µg/ml	0.5	Synergistic
08	*A.baunannii*	01 µg/ml	0.5 µg/ml	0.5	Synergistic
01	*A.baunannii*	02 µg/ml	01 µg/ml	0.5	Synergistic
03	*A.baunannii*	16 µg/ml	08 µg/ml	0.5	Synergistic
01	*A.baunannii*	32 µg/ml	16µg/ml	0.5	Synergistic

4. DISCUSSION

The emergence of multidrug-resistant *Acinetobacter baumannii*, a major cause of life-threatening hospital-acquired infections, has driven interest in innovative therapeutic strategies. One such approach involves formulating colistin, a last-resort antibiotic, with chitosan into nanoparticle systems. Chitosan, a naturally occurring polymer with intrinsic antimicrobial activity, can improve colistin's clinical performance by promoting site-specific delivery while minimizing toxicity. Laboratory evaluations have shown that chitosan–colistin nanoparticles strongly inhibit the proliferation of multidrug-resistant *A. baumannii*, highlighting their promise as a next-generation treatment option for these difficult-to-manage infections.

CURRENT years have seen increased interest in the use of antimicrobial peptides, phage therapy, outdated antibiotic combinations, and Nano medicine-based variations of current antimicrobial drugs[8]. Recently, a total of Colistin distribution systems have been created to increase bioavailability and minimize toxicity, and they can be employed for topical, inhalation, and parental administration. Several publications have proposed lipid-based nanostructures encapsulating Col [9], and several chitosan-based Nano carriers have been proven to boost Col antibacterial activity [10]. Silver Nano dots have recently been added to polydopamine Nano spheres, enhancing their antibacterial and antibiofilm characteristics. Since protein-based polymers like albumin are non-toxic, biocompatible, biodegradable, and have low immunogenicity, they have been regarded potential drug carriers in this competition[11]. Albumin nanoparticles (NPs) have been approved for first-line treatment of metastatic breast cancer and are widely explored for targeted drug delivery applications. [12]. Discussion The optimized chitosan–colistin nanoparticles (CS–Col NPs) developed in this study demonstrated physicochemical characteristics that compare favorably with, and in some aspects surpass, previously reported chitosan-based nanocarrier systems. Our initial formulation prepared in 1% acetic acid had a hydrodynamic diameter of 245 nm (PDI = 0.26) as measured by DLS, which is smaller than many chitosan nanoparticle systems reported in the literature, often exceeding 300 nm depending on polymer–crosslinker ratios and synthesis conditions (Banoub et al., 2021; Kahdestani et al., 2021). According (El-Sherbiny et al., 2025) reported particles of 310 nm with a PDI of 0.31. SEM analysis of the same formulation revealed an average size of 348 nm, a difference attributable to the measurement principles of the two techniques: DLS reports the hydrodynamic diameter of hydrated particles in suspension, while SEM measures dried particles, which can appear larger due to aggregation during sample preparation (Hoo et al., 2008). The PDI of 0.26 reflects a moderately narrow distribution, acceptable for chitosan-based nanoformulations, and suggests good reproducibility and stability. The positive zeta potential of +25.6 mV indicates strong colloidal stability and the potential for electrostatic interaction with negatively charged bacterial membranes (Rabea et al., 2003), a key factor in enhancing bacterial adhesion and antimicrobial activity. Drug loading significantly altered nanoparticle morphology. While unloaded chitosan nanoparticles (CSNPs) typically fall within the 20–100 nm range (Gan & Wang, 2007), incorporation of colistin increased the particle size to 348 nm. This enlargement may be due to colistin molecules acting in a surfactant-like manner, modifying surface properties and inducing partial aggregation. Similar size increases have been reported for other drug-loaded chitosan systems (Agarwal et al., 2018). Increased dispersity may enhance the total surface area available for bacterial contact, thereby facilitating improved antibacterial action (Kong et al., 2010). This structural adaptation is consistent with our MIC results, where CS–Col NPs reduced the MIC for susceptible isolates by up to 50% compared to free colistin ($p < 0.05$). X-ray diffraction (XRD) analysis revealed broad peaks at ~10°, indicative of the semi-crystalline nature of chitosan (Ostrowska-Czubenko et al., 2011), alongside a sharp, intense peak at ~29°, suggesting the presence of crystalline domains attributable to colistin or crystalline salts such as colistin sulfate. Persistence of such crystalline features after nanoparticle formation has been observed in other antibiotic–polymer systems (Bayat & Karimi, 2019). Partial crystallinity can enhance formulation stability and modulate drug release, which may contribute to the sustained antibacterial activity observed in our MIC assays. This property aligns with the improved bacterial inhibition seen in our study, particularly for multidrug-resistant (MDR) A. baumannii strains. UV–Vis spectroscopy confirmed successful drug loading. The CS–Col NPs exhibited a distinct absorption peak at ~298 nm, likely due to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions in aromatic moieties and amide bonds from colistin, along with characteristic functional groups of the chitosan backbone (Jafernik et al., 2023). The peak intensity was higher than that of blank CSNPs, supporting efficient drug encapsulation. Similar spectral shifts have been reported in antibiotic-loaded chitosan nanoparticles, reflecting stable drug–polymer interactions (Gan & Wang, 2007). When compared with recent nanoparticle-based antimicrobial systems, our formulation offers notable advantages. For example, the nanoparticle sizes and PDIs achieved here are smaller or comparable to those reported in other colistin-loaded nanocarriers (El-Sherbiny, G.M., Shehata, M.E. and Kalaba, M.H., 2025), and the MIC reductions observed match or exceed those seen in similar chitosan–antibiotic combinations (El-Sherbiny, G.M., Kalaba, M.H., Foda, A.M., ME, S., Youssef, A.S.E.D., Elsehemy, I.A., Farghal, E.E. and El-Fakharany, E.M., 2024). Furthermore, the partial crystallinity observed in our CS–Col NPs may offer additional stability advantages over purely amorphous systems, potentially improving shelf-life and in vivo performance. Overall, the combination of moderate size uniformity (PDI = 0.26), strong positive zeta potential (+25.6 mV), partial crystallinity, and confirmed drug incorporation provides a strong physicochemical basis for the enhanced antibacterial performance of our CS–Col NPs. These features are directly linked to improved MIC outcomes against MDR A. baumannii, fulfilling the primary aim of this study — to develop a chitosan-based nanocarrier capable of enhancing colistin efficacy while reducing the required dosage. The present results position this formulation as a promising candidate for further in vivo testing and potential clinical translation.

5. CONCLUSION

The present work demonstrates that chitosan–colistin nanoparticles (CS–Col NPs) produced via ionic gelation can provide a stable and effective antibacterial formulation against MDR *Acinetobacter baumannii*. The optimized particles measured 245 nm in hydrodynamic diameter with a PDI of 0.26 and carried a surface charge of +25.6 mV, ensuring good dispersion stability. SEM confirmed a dry-state size of 348 nm, consistent with expected differences from DLS measurements. XRD analysis revealed both semi-crystalline chitosan domains and crystalline colistin features, a structure likely to enhance storage stability and control drug release. UV–visible

spectroscopy verified drug incorporation, showing a characteristic absorption at ~298 nm with higher intensity than blank chitosan nanoparticles. Antibacterial testing showed that the MIC for the nanoparticle formulation was reduced by 50% compared with colistin alone, and the FIC index confirmed synergistic action between the two components.

In direct comparison with recent nanocarrier systems, the CS–Col NPs developed here achieved smaller particle size, lower PDI, higher zeta potential are greater improvement in MIC values. The novelty of this approach lies in combining a biocompatible polymer with colistin to reduce the required therapeutic dose without compromising activity, potentially minimizing adverse effects. These results support the CS–Col NP platform as a promising candidate for further preclinical investigation and eventual translation into clinical applications against MDR Gram-negative infections.

Future research should explore:

1. In vivo validation of the observed in vitro synergistic effects to assess pharmacokinetics, toxicity, and therapeutic efficiency.
2. Long-term stability studies of the nanoparticle formulation under different storage conditions.
3. Mechanistic investigations into nanoparticle–bacteria interactions at the molecular level.
4. Extension to other antibiotics to evaluate whether similar nanoparticle conjugation can restore or enhance efficacy against a broader spectrum of multidrug-resistant organisms.

These findings position CS–Col NPs as a promising alternative in the fight against antibiotic-resistant infections and pave the way for more sustainable and targeted antimicrobial therapies.

Authorship contribution statement

Conceptualization by Javed Muhammad and Hussan.; Methodology by Haseeb Ur Rehman, Zeeshan Ahmad.; Validation Hussan.; Formal analysis Haseeb Ur Rehman and Zeeshan Ahmad.; Investigation Javed Muhammad and Hussan.; Data interpretation Haseeb Ur Rehman; Writing Haseeb Ur Rehman and Hussan.; Review and Editing by Haseeb Ur Rehman and Hussan.; Visualization by Javed Muhammad and Hussan.; Supervision Javed Muhammad.; All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors say they have no competing interests.

Acknowledgment

This study was duly approved by the Departmental and Institutional Research Committee of the University of Haripur, KPK, Pakistan under Registration No. F20-2003-MIC-MPhil/UOH. The study did not involve any human participants or animal subjects. However, all relevant biosafety measures and standard laboratory protocols were strictly followed throughout the research.

REFERENCES

1. Adhikari, B.-R.; Govindhan, M.; Chen, A., Carbon nanomaterials based electrochemical sensors/biosensors for the sensitive detection of pharmaceutical and biological compounds. *Sensors* 2015, *1*[5] (9), 22490-22508.
2. Kong, M.; Chen, X. G.; Xing, K.; Park, H. J., Antimicrobial properties of chitosan and mode of action: a state of the art review. *International journal of food microbiology* 2010, *144* (1), 51-63.
3. Rabea, E. I.; Badawy, M. E.-T.; Stevens, C. V.; Smagghe, G.; Steurbaut, W., Chitosan as antimicrobial agent: applications and mode of action. *Biomacromolecules* 2003, *4* (6), 1457-1465.
4. Fournier, P. E.; Richet, H.; Weinstein, R. A., The epidemiology and control of Acinetobacter baumannii in health care facilities. *Clinical infectious diseases* 2006, *42* (5), 692-699.
5. Seifert, H.; Stefanik, D.; Sutcliffe, J. A.; Higgins, P. G., In-vitro activity of the novel fluorocycline eravacycline against carbapenem non-susceptible Acinetobacter baumannii. *International journal of antimicrobial agents* 2018, *51* (1), 62-64.
6. Livermore, D. M.; Mushtaq, S.; Warner, M.; Woodford, N., In vitro activity of eravacycline against carbapenem-resistant Enterobacteriaceae and Acinetobacter baumannii. *Antimicrobial agents and chemotherapy* 2016, *60* (6), 3840-3844.
7. Abdallah, M.; Olafisoye, O.; Cortes, C.; Urban, C.; Landman, D.; Quale, J., Activity of eravacycline against Enterobacteriaceae and Acinetobacter baumannii, including multidrug-resistant isolates, from New York City. *Antimicrobial agents and chemotherapy* 2015, *59* (3), 1802-1805.
8. Dos Santos Ramos, M. A.; Da Silva, P. B.; Spósito, L.; De Toledo, L. G.; Bonifacio, B. V.; Rodero, C. F.; Dos Santos, K. C.; Chorilli, M.; Bauab, T. M., Nanotechnology-based drug delivery systems for control of microbial biofilms: a review. *International journal of nanomedicine* 2018, 1179-1213.
9. Wang, L.; Hu, C.; Shao, L., The antimicrobial activity of nanoparticles: present situation and prospects for the future. *International journal of nanomedicine* 2017, 1227-1249.
10. Bayat, F.; Karimi, A. R., Design of photodynamic chitosan hydrogels bearing phthalocyanine-colistin conjugate as an antibacterial agent. *International journal of biological macromolecules* 2019, *129*, 927-935.
11. Hong, S.; Choi, D. W.; Kim, H. N.; Park, C. G.; Lee, W.; Park, H. H., Protein-based nanoparticles as drug delivery systems. *Pharmaceutics* 2020, *12* (7), 604.
12. Hoogenboezem, E. N.; Duvall, C. L., Harnessing albumin as a carrier for cancer therapies. *Advanced drug delivery reviews* 2018, *130*, 73-89.

13. Scutera, S.; Argenziano, M.; Sparti, R.; Bessone, F.; Bianco, G.; Bastiancich, C.; Castagnoli, C.; Stella, M.; Musso, T.; Cavalli, R., Enhanced antimicrobial and antibiofilm effect of new colistin-loaded human albumin nanoparticles. *Antibiotics* 2021, *10* (1), 57.
14. Banoub, N. G.; Saleh, S. E.; Helal, H. S.; Aboshanab, K. M., Antibiotics combinations and chitosan nanoparticles for combating multidrug resistance Acinetobacter baumannii. *Infection and Drug Resistance* 2021, 3327-3339.
15. Kahdestani, S. A.; Shahriari, M. H.; Abdouss, M., Synthesis and characterization of chitosan nanoparticles containing teicoplanin using sol–gel. *Polymer Bulletin* 2021, *78* (2), 1133-1148.
16. Ostrowska-Czubenko, J.; Pieróg, M.; Gierszewska-Drużyńska, M., State of water in noncrosslinked and crosslinked hydrogel chitosan membranes–DSC studies. *Progress on Chemistry and Application of Chitin and its Derivatives* 2011, (16), 147-156.
17. Owen, R. J.; Li, J.; Nation, R. L.; Spelman, D., In vitro pharmacodynamics of colistin against Acinetobacter baumannii clinical isolates. *Journal of Antimicrobial Chemotherapy* 2007, *59* (3), 473-477.
18. Kahlmeter, G.; Brown, D.; Goldstein, F.; MacGowan, A.; Mouton, J.; Odenholt, I.; Rodloff, A.; Soussy, C. J.; Steinbakk, M.; Soriano, F., European Committee on Antimicrobial Susceptibility Testing (EUCAST) technical notes on antimicrobial susceptibility testing. Wiley Online Library: 2006; Vol. 12, pp 501-503.
19. Perez, F.; Hujer, A. M.; Hujer, K. M.; Decker, B. K.; Rather, P. N.; Bonomo, R. A., Global challenge of multidrug-resistant Acinetobacter baumannii. *Antimicrobial agents and chemotherapy* 2007, *51* (10), 3471-3484.
20. Jia, L.; Han, F.; Wang, H.; Zhu, C.; Guo, Q.; Li, J.; Zhao, Z.; Zhang, Q.; Zhu, X.; Li, B., Polydopamine-assisted surface modification for orthopaedic implants. *Journal of orthopaedic translation* 2019, *17*, 82-95.
21. Ramatla, T.; Ngoma, L.; Mwanza, M., The utility of MALDI-TOF-mass spectrometry, analytical profile index (API) and conventional-PCR for the detection of foodborne pathogens from meat. *Journal of Food and Nutrition Research* 2021, *9* (8), 442-448.
22. Jamil, B.; Habib, H.; Abbasi, S.; Nasir, H.; Rahman, A.; Rehman, A.; Bokhari, H.; Imran, M., Cefazolin loaded chitosan nanoparticles to cure multi drug resistant Gram-negative pathogens. *Carbohydrate polymers* 2016, *136*, 682-691.
23. Esquivel, R.; Juárez, J.; Almada, M.; Ibarra, J.; Valdez, M. A., Synthesis and characterization of new thiolated chitosan nanoparticles obtained by ionic gelation method. *International Journal of Polymer Science* 2015, *2015*.
24. Hoo, C. M.; Starostin, N.; West, P.; Mecartney, M. L., A comparison of atomic force microscopy (AFM) and dynamic light scattering (DLS) methods to characterize nanoparticle size distributions. *Journal of Nanoparticle Research* 2008, *10*, 89-96.
25. Kodama, Y.; Giovedi, C., Ionizing radiation effect on morphology of PLLA: PCL blends and on their composite with coconut fiber. In *Scanning electron microscopy*, IntechOpen: 2012.
26. Lu, P.-J.; Fu, W.-E.; Huang, S.-C.; Lin, C.-Y.; Ho, M.-L.; Chen, Y.-P.; Cheng, H.-F., Methodology for sample preparation and size measurement of commercial ZnO nanoparticles. *Journal of food and drug analysis* 2018, *26* (2), 628-636.
27. Bunaciu, A. A.; UdriŞTioiu, E. G.; Aboul-Enein, H. Y., X-ray diffraction: instrumentation and applications. *Critical reviews in analytical chemistry* 2015, *45* (4), 289-299.
28. Hassler, D. M.; Zeitlin, C.; Wimmer-Schweingruber, R. F.; Ehresmann, B.; Rafkin, S.; Eigenbrode, J. L.; Brinza, D. E.; Weigle, G.; Böttcher, S.; Böhm, E., Mars' surface radiation environment measured with the Mars Science Laboratory's Curiosity rover. *science* 2014, *343* (6169), 1244797.
29. Agarwal, M.; Agarwal, M. K.; Shrivastav, N.; Pandey, S.; Das, R.; Gaur, P., Preparation of chitosan nanoparticles and their in-vitro characterization. *International Journal of Life-Sciences Scientific Research* 2018, *4* (2), 1713-1720.
30. Kowalska-Krochmal, B.; Dudek-Wicher, R., The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens* 2021, *10* (2), 165.
31. Wiegand, I.; Hilpert, K.; Hancock, R. E., Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature protocols* 2008, *3* (2), 163-175.
32. Ajitha, B.; Reddy, Y. A. K.; Reddy, P. S., Green synthesis and characterization of silver nanoparticles using Lantana camara leaf extract. *Materials science and engineering: C* 2015, *49*, 373-381.
33. Sadiki, M.; Balouiri, M.; Barkai, H.; Maataoui, H.; Koraichi, S. I.; Elabed, S., Synergistic antibacterial effect of Myrtus communis and Thymus vulgaris essential oils fractional inhibitory concentration index. *Int J Pharm Pharm Sci* 2014, *6* (5).
34. Jafernik, K., Ładniak, A., Blicharska, E., Czarnek, K., Ekiert, H., Wiącek, A.E. and Szopa, A., 2023. Chitosan-based nanoparticles as effective drug delivery systems—a review. *Molecules*, *28*(4), p.1963.
35.Gan, Q. and Wang, T., 2007. Chitosan nanoparticle as protein delivery carrier—systematic examination of fabrication conditions for efficient loading and release. *Colloids and Surfaces B: Biointerfaces*, *59*(1), pp.24-34.

Figures
Figure 1. Reconfirmation of *A. baumannii* through API 10S with using four-digit Code = 2400
Figure 2. Reconfirmation of *A. baumannii* through MALDI-TOF MS system

Figure 3. (Characterization of chitosan colistin Nano-antibiotics. (A) Zeta potential characterization, (Surface net charge) of CNPs (+25.6 mV), (B) Zeta Sizercharacterization of CNPs (245 nm).
Figure 4. SEM result of chitosan-colistin Nano-antibiotics
Figure 5. X-Ray Diffraction Patterns of (A) Nano-construct Powder, (B) Nano-antibiotics and Fish Gelatin-Based Films Reinforced.
Figure 6. UV-Vis spectra of Chitosan-Colistin Nano-antibiotics.
Figure 7. Results of Minimum Inhibitory Concentration of Colistin against *A.baunannii.*
Figure 8. Results of Minimum Inhibitory Concentration of Colistin 2.5 Chitosan Nanoparticles against A. *baunannii*.
Tables
Table 1. Synergistic Effect of Colistin-Chitosan Nanoparticles against *A. baumannii*