pH-responsive gelatin nanoparticles for targeted delivery of ciprofloxacin against bacterial infections

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"It always seems impossible until it's done".

~Nelson Mandela

This dissertation is dedicated to all the young girls around the world from disadvantaged backgrounds. I hope you thrive academically, professionally, and personally. I know the pressure mounts on your shoulders, but kings can carry the burden that others never knew existed. And you exist. Show your beauty. Show your cool. Ukubekezela kuzala impumelelo. I hope I have made you proud.

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Abstract

Background: Given the rise in antimicrobial resistance and challenges associated with traditional antibiotic dosage forms, there is an urgency to develop drug delivery systems that improve, safeguard, and augment the present antibiotics on the market. Further research is required to maximize extended and targeted drug release, which can be accomplished via stimuli-responsive approaches such as pH-responsive nano-drug delivery systems. Furthermore, these pH-responsive nanosystems may be employed as carriers for antimicrobial drugs, which could be beneficial against antimicrobial resistance.

Aim: The aim of this study was to prepare novel pH-responsive gelatin nanoparticles to function as delivery agents of Ciprofloxacin (CIP) to enhance their antibacterial effectiveness against methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods: CIP-GNPs were prepared using a two-step desolvation method. The particle size, polydispersity index (PDI) and zeta potential (ZP) of CIP-GNPs were determined using the dynamic light scattering technique. Transmission electron microscopy analysis was conducted to confirm particle size and visualize the morphology of CIP-GNPs. The entrapment efficiency (EE %) of CIP-GNPs was determined using the ultrafiltration method and was quantified using High-Performance Liquid Chromatography (HPLC*). In vitro* drug release of CIP-GNPs was conducted using the dialysis bag technique and CIP released was quantified using HPLC. Drug release dissolution factors were analysed using the DDSolver program. Hemocompatibility of CIP-GNPs was performed using sheep blood. *In vitro* antibacterial activity of CIP-GNPs was determined using micro broth assay against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli (E. coli*), MRSA, and *P. aeruginosa*. Bacterial killing kinetics were performed against MRSA and *P. aeruginosa* using the plate colony counting method. MRSA and *P. aeruginosa* biofilm inhibition of CIP-GNPs was evaluated using the microtiter method.

Results: CIP-GNPs had a particle size, polydispersity index, zeta potential, and entrapment efficiency of 212.3 ± 1.739, 0.259 ± 0.023, +4.58 ± 0.148 mV and 38.1 ± 3.85%, respectively. *In vitro,* biosafety testing identified CIP-GNPs as non-hemolytic. The CIP-GNPs demonstrated pH responsiveness with an increase in particle size from 204.1 ± 0.100 to 226.4 ± 0.451 nm and a charge switch on the zeta potential from -3.59 ± 0.428 to 1.06 ± 0.271 mV, followed by a significantly faster release of CIP at pH 6.0 compared to 7.4. The *in vitro* antibacterial activity of CIP-GNPs showed 2-fold lower minimum inhibitory

concentration values compared to bare ciprofloxacin against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). Moreover, the bacterial-killing kinetic test showed 100% elimination of MRSA and *P. aeruginosa* within eight and one hour(s) of treatment with CIP-GNPs, respectively. In contrast, 100% elimination of MRSA and *P. aeruginosa* was observed within 24 and 12 hours of treatment with bare ciprofloxacin, respectively. CIP-GNPs eliminated 3,75-fold MRSA biofilm compared to bare ciprofloxacin, whereas 1.4-fold *Pseudomonas aeruginosa* biofilms were eliminated.

Conclusion: CIP-GNPs could effectively treat MRSA infections at a faster rate as compared to bare CIP. Therefore, this novel pH-responsive CIP-GNPs may serve as a promising nanocarrier for enhancing antibiotic delivery and antibacterial activity.

Keywords: pH-responsive, Gelatin nanoparticles, ciprofloxacin, bacterial infections

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Table of abbreviations

ABS	Absorbance	*P. aeruginosa*	*Pseudomonas aeruginosa*
CHL	Chloramphenicol	PBS	Phosphate buffer saline
CIP	Ciprofloxacin	PDI	Polydispersity index
CNM	Clindamycin	QRDR	Quinolone resistance determining region
DLS	Dynamic Light Scattering	S. aureus	Staphylococcus aureus
DNA	Deoxyribose Nucleic Acid	SD	Standard deviation
E. coli	*Escherichia coli*	SPEC	Spectinomycin
EE	Entrapment efficiency	TEM	Transmission electronic microscopy
ERY	Erythromycin	UV	Ultraviolet
FIC	Fractional inhibitory concentration	VCM	Vancomycin
GNP	Gelatin nanoparticle	ZP	Zeta potential
HPLC	High-Performance Liquid Chromatography		
Mfx	Moxifloxacin		
MIC	Minimum inhibitory concentration		
MHA	Müller Hilton Agar		
MHB	Müller Hilton Broth		
MRSA	Methicillin-resistant *Staphylococcus aureus*		

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CHAPTER 1: INTRODUCTION

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1.1 Introduction

This chapter provides a brief background to this study, highlighting the prevalence of bacterial infections, the multiple challenges encountered with the applications of antibiotics, and bacterial growth which has enhanced resistance. Moreover, this chapter outlines strategic approaches for further improving antibiotic nano-drug delivery systems and eliminating problems initiated by antibiotic resistance. This is followed by the aims, objectives novelty, and significance of the study, and concludes with an overview of the thesis.

1.2 Background

Severe infections caused by bacteria are recurrent reasons for hospitalization and are linked to detrimental effects like sepsis and death (1). Bacterial infections continue to be a major global public health menace, resulting in over 13 million fatalities annually (2). It is predicted that organisms resistant to drugs cause 700,000 deaths worldwide yearly, and should necessarily precautions and management strategies not be implemented, these numbers could potentially increase to 10 million fatalities which could result in a worldwide financial loss (3). Furthermore, the danger of bacterial infections has been growing since the early years of the modern age while the rate of infections brought on by drug-resistant bacteria also continues to increase rapidly (4). This highlights the need to enhance existing approaches or develop new strategies for the management and treatment of infectious diseases.

More than 70 years have passed since the discovery of antibiotics, which are bioactive compounds with a wide range of therapeutic uses (5). After the emergence of antibiotics, bacterial infections were controlled and prevented (4, 6). However, current antibiotic dosage forms are challenged by limitations such as insufficient concentrations at targeted areas which necessitates large and frequent dosage administration to achieve the intended therapeutic outcomes (7). Moreover, the excessive use of antibiotic dosages can negatively affect cells or tissues that are untargeted (8). These limitations together with the uncontrolled, incorrect usage of antibiotics has led to the crisis of antimicrobial resistance (9).

Antimicrobial resistance (AMR) refers to when bacteria, viruses, fungi, and other microbes adapt over time to the degree whereby they can withstand lethal concentrations of various antimicrobial agents such as antibiotics (10). Worldwide projections indicate that over 1.2million deaths were directly related to AMR in the year 2019, and if no effort is made to prevent AMR, the amount will likely increase to almost 10 million fatalities annually by 2050

(11). Multidrug-resistant (MDR) bacteria pose a significant threat to public health due to the decreased availability of antibiotics to effectively treat bacterial infections resulting in mortality (12). Numerous reports have indicated that the misuse of antibiotics has a significant role in the development of bacterial resistance (4, 5), and the more frequently antibiotics are administered the more likely it is that bacteria with resistance will emerge (13). The strains of Methicillin-resistant *Staphylococcus aureus* (MRSA) were discovered in 1961 and are regarded as the first MDR bacteria (14). MRSA is a prevalent pathogen resulting in sepsis, infections within bones and skin, as well as endocarditis in both public and hospital-acquired infections (15). This highlights the necessity of strategically introducing new ways to reduce the spread of antibiotic-resistant bacteria and the drawbacks of traditional antibiotics.

Due to the limitations of conventional dosage forms, extensive research efforts have been directed towards the discovery of measures to effectively deliver therapeutic agents to targeted sites for improved treatment of different bacterial infections and thereby overcome the AMR challenge (16). Among these different measures, nano drug delivery systems (NDDS) are proposed as better vehicles for a broad range of antibiotics. NDDS are rationally designed to improve the delivery and effectiveness of current drugs in comparison to conventional systems (17). They utilize advanced methodologies which include surface functionalization to target, control, and modify drug delivery (18). These nanosystems present a significant potential to enhance the concentration of the drug at the target sites (19), increasing their drug efficiency (20), and decreasing the undesirable effects of the drug that has been encapsulated (21). Furthermore, they improve drug bioavailability, solubility, and stability (22). Nevertheless, nanosystems also face challenges such as sub-optimal targeting to the infection site, pre-mature drug release before reaching the infection site, sub-optimal drug release at the infection site (23), costly manufacturing, brief half-lives, and lack of solubility (24). Thus, optimization of nanosystems that overcome these challenges are still required.

There has been an increasing pursuit of stimuli responsive NDDS which has prompted developments in the drug delivery and pharmaceutical industry to increase the NDDS's efficacy and potency (25). To improve antibiotic release at targeted sites, enhanced aggregation, and greater adsorption at infection sites (26). Stimuli-responsive delivery systems are engineered to respond optimally to both endogenous (such as pH levels, enzymes, etc.) and exogenous (such as temperature, light, etc.) stimulation at sites of infection (27). The relevance of pH-responsive NDDS is based on their capacity to administer antibiotics in response to subtle pH

shifts that correspond with the disease progression, which improves the effectiveness of the treatment and patient adherence (28). In contrast to the physiological pH of 7.4, diseases like cancer and infectious illnesses caused by bacterial infections exhibit shifts towards a pH that is acidic (29). Anti-bacterial and anti-cancer drugs have been reported to have been delivered to specific infection areas using pH-sensitive NDDS (30). Bacterial metabolism creates lactic acid and acetic acid, therefore decreasing the local pH from 7.4 to 6.0 or below can cause antibiotics to be released. The pH-sensitive NDDS of therapeutic drugs to bacterial infection sites, particularly MRSA, has obtained inadequate research attention (31), this could significantly impact the treatment of infectious diseases by enabling more precise and enhanced drug delivery to infection sites, thereby reducing the side effects of traditional antibiotics (32). Thus, pH-responsive nanosystems can maximize antibiotic efficacy.

Gelatin nanoparticles (GNPs) are highly effective for the delivery and controlled release of drugs. GNP-based delivery systems are biocompatible, biodegradable and do not generate harmful byproducts in the body (33). Additionally, they display biological properties (34). Several methods can be employed to prepare GNPs, one of which is a two-step desolvation which allows the manufacturing of GNPs with a decreased inclination for aggregation (35). However, when GNPs are expose to an aqueous environment, they lose structural integrity and disassemble. As a result, they must be stabilized to attain physicochemical and mechanical stability (36). Crosslinking serves as a critical step in the production of stable GNPs. Glutaraldehyde, glyoxal and water-soluble carbodiimide have all been utilized as cross-linkers (37). GNPs have been used for the delivery of various antimicrobial drugs such as Ciprofloxacin (CIP) (38). CIP is a fluoroquinolone hydrophobic antibiotic that has antimicrobial properties and high solubility at normal pH (39). Due to emerging resistance, CIP has been reported for various pH-responsive nanosystems such as nanofibers (40), hydrogels (41) and bimetallic nanoparticles (42). To date, no pH-responsive GNP has been reported for CIP to target bacterial infections.

Herein, we report pH-responsive GNPs nanoformulation prepared using the two-step desolvation method, with glutaraldehyde employed as a cross-linker. The nanoparticles will be evaluated for their ability to enhance the antibacterial efficiency of CIP against MRSA, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, as well as inhibition of biofilm formation.

1.3 Problem statement

Bacterial infections caused by resistant bacteria have been recently identified as a leading cause of death worldwide. Another source of concern is the decreased effectiveness of conventional antibiotic therapy towards bacterial infections due to inadequate antibiotic bioavailability at areas of infection and, weak penetration of antibiotics to infected sites which results in subtherapeutic antibiotic levels being delivered. Therefore, a greater therapeutic dose is needed, which may contribute to dose-dependent side effects and toxicity. The development of new antibiotics is stalling, necessitating the design and formulation of novel methods to enhance antibiotic therapy. This foregrounds the need for novel nano-drug delivery systems that efficiently deliver antibiotics directly to infection sites. This method successfully eliminates bacteria and contributes to avoiding the emergence of resistance. Nevertheless, present nanodrug delivery methods offer an avenue for additional advancement. Further investigations are required to maximize prolonged and targeted drug release, this can be achieved via stimuli responsive techniques such as pH-responsive nanosystems. Furthermore, these pH-responsive nanosystems may be used as delivery vehicles for antimicrobial agents such as Ciprofloxacin. This way, further research, and optimization are needed in the formulation of pH-responsive nanosystems to improve bacterial infection treatment.

1.4 Aims and Objectives

The study aimed to prepare novel pH-responsive gelatin nanoparticles to function as delivery agents of Ciprofloxacin (CIP) to enhance their antibacterial effectiveness against methicillin resistant *Staphylococcus aureus* (MRSA).

To achieve this aim, the objectives were as follows:

1. To formulate and optimize pH-responsive CIP-loaded gelatin nanoparticles.
2. To optimize and characterize CIP-GNPs in terms of particle size, polydispersity index, zeta potential, pH responsiveness, entrapment efficiency, and morphology.
3. To investigate the hemocompatibility of CIP-GNPs.
4. To investigate the *in vitro* drug release of CIP-GNPs.
5. To determine the *in vitro* antibacterial, and antibiofilm activities as well as the bacterial killing kinetics of CIP-GNPs.

1.5 Hypothesis

We hypothesize that delivering CIP via a pH-responsive GNP formulation could enhance its antibacterial activity.

1.6 Novelty of study

The research conducted in this study is novel for the following reasons:

1. This study reports the formulation and characterization of GNPs designed for pH responsive delivery of CIP for the treatment of bacterial infections, which has not been reported in the literature before.

1.7 Significance of study

The reported pH-responsive GNPs for delivery of CIP offer a new promising method that focuses on acidic infection sites, which improves drug localization within target tissues. This allows for, lowering concentrations of the dosage required for effective treatment, improving antibiotic activity, and hindering the development of bacterial resistance. This has few side effects and enhances patient compliance with therapy. The following is an outline of the possible significance of this study:

New pharmaceutical products

Academic researchers and the pharmaceutical industry can collaborate to create superior and side-effect-free drugs by translating the stated nanosystem into commercially viable products. This can also result in greater job possibilities and improvement of the local community.

Improved patient therapy and disease treatment

This new nanosystem has the potential to improve the treatment of bacterial infections by enabling targeted and controlled release at infection sites, improving drug localization and bioavailability at acidic infection sites, contributing to the improvement of antibacterial properties, reducing dosage frequency, and having minimal side effects, which ultimately improves patient adherence and counters threats from antibacterial resistance.

Stimulation of new research

This work could lead to new research areas for:

- The development of various pH-responsive nanosystems for administering multiple antibiotic categories and treating other ailments.
- Additional research into other bacterial behavioural characteristics has the likelihood for enhancing disease treatment and extend the lifespan.

Enhanced treatment of bacterial infections

This novel pH-responsive gelatin nanoparticle will maintain drug release profiles while inhibiting antibacterial action, resulting in increased drug concentration at the infection site. Other advantages of these advancements include preventing the development of resistance to certain antibiotic classes.

1.7 Overview of the dissertation

The research is presented in the following chapters:

Chapter One: Introduction

This chapter outlines the current state of bacterial infections as well as problems associated with conventional antibiotic treatment. Moreover, it discusses the advantages of pH-responsive nano drug delivery systems and their role towards curbing antibiotic resistance. It also provides the aims, novelty and significance of the study and concludes with an overview of the dissertation.

Chapter Two: Literature Review

This chapter presents an overview of the current state of infectious diseases in the medical sector, as well as limitations associated with antibiotic therapy which has contributed to the emergence of bacterial resistance. It also covers an overview on biopolymeric nanoparticles, gelatin nanoparticles characteristics, methods of preparation, as well as the usage of pH responsive NDDS as an approach to improving the treatment of infectious diseases. It concludes with an overview of ciprofloxacin as a drug model.

Chapter Three: Experimental paper

This chapter is presented in the required format of the journal. It presents the design and preparation of novel CIP-GNP. It also highlights the formulation of the pH-responsive CIP-

GNP for targeted delivery of CIP, and characterization of its physical and antibacterial studies via *in vitro* studies.

Chapter Four: Conclusion

This chapter includes the overall conclusions reached in achieving the aim of the study, objectives, outlines the significance of the findings and presents future recommendations for further research work in the field of strategic solutions to curb bacterial resistance to antibiotics.

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CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

This chapter presents an overview of the status of infectious diseases and limitations related to conventional antibiotic dosage forms, resulting in antimicrobial resistance challenges. It highlights the severity of antibiotic resistance and biofilm formation as an additional survival mechanism. Consequently, it outlines the prospects of nano-drug delivery systems as an innovative approach to overcome the limitations of conventional dosage forms and antibiotic resistance. It further provides a comprehensive insight into biopolymeric nanoparticles and concludes by describing ciprofloxacin as a model drug.

2.2 Current status of infectious diseases and limitations associated with anti-bacterial therapy

Infectious diseases (IDs) continue to cause substantial concern on a global scale despite advances in scientific research (1). IDs have consistently been the leading cause of morbidity and fatalities in humans (2). Bacterial infections make up one of the infectious diseases that pose a serious threat because of their likelihood to result in early death (3). Based on research, 7.7 million deaths worldwide were due to IDs in 2019, which equates to 1 in 8 of the total deaths globally (4). Additionally, this ranks bacterial infections as the second leading cause of death worldwide (5). Bacterial infections can cause minor infections and severe illnesses including sepsis, pneumonia, and tuberculosis to name a few. Therefore, such patients must have an appropriate treatment plan to prevent death from bacterial infections (6).

Over the years, modern medicine transformed, and the treatment perspective altered by the discovery, production, and regular administration of antibiotics to treat infections (7). Complicated medical procedures have highlighted the need for antibiotics to ensure patients do not develop infections prior to surgery. This is particularly important for cancer patients who have compromised immune systems due to treatment regimens being received (7). However, this therapeutic success is currently in jeopardy due to the notable rise in antimicrobial resistance (AMR) amongst prevalent bacterial pathogens, which endangers the positive results of patients in life-threatening conditions (8). Infections brought by AMR microorganisms are not only challenging to eradicate but there is always a greater possibility of severe complications and even death (9). Moreover, following the growing usage of antibiotics, particularly in countries that are still developing, bacteria have a high potential for developing AMR with major repercussions including increased high mortality and morbidity (8). The

World Health Organization (WHO) has classified AMR as one of the top three public health hazards. Studies have reported that AMR diseases caused approximately 1.27 million fatalities in 2019, while over 5 million fatalities were linked to drug-resistant infections (10). Additionally, the rate and severity of AMR bacterial infections have reached staggering levels, and endanger worldwide public health as a silent pandemic, necessitating urgent interventions (11).

In addition to the points mentioned above, biofilms significantly contribute to antimicrobial resistance, thus promoting resistance (12). Biofilms are cells of bacteria that are enveloped in an extracellular polymeric material and are stably adhered to a surface (13). Contrary to their planktonic substituents, bacteria that exist in biofilms might have antibiotic resistance that is 10-1000 times greater (14). Recent studies have shown that biofilms are a source of more than 80% of long-term infectious disorders, and it has been proven that the current antimicrobial drugs do not effectively treat infections produced by biofilms (15). This was also confirmed by a study reported by Backman *et al*,. (16). Thus, systems that overcome resistance at a molecular level are required.

While certain strains of bacteria possess numerous resistance genes that result in multi-drug resistance (MDR), others only have one antibiotic-resistant gene (17). *Mycobacterium* Tuberculosis*,* methicillin-resistance *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* (*P. aeruginosa*) are among the incurable MDR strains that are linked to increased mortality and morbidity, making them a major threat to global health (18). Furthermore, according to the World Health Organization (WHO), one of the biggest challenges to public health in the 21st century is antibiotic resistance (19). Additionally, based on recent analysis, antibiotic resistance is expected to be responsible for 300 million fatalities by 2050, as shown in (Figure 1) which demonstrates the timeline depicting the progression of antibiotic resistance (7).

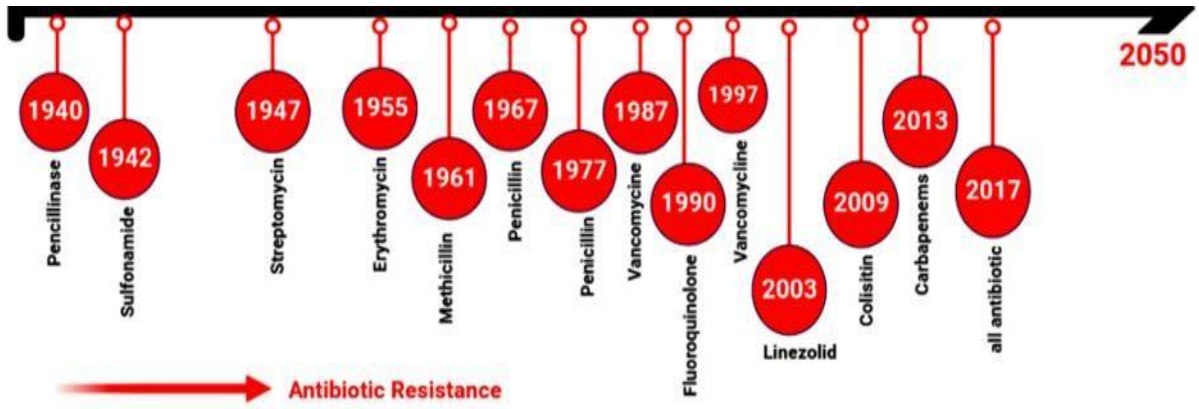

Figure 1: Timeline depicting the progression of antibiotic resistance (20).

Amongst other factors that contribute to the development of resistance are conventional antibiotic dosage forms (21). The constraints resulting from sub-optimal levels at bacterial infected areas pose a challenge to the present traditional dosage forms of antibiotics (22). Ineffective targeting (23), inadequate bioavailability (24), brief circulation time (25), early drug release (26), low cell/tissue permeability (27) and subpar pharmacokinetics profiles are amongst some of those (27). Furthermore, to fully utilize the antibiotic's demonstrated therapeutic effect, excessive amounts are administered regularly (28), which might contribute to the possibility of negative side effects and low adherence from patients (29). The development of antibiotic resistance is mostly caused by the limitations of antibiotics as well as their rampant use (30). Therefore, solutions are required to overcome limitations of conventional dosage forms.

2.3 Antibiotic therapy using nanoengineered drug delivery systems

Notwithstanding the antibiotic's apparent favourable outcomes, improved antimicrobial drugs and innovative approaches are vital in effectively preventing and treating infections (31). A viable way to enhance therapy with antibiotics and lessen antimicrobial resistance is through the application of nanotechnology, considering the present problems associated with conventional dose forms of antibiotics and antimicrobial resistance (32). Nanotechnology pertains the design, development and utilization of nanosized materials, and is considered as an innovative approach for maximizing effectiveness in infectious diseases therapy (33). The usage of novel nano-drug delivery systems (NDDS) in antibiotic treatments is advantageous since they may tackle bacterial infections and serve as vehicles of antibiotics and naturally occurring antibacterial agents (34). Following their emergence, various nanosystems have been designed and loaded with different antibiotics for the treatment of several diseases. Because of

their excellent physical and therapeutic features (35). A substantial surface-to-volume ratio is one of the desirable physical characteristics of nanosystems allowing for greater interaction with bacteria (36). These drug vehicles are designed to navigate past physiological barriers to reach the target sites through their micro size and appropriate surface modifications (37).

Compared to traditional antibiotics, nanosystems offer several therapeutic benefits, such as enhancement of antibacterial effectiveness, increased adherence by patients, fewer adverse effects, and increased cellular digestion and solubility (38). Additionally, increased stability of the drug, aimed and prolonged drug release, and specific targeting of tissues and cells further improves the active concentrations of antibiotics at the bacterial infection sites (39). These nanosystems also combat antimicrobial resistance following an increase in the number of bacteria that are resistant to antibiotics and have established resistance mechanisms (40). Therapeutic concentrations in the infected areas, shielding encapsulated medications against bacterial enzymatic deactivation, excellent entrapment of drugs, and anti-biofilm efficacy are some of the mechanisms through which nanosystems can combat antimicrobial resistance (41)

For the development of NDDS, a variety of materials with unique structural features are coupled with therapeutics (42). As per present methods, the most widely employed drug delivery carriers comprise nanoparticle biopolymers (43), polymeric nanoparticles (44), micelles (45), dendrimers (46), nanostructured lipid carriers, etc. as shown in (Figure 2) (47). Numerous preliminary and clinical research underpins their appropriateness in treating a range of diseases (48). There is an expanding array of nanomaterials being developed for the delivery of drugs, and these materials offer excellent treatment and detection properties (49).

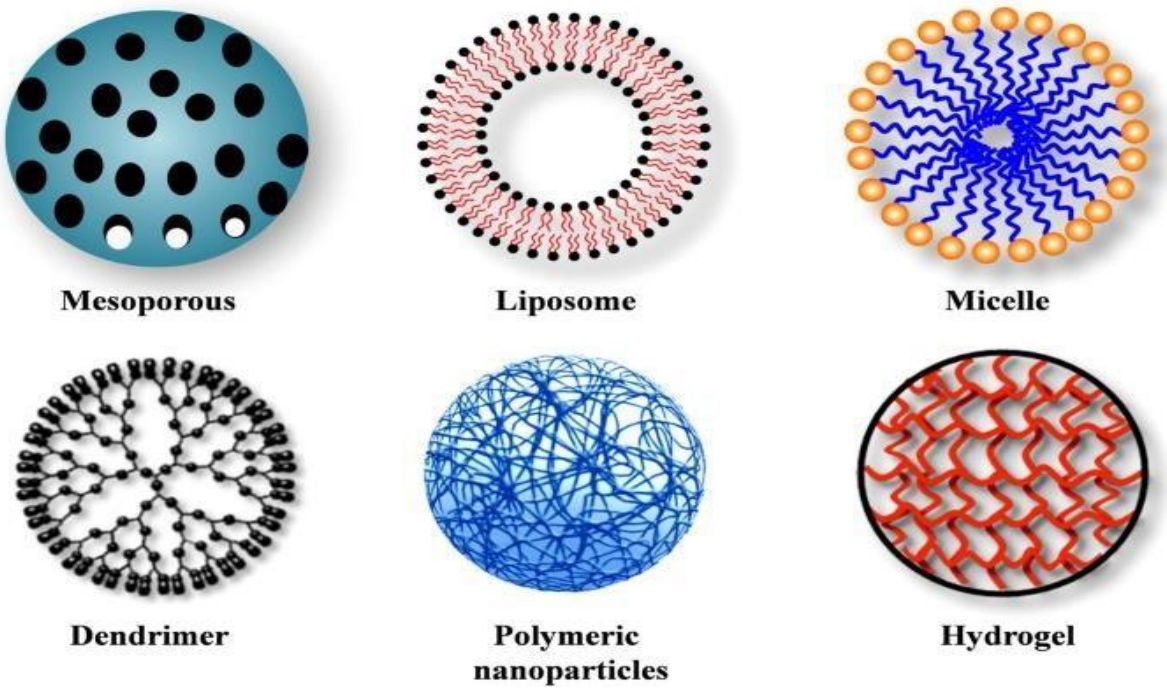

Figure 2: Examples of different nano drug delivery systems (50)

2.3.1 Biopolymeric nanoparticles.

Biopolymeric nanoparticles are biodegradable particles made of natural polymers that exist in biological species (for example: gelatin, collagen, and albumin) and polysaccharides (for example: chitosan, heparin, and starch) (51). The potential for treatment of the biodegradable colloidal particles has been examined for a variety of uses (52). Despite the intriguing findings described in the literature, these systems may also be linked to toxicological issues (53). A further limitation exists in the bionanoparticle-based delivery of hydrophilic substances such as proteins, peptides, and nucleic acids, which have been shown to have tremendous therapeutic promise (54). This drawback stems mostly from the hydrophobic nature of the polymers used to make these nanoparticles (55). This makes it difficult to properly encapsulate the drug and protect it from enzymatic breakdown (56). As a result, the synthesis of nanoparticles with more hydrophilic and naturally occurring substances needs to be investigated (57).

It was recognized that biodegradable nanoparticles (proteins, liposomes, virus-like particles etc.) may be utilized as effective drug-delivery vehicles (58). The rationale for this is that, alongside the wide range benefits of nanoparticles, biopolymeric nanoparticles specifically have several advantages, including the ease of preparation, well-known biodegradable

polymers and their excellent durability and stability in biological solutions and during storage (59).

Nanoparticles consisting of biodegradable polymers can operate as efficient drug delivery vehicles for prolonged, regulated, and targeted release, to improve the therapeutic effectiveness and reduce the adverse effects of the drugs (60). Nanoparticles can produce mediators at the final retention site in the target organs, activating inflammatory and immune responses (61). For these reasons, biopolymer-based nanoparticles of specified sizes constitute as one of the essential factors for delivery agent utilization (62). Surface charge, particle shape, and surface features, all crucial play roles in intercellular transport since they all influence the method of cellular ingestion via endocytosis (63). Furthermore, to ensure site specific administration and release of biologically active drugs at the appropriate amount, factors such as the particle size, biodegradability, and surface characteristics must be considered (64).

Biopolymeric nanoparticles can be broken down into compounds that can be harmless for consumption in the human system (65). Various biopolymeric nanoparticles have been utilized for encapsulation (66). To accomplish this, a biopolymer has to satisfy specific requirements, (i) robust and not reacting with the drugs (67), (ii) not detrimental to the function and cellular survival (68), (iii) environmentally friendly and inexpensive (69), (iv) the entirety of the biopolymer and its byproducts ought to be non-antagonistic to the host (70), (iv) the molecular size, dissolution features, chemical features must permit for suitable drug diffusion and release (71), (v) biocompatible and biosafe (72), and lastly (vi) when biocompatibility requires to be enhanced, the biopolymer must be paired with other compounds for an additive effect (73). Several variables impact the rate at which degradation of biopolymer-based nanoparticles occurs, including internal characteristics such as particle size, structure, and molecular weight, along with external factors such as pH and temperature which both contribute to drug loading release profiles (74). Synthetic biopolymers have a longer drug release profile than natural biopolymers. However, depending on the materials used, certain nanoparticles may have drawbacks such as limited drug loading ability, instability, and enhanced frailty (43).

The existence of a variety of advantages and disadvantages of biopolymeric nanoparticles necessitates careful attention when developing them (75). **Table 1** highlights some of the advantages and disadvantages of biopolymeric nanoparticles.

Table 1: Summary of advantages and limitations of biopolymeric nanoparticles.

Biopolymeric nanoparticles	
Advantages	**Limitations**
• Biocompatibility (76) • Poor immunogenicity (77) • Gradual and controlled release of the encapsulated drug (78) • Targeted delivery (79) • Increased therapeutic effectiveness (80) • Reduced undesirable effects and cytotoxicity (81) • Configurable size and surface for superior drug release profile and targeting precision (76)	• Unstable without modification (82) • Elevated cost of particle fabrication (83) • Difficult to produce particles with consistent form and size (84) • Low drug encapsulation efficiency (84) • Difficulty in large-scale manufacturing and sterilizing (77)

Recently, biopolymer-based nanoparticles such as protein nanoparticles have been extensively utilized as pharmacological and therapeutic tools because of the low toxicity as demonstrated by the studies (85). Proteins have distinct properties that are utilized in biological materials and manufacturing, and they can be employed as building blocks for nanoparticle development (86). Protein nanoparticles have numerous benefits as drug delivery vehicles, including particle surface modification, simplicity of particle size management and decreasing toxicity (87). More particularly, preserving the drug from enzymatic breakdown which can help in improving its stability and half-life (87). Albumin and gelatin, two naturally occurring proteins, were the first materials used to make nanoparticles (55). In this study, gelatin nanoparticles were the focus and will be expanded in the following section.

2.3.2 Gelatin nanoparticles

Gelatin is a natural and biodegradable polymer that is made up of peptides, proteins, and amino acids (Figure 3) (88). It has recently gained interest due to its numerous application capabilities in bioengineering and pharmaceutical industries. Moreover, it has distinctive qualities that make it an appealing material such as high biocompatibility, which means it may combine with

live tissues without creating any harmful responses (89). In addition, its biodegradability enables it to degrade organically, limiting the possibility of contamination and waste development (90).

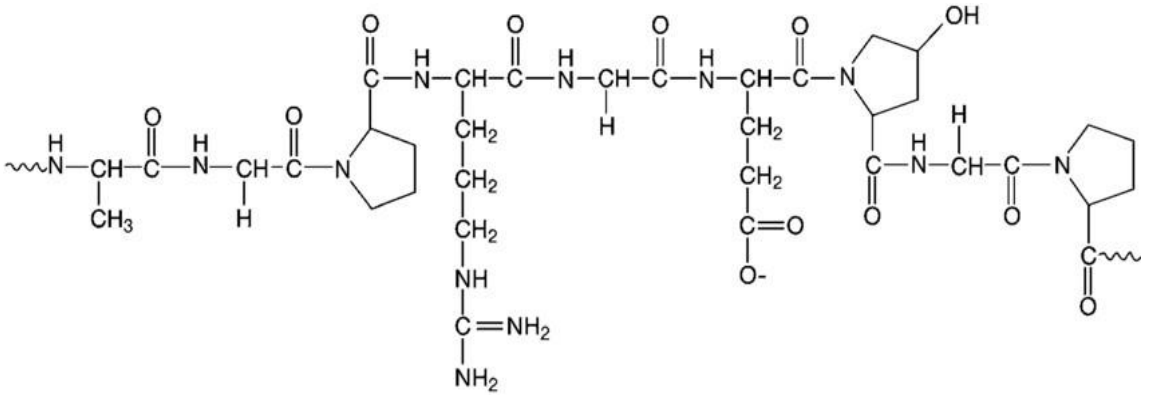

Figure 3: Basic chemical structure of gelatin (91)

Enzymatic hydrolysis naturally breaks down gelatin in the human body and proteins and are subsequently degraded by a variety of enzymes (92). For example, the peptide bonds that link amino acids in a gelatin molecule can be cleaved by protease enzymes (93). This technique efficiently breaks down gelatin into its constituent amino acids, which allows it to be easily absorbed by the body (94). Moreover, gelatin has thermo-reversible gelation qualities that increase its adaptability by enabling it to change from a liquid to a gel form at various temperatures (95). Various desired gelatin nanoparticles (GNPs) can be produced by utilizing gelatin's physical and chemical properties (95).

Of its various applications, GNPs have shown several benefits when utilized as drug delivery systems. A range of therapeutics, inclusive of both hydrophobic and hydrophilic agents can be encapsulated using GNPs (96). They can therefore be used to administer a variety of drugs (97). Furthermore, by altering the method of preparation, their characteristics, including particle size, profile of drug release and surface charge may be readily customized to fit certain applications (64). GNPs can improve the durability of drugs that are encapsulated, shielding them against degeneration (98). This is advantageous for delicate drugs which are prone to premature breakdown since it enables them to reach the sites of the target while they are still active (99). Moreover, GNPs are capable of being engineered to deliver drugs to specified regions in the body, utilizing targeting ligands that attach to receptors on the target cells allowing for their surface modification (100). Through more concentrated drug administration, adverse effects can be reduced, and the therapeutic effect can be increased at the target site (101). With the help of GNPs, the encapsulated drug can be released in a regulated and maintained manner,

enabling the concentration of the drug to remain in the range of therapeutic effect for a prolonged duration thus enhancing therapeutic results and patient adherence (102).

Based on the abovementioned advantages, together with their versatility and easy-to-prepare qualities, GNPs offer great potential as building blocks for effective and secure systems of delivering drugs (103). As a foundation for innovative alternatives to therapy, present studies continue to be centred on identifying new ways to enhance these advantages. Namely, GNPs may be utilized in the treatment of cancer for the delivery of chemotherapeutic agents efficiently into cancerous cells (104). Using this strategy reduces harmful negative effects on normal cells while increasing the drug's effectiveness (105). To increase the treatment's specificity and efficacy, their surfaces can be modified using antibodies or alternative targeted ligands that bind with the receptors on malignant cells that are overproduced (106).

GNPs have been further explored for oral administration of different drugs (107). By expanding the drug's duration in the intestines, they may improve the digestion of drugs and prevent the enclosed drug from breaking down in a hostile environment of the intestinal tract (107). Additionally, some research indicates that GNPs may improve the oral absorption of insoluble drugs (108). Also, GNPs have demonstrated potential as non-viral gene delivery systems (109). Various plasmids may become encapsulated by them, and through inhibiting nuclease-induced degradation, the efficacy of the encapsulated DNA can be increased (110). GNPs have been associated with agents that promote receptor-mediated endocytosis (111). Additionally, the biological activity of long-circulating PEGylated nanoparticles can be enhanced by their utilization (112).

2.3.2.1 Factors influencing GNPs characteristics

GNP characteristics including size, shape, surface charge and drug loading capacity are essential for evaluating their efficacy as drug delivery agents. These features are controlled by a variety of variables upon preparation, namely the method of preparation, gelatin concentration, pH and temperature of the solution, and cross-linking method (113). Recognizing how these parameters affect the characteristics of GNPs is critical for customizing them to specific uses. GNPs with desired features can be produced by precisely regulating these variables, maximizing their potential as drug delivery systems. Current research in this field continues to provide an understanding of these interactions, opening the path for the creation of more advanced and effective GNP-based drug delivery systems (114).

Method of preparation

The method of preparation has a significant impact on the features of GNPs. The most often utilized methods include desolvation, emulsion, coacervation and nanoprecipitation. Each method produces nanoparticles with varied properties. For examples, the desolvation methos may necessitate further procedures, such as cross-linking to stabilize the nanoparticles. Coacervation usually yields bigger particles than nanoprecipitation. Emulsion method may accept both hydrophobic and hydrophilic drugs, which influences the drug loading efficacy and the release behaviour of the nanoparticles (115).

Concentration of gelatin

The amount of gelatin in the initial formulation has an enormous effect on the particle size and drug-loading capability of the generated nanoparticles. A high gelatin concentration often produces bigger nanoparticles and can improve drug encapsulation efficiency. Nevertheless, this may result in the agglomeration of nanoparticles, which might be unfavourable in some applications (116).

pH and temperature

The properties of gelatin can be influenced by pH and temperature of the solution upon the fabrication process. Gelatin has an isoelectric point of roughly pH 5, at which the gelatin molecules possess no net electrical charge and frequently agglomerate, producing bigger nanoparticles. Additionally, temperature may impact the solubility and configuration of gelatin molecules, which affects the size and stability of the generated nanoparticles (117).

Cross-linking

The cross-linking technique used for stabilizing GNPs can have a considerable impact on their characteristics. The type of cross-linking agents utilized, the level of cross-linking, and the length of time of the cross-linking process can all have an impact on the size, stability, drug loading capacity, and drug release behaviour of the nanoparticles. For example, an elevated amount of cross-linking can improve the stability and manage the release of the encapsulated drug, yet it can also decrease drug loading capacity (118).

2.3.2.2 Methods Used for GNP Preparation.

Desolvation, coacervation methods, nanoprecipitation, solvent/emulsification evaporation, and reverse phase methods are some of the techniques used for preparing GNPs (119). Below is a description of them:

Two-step desolvation

This method involves adding a desolvating agent into an aqueous solution. The low molecular weight of gelatin is removed, the remaining high molecular weight is dissolved in water, and acetone is steadily added to the mixture at a pH that is regulated. A cross-linker is added while stirring (120).

Nanoprecipitation

In nanoprecipitation or solvent displacement, the water-soluble gelatin solution is added to an organic solvent such as ethanol, which has a poloxamer added as a stabilizer. This method dissolves the drug molecules and gelatin in water, which serves as a solvent phase. Due to interface instability created by nanoprecipitation, the GNPs are produced at the interface of water and ethanol which are cross-linked (121).

Coacervation method

Durable, small nanoparticles are produced by coacervation methods. Two apparent inseparable phases are produced by the polymer settling down in the solution during the liquid-liquid separation phase (122). To obtain nanoparticles, organic salts including sodium chloride or sodium sulfate, or alcohols such as ethanol are typically utilized. Nonetheless, multi-modal coacervation, one of inversely charged macromolecules such as proteins or polyelectrolytes are also recommended. Ultimately, gelatin molecules undergo dehydration to generate GNPs, and these are subsequently cross-linked (123).

Emulsification evaporation

To produce GNPs, this method utilizes either single or double emulsions. After homogenizing gelatin and drugs in the aqueous stage using an oil phase (paraffin oil) or genipin is used to cross-link the mixture. The solvent then evaporates. Once the nanoparticles have formed, they are gathered, rinsed with distilled water to get rid of any additives such as surfactants, and finally preserved (124).

Reverse phase method

The aqueous gelatin solution is mixed with a surfactant solution [sodium bis (2-ethylhexyl) sulfosuccinate] in n-hexane to create a microemulsion. A cross-linker is then added and when n-hexane evaporates, GNPs are extracted. Since microemulsion may regulate the nanoparticle's site, it is thought to be beneficial (125).

2.3.2.3 Optimization of GNPs for drug delivery uses

GNPs' efficacy as drug carriers is depended on their capacity to preserve the enclosed drug, deliver it to the targeted site, and release it in a regulated manner. These capacities are heavily influenced by the nanoparticles' features, such as size, surface charge, drug loading capacity, and release profile which will be explained further below. To maximize GNPs for delivery of drug uses, these attributes can be cautiously monitored and customized to meet the unique needs of the application (126).

Size

The size of nanoparticles affects their dispersion, penetration into cells, and elimination in the body. Smaller nanoparticles are favoured for widespread purposes as they can bypass the reticuloendothelial system and enter deeper into tissues. GNPs' size can be adjusted during the manufacturing process by altering parameters such as gelatin concentration, stirring rate, and temperature (127).

Surface charge

The charge of a nanoparticles' surface influences its stability, cell absorption, and attachment to biological systems. GNPs often have a positive surface charge owing to the presence of amino groups in gelatin. This positive charge increases how it interacts with negatively charged cellular membranes, hence increasing their absorption. The surface charge can be changed by altering the pH during the production process or by adding charged molecules (128).

Drug loading capacity

The drug loading capability regulates how much drug can be supplied per nanoparticle. This can be enhanced by increasing the amount of gelatin or altering the gelatin to enhance its ability to interact with the drugs. The drug's absorption in the gelatin matrix, as well as the drug's compliance with gelatin may additionally impact the drug loading capacity (129).

Release profile

The drug's release profile from the nanoparticles is critical for establishing therapeutic efficacy. Ideally, the drug ought to be released in a regulated and maintained manner such that its concentration remains inside the therapeutic range for a prolonged time. This can be accomplished by varying the amount of cross-linking in nanoparticles, which controls their disintegration rate and permeability. Furthermore, by including responsive components in nanoparticles that respond to specific triggers, such as changes in pH or temperature, the release profile can be altered (130). Considering and regulating these features allows for the optimization of GNPs for a variety of applications involving drug delivery. Different applications may have unique requirements; therefore, a one-size-fits-all strategy may not be acceptable. Instead, a comprehensive design and optimization procedure specific to the application is required. Current research in this area involves developing novel strategies and methodologies for improving GNPs, which will help advance nanoparticle-based drug delivery systems (94).

2.3.2.4 Overview of GNPs in antibiotic therapy

Antibiotics have been successfully delivered via GNPs. These nanoparticles shield the drugs from deterioration in the body's natural environment and enable targeted, regulated release which may increase their efficacy and lessen adverse effects (131). Chloramphenicol (CHL), Spectinomycin (SPEC) (127), and Vancomycin (VCM) (132) are among the antibiotics that have been effectively loaded in GNPs for specific administration to infected areas, showing improved antibacterial properties (133). **Table 2** shows various methods for developing GNPs and antibiotics utilized to treat bacterial infections.

Li *et al.,* 2023 reported a method of delivering antibiotics flexibly by using red blood cells that were membrane-coated and loaded with vancomycin supramolecular GNPs to circumvent the immune clearance conducted via macrophages. Red blood cell membranes also operate as purifying agents, by absorbing the exotoxins generated by microorganisms and reducing the inflammation that the bacteria cause (131). Meanwhile, the exceptionally amplified bacterial gelatinases within the infectious milieu can effectively dissolve supra-molecule gelatin nanoparticles into minuscule particles. Through this, the encapsulated VCM is released which leads to the immediate elimination of pathogenic microorganisms. Antibiotics can therefore be used cautiously to treat bacterial infections attributable to the mimicry antibiotic delivery method (134).

Towards increasing the bactericidal and antifungal efficiency of insufficient cell penetration of antibacterial agents such as SPEC and CHL, a study was conducted by Ibrahim *et al*., 2023 in which they prepared, described, and employed GNPs for intracellular delivery. The desolvation technique was utilized by the investigators to produce GNPs, which were subsequently filled with cellulosic cotton medicinal cloth, SPEC, and CHL (127). GNPs loaded with the antibiotics SPEC and CHL were discovered to exhibit improved antimicrobial activity against *S. aureus* and *E. coli* when pretreated cellulosic cotton cloth was used (135). Scharade *et al*., 2022 designed a two-stage drug mechanism to release cartilage morphogenetic protein-2 and Clindamycin (CNM) in a different experiment. Hydrogels and GNPs were both present in this disintegrating system. CNM was released over 28 days at a dosage which has 25 times greater than the minimal inhibitory concentration (MIC) required to eradicate *S. aureus* (136).

The nanoprecipitation approach was applied by Fatollahipour *et al.,* 2016 to develop and evaluate GNPs loaded with Erythromycin (ERY). ERY-loaded GNPs demonstrated antimicrobial activity toward *S. aureus* and *P. aeruginosa* (137). Remarkably, Li *et al.,* 2022 produced GNPs and then attached AMP-Cypate, and antimicrobial photothermal peptide to form AMP-Cypate GNPs. It was discovered that this mixture eliminated bacterial infections and stimulated thorough wound rehabilitation (138). Mahor *et al.,* 2016 synthesized highly charged GNPs preloaded using Moxifloxacin (VC) for effective ophthalmic administration and regulated release throughout the corneal layer of the eye in a separate study. When evaluated *in vivo* upon *S. aureus*, the produced nanosuspensions demonstrated greater antibacterial effects (139).

Table 2: Summary of various methods for developing GNPs and antibiotics utilized to treat bacterial infections.

Infection type	**Type of drug encapsulated in GNPs**	**Preparation method**	**Results**	**References**
Bacterial infections	VCM	Desolvation	Improved the effectiveness of the antibiotic's antibacterial properties thereby rendering the ability to treat infections caused by bacteria with less dose.	(131)

Bacterial infections	CHL and SPEC	Desolvation	Enhanced antibacterial efficacy against *S. aureus* and *E. coli*.	(127)
Cartilaginous infections	CNM	Desolvation	The dosage of CNM that was released was twenty-five times more than the MIC required to eradicate *S. aureus*.	(140)
Bacterial infections	ERY	Nanoprecipitation	Demonstrated antibacterial effects towards *P. aeruginosa* and *S. aureus*.	(137)
Wound rehabilitation	Antibacterial photothermal peptide AMPcryptate	Desolvation	Eliminated the infection of bacteria enabling the wound to heal completely.	(131)
Ophthalmic infections	Mfx	Desolvation	When contrasted to the market product, the nanosuspensions demonstrated greater in vivo antibacterial efficacy towards *S. aureus*.	(139)

CHL= Chloramphenicol, CNM= Clindamycin, ERY= Erythromycin, Mfx= Moxifloxacin, MIC= Minimal Inhibitory Concentration, SPEC= Spectinomycin, VCM= Vancomycin

2.3.2.5 pH-responsive GNPs

To further enhance nanoparticles efficacy for treatment, research has started investigating the use of environmentally sensitive nanoparticles, that when subjected to external stimuli, create physical alterations which promote drug release in the area of interest (141). External stimuli include (i) physical signals such as temperature, electric field, magnetic field, and ultrasound; and (ii) chemical signals like pH, ionic charge, redox capability, and enzyme activity (142). Amongst these environmental cues, pH gradients have become frequently utilized for developing novel, sensitive nanosystems (143). The chemical synthesis of pH-responsive nanoparticles is flexible, enabling them to incorporate various pH-responsive groups or bonds for regulating drug release in an acidic environment of bacterial infections (142). Infected areas in humans have been discovered to have acidic pH levels ranging from pH 5.7-7.8 (144). The

acidity in infection site microenvironments is induced partially by lactic acid production in rapidly developing infectious cells, which have higher rates of glucose intake but lower rates of oxidative phosphorylation (145). The persistence of elevated lactate generation by infectious sites in the presence of oxygen gives infected cells an advantage *in vivo* (146). Furthermore, most infected locations have limited availability of blood and poor lymphatic drainage, which contributes to the acidity of the infectious milieu (147). Thus, pH responsive nanosystems that can enhance antibiotic efficiency are required.

Standard pH-responsive nanosystems have distinct response modes: (i) Physical dissociation, (ii) chemical bond cleavage-induced nanoparticle dissociation, and (iii) nanoparticle swelling (148). These responses will be explained further below:

i) Physical dissociation

The physical dissociation mechanism encompasses the reaction behaviour of nanoparticles to a particular pH environment, ultimately resulting in hydrophobic core protonation or ionization, triggering the physical dissociation of nanoparticles (149). Physical disintegration nanoparticles responsive to acidic environments are composed of polymers containing ionizable groups that act as proton acceptors or donors in reacting to pH variations in the environment (150). In an acidic environment, groups like acrylic acid, sulfonamide, and tertiary amine undergo protonation or charge reversal, enhancing water solubility or mutual repulsion and thereby disintegrating the polymer and delivering the drug (151). The protonation of the nitrogen on the hydrophobic surface causes electrostatic repulsion, resulting in nanoparticle dissolution (152).

ii) Chemical bond cleavage-induced nanoparticle dissociation

In a chemical bond cleavage-resulting nanoparticle dissociation system, acid-labile chemical bonds connect hydrophilic and hydrophobic segments of amphiphilic block copolymers, and drugs are contained by self-assembling nanoparticles. At physiological pH parameters, the nanoparticles continue to be robust, and the encapsulant is not discharged. When H^+ penetrates nanoparticles in a state of acidity, the acid-labile link breaks, causing the polymer to dissolve and the drug to be released (153).

iii) Nanoparticle swelling

Hydrophobic segments in suitable amounts are attached to lengthy hydrophobic chains by acid labile chemical bonds, and the hydrophilic-hydrophobic component ratio is adjusted to allow

nanoparticles to form via self-assembly in aqueous solution at normal physiological pH (154). As soon as nanoparticles get an acidic environment, such as infectious tissues, H^+ passes through the nanoparticles and facilitates the destruction of the acid-labile chemical bonds, triggering hydrophobic fragments to separate, thus, decreasing hydrophobic fragments and enhancing solubility, resulting in nanoparticle size enlargement and drug release (142). Thus, pH-responsive nanosystems can utilize either of these above-mentioned responses for drug release under acidic conditions.

Antibacterial drugs can be encapsulated inside the pH-responsive nanoparticles as a viable way of extending the duration of drug circulation and remaining within the nanoparticles at pH conditions synonymous with the diseased area (155). Additionally, the mechanism of action and biodistribution of the drug can be enhanced by pH-responsive nanoparticles. Delaying the metabolism and consequent release of drugs is crucial (156). Moreover, after the drug is delivered to the intended location, pH-responsive nanoparticles enable the regulated release of the drug which is encapsulated at an acidic pH (157). These findings have rendered pHresponsive nanosystems extremely intriguing to investigate as drug delivery systems. **Table 3** shows a summary of pH-responsive nanosystems that have been reported, as well as their main findings.

Table 3: Summary of various pH-sensitive nanosystems as well as their main findings.

pH-responsive nanosystem	Materials used	Main findings	References
Hydrogel beads	Alginate and chitosan which contained ethanolic extract of propolis.	In comparison to the drug's free form, the hydrogels loaded with the drug demonstrated excellent antibacterial properties. Abrupt release of the drug was observed at pH 6.8, suggesting that the drug is being released under regulated conditions for the treatment of gastric cancer, ulcerative stomach, and oral mucositis.	(158)

Polymeric nanoparticles	Acrylate-based hydrophobic polymers with hydroxyl groups that were masked by pH-labile protecting groups.	At neutral pH, the NPs remained robust. However, at moderately acidic pH (~pH 5), the protective group was broken down, exposing the hydroxyl groups. The hydrophobic-to-hydrophilic transition produced swelling of the NPs and consequently drug release. Paclitaxel release was low at pH 7.4 (< 10%), while almost all the treatments were released after 24 hours at pH 5. These acrylate-based, pH-sensitive NPs were found to inhibit the rapid growth of LLC tumours in C57Bl/6 mice when contrasted with non-responsive NPs or paclitaxel in solution, implying that pH-responsive drug release could potentially be advantageous for drug delivery to tumours.	(159)
Core shell nanoparticles	poly (ethylene glycol)poly(L-histidine)-poly(L-lactide) (PEGPH-PLLA) core-shell NPs as antitumor drug. carriers encapsulated DOX	The findings indicated that blank and drug-loaded nanoparticle sizes were smaller at pH 7.4 than at pH 5.0, and DOX was released faster at pH 5.0 than at pH 7.4. *In vitro* tests demonstrated that the produced nanoparticles were harmless on both NIH 3T3 fibroblasts as well as HepG2 cells.	(160)
Magnetic nanoparticles	Chromone linked to magnetic Fe_3O_4 nanoparticles through a Schiff base.	A Schiff-base link increased chromone release by fourfold at pH 5 compared to pH 7.4, increased chromone solubility in buffer solutions from 2.5 to 633 μg/mL, and improved cytotoxicity *in vitro*.	(161)

Cisplatinpolymeric nanoparticles	Cisplatin–polymer conjugated nanoparticles using hydrazone crosslinkers.	Cisplatin was released at pH less than 6 via hydrazone hydrolysis, rather than poly (lactic acid) (PLA) breakdown, which led to increased cellular cytotoxicity *in vitro* compared to free cisplatin.	(162)
Micelles	PEG-poly (β-amino ester) polymers that had a pKb of roughly 6.5	Amine protonation enhanced polymer solubility at pH 6.4-6.8 and produced a rapid micellization-de micellization switch for drug release.	(163)

DOX= Doxorubicin

pH-responsive GNPs have been prepared using different materials for the delivery of therapeutics in the treatment of different diseases (164). Khodashenas *et al.,* produced the pHresponsive gelatin-coated gold nanoparticle methotrexate drug delivery system. The findings demonstrated that methotrexate (MT) was released from the drug carrier at two different pH levels (5.4 and 7.4) at varying rates. The maximum drug rate of release was found at pH 5.4, making this drug delivery system appropriate for application in breast tumour environments. Moreover, gelatin-based nanodrug-loaded particles enhanced the therapeutic effect of MT, showing a mutually beneficial relationship between the drug transporter and the drug (165). Moreover, Anhui *et al.,* fabricated a new type of magnetic gelatin-encapsulated nano assembly which was developed as a multimodal drug delivery system with pH stimuli-responsive drug release for cancer theragnostic. Cysteamine-modified gelatin was employed to encapsulate doxorubicin (Dox), an anticancer drug, and Fe_3O_4, a magnetic resonance imaging (MRI) contrast agent, which were then assembled into a nano assembly by the production of an oil in-water microemulsion (166). However, pH-responsive GNPs prepared using two-step desolvation method for enhanced delivery of CIP in treatment of bacterial infections has not been investigated before, thus, this study is intended to accomplish this.

2.4 Ciprofloxacin as a drug model for antibiotic therapy.

Ciprofloxacin is an antibiotic belonging to the fluoroquinolone group. It treats bacterial infections, including pneumonia and urinary tract infections (Figure 5) (167). It has also been regulated to cure acute cases of otitis externa spurred by susceptible strains of *S. aureus* or *P.*

aeruginosa. This versatile antibiotic with a range of actions inhibits the replication of DNA in various aerobic bacteria, both gram-negative and positive. (168).

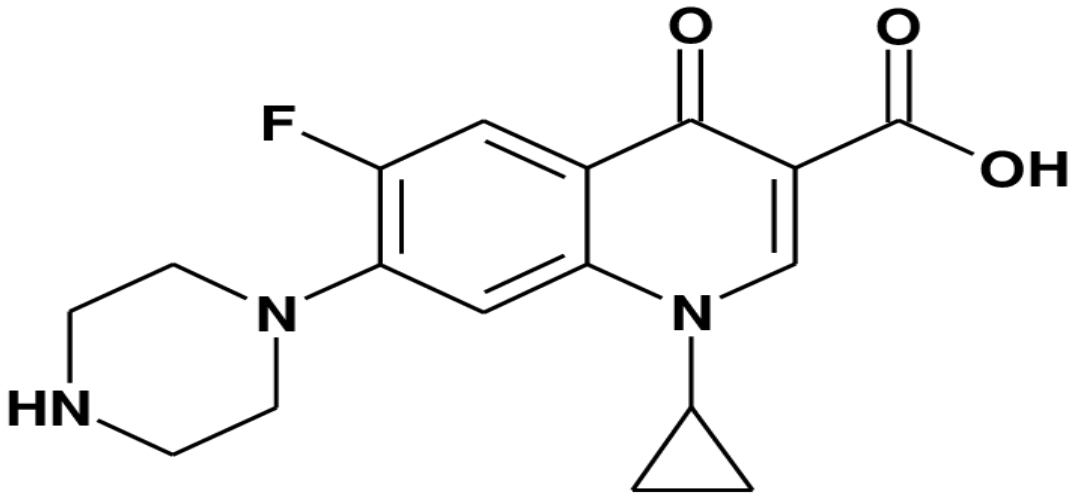

Figure 4: Structure of Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) (169)

2.4.1 Mechanism of action and current limitations of CIP

Topoisomerase II and Topoisomerase IV are two types of DNA gyrases that are inhibited via this broad-spectrum antibiotic, CIP. Subunits A and B are components of DNA gyrase (170). It appears that quinolones, notably CIP, inhibit subunit A from closing the double-stranded DNA. Consequently, single-stranded DNA might be broken down exonucleotically (171). An amino acid alteration in topoisomerase IV or gyrase may lead to CIP resistance (172). These mutations that confer resistance are mostly found in the amino-terminal domains of *GyrA* or *ParC*, which have been covalently linked to DNA in an enzyme intermediate, where the tyrosine site of activity is situated close by (173). The component is known as *GyrA* and *ParC'*s quinolone resistance determining region (QRDR) (Figure 6) (174). Modifications in certain domains of *GyrB* and *ParE* are additionally associated with quinolone resistance in clinical bacterial isolates (175). Thus, novel approaches are required to overcome these limitations and mechanisms to increase the effectiveness of CIP.

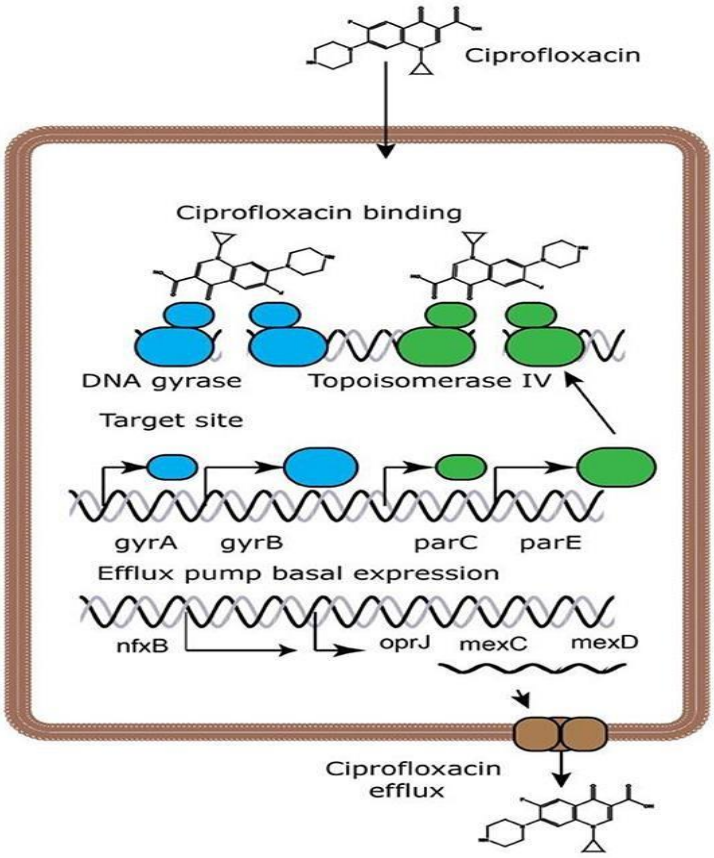

Figure 5: Mechanism of action for CIP (176)

2.4.2 Overview of pH-responsive CIP nanosystems.

Nano-drug delivery systems have drawn growing interest in both disease diagnosis and treatment. Nevertheless, current imaging tests for accurate imaging of disease possess certain prevalent problems with low sensitivity or inadequate specificity (177). Conventional nanodrug delivery systems, meanwhile, are typically unable to accomplish all the objectives for the treatment of illnesses, such as elevated drug levels in the targeted sites, prolonged retention time *in vivo*, and fewer adverse effects (178). Many stimuli-responsive and targeted nanosystems that take advantage of variations in ATP, pH, redox, temperature, and reactive oxygen species levels between healthy and pathological conditions are being employed to address these issues (179). Nanosystems such as hydrogels, cryogels, nanofibers, biopolymers, liposomes, nanogels, and micelles enhance the current therapies due to their modified pharmacokinetic and biodistribution patterns (180).

Table 4: Examples of CIP-loaded pH-responsive nanosystems in nano drug delivery.

Nanosystem	Entrapment efficiency	Key outcomes	References
Hydrogel	83.6%	Nanocarriers loaded with CIP exhibited antibacterial efficacy against *S. aureus* and *E. coli*.	(181)
Nanofibers	-	Diffusion disk tests on *Staphylococcus epidermidis* and *E. coli* showed improved bacterial activity.	(182)
Nanogels	95%	The MICs of CIP demonstrated an increased antibacterial activity when it was released from the nanoparticles.	(183)
Cryogels	-	The cryogels that were evaluated showed excellent cytocompatibility and sufficient cell survival. These systems show promise carriers of drugs for antibiotic	(184)
		formulations due to their pH responsiveness and biocompatibility.	
Micelles	44%	When contrasted with biofilms treated with CIP only, bare pH-responsive micelles demonstrated a decrease in *MRSA* biofilm bacteria that was either similar or higher.	(185)
Biopolymeric nanoparticles	65%	Biopolymer nanocomposites were found as an effective treatment for chronic infections caused by multi-resistant and unidentified microbes.	(186)
Liposomes	-	CIP-loaded liposomes had excellent therapeutic efficacy in mice.	(187)

2.5 Conclusion

This chapter reviewed the research findings in the literature regarding the status of infections caused by bacteria, as well as treatment difficulties brought on by the ever-evolving mechanisms of antibiotic resistance. It further establishes the potential of pH-responsive gelation nanoparticles in enhancing antibiotic accumulation and countering multi-drug resistance. Ciprofloxacin has also been introduced in this chapter as a model drug linked to treatment failures, emphasizing the necessity for creative approaches to improve their efficacy.

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CHAPTER THREE: MANUSCRIPT

3.1 Introduction

3.2 Author contributions

3.3 Manuscript

3.1 Introduction

This chapter is a first-authored experimental article that presents pH-responsive gelatin nanoparticles for targeted delivery of antibiotics against bacterial infections, as described in **CHAPTER 1.** This article describes the various *in vitro* characterization properties of the CIP-GNPs including antibacterial efficacy, and pH-responsive release of ciprofloxacin and concludes with the summarized findings of the study.

3.2 Author Contributions

In this paper, Miss Minenhle Hlabisa performed the literature searches that contributed to the conceptualization, planning, and implementation of this project. Moreover, Miss Minenhle Hlabisa was responsible for the design and characterization of the nanosystem, as well as the analysis and interpretation of all data and writing of all draft versions, and revisions according to the suggestions of the co-authors. Mr. Sbongumusa Dlamini assisted with *in vitro* antibacterial activity studies, analysis, and interpretation of the data. Mr. Mohammed A. Gafar, Miss Eman A. Ismail, and Mrs. Eman Elhassan assisted with the implementation, planning, and problem-solving involving the experiments. Prof. Calvin A. Omolo contributed to conceptualization, planning, and problem solving as well as co-supervision of the study. Prof. Thirumala Govender served as the supervisor and was responsible for conceptualization of the project, problem-solving, editing of papers and abstracts as well as general supervision of all the experiments.

3.3 Manuscript

pH-responsive gelatin nanoparticles for targeted delivery of ciprofloxacin against bacterial infections.

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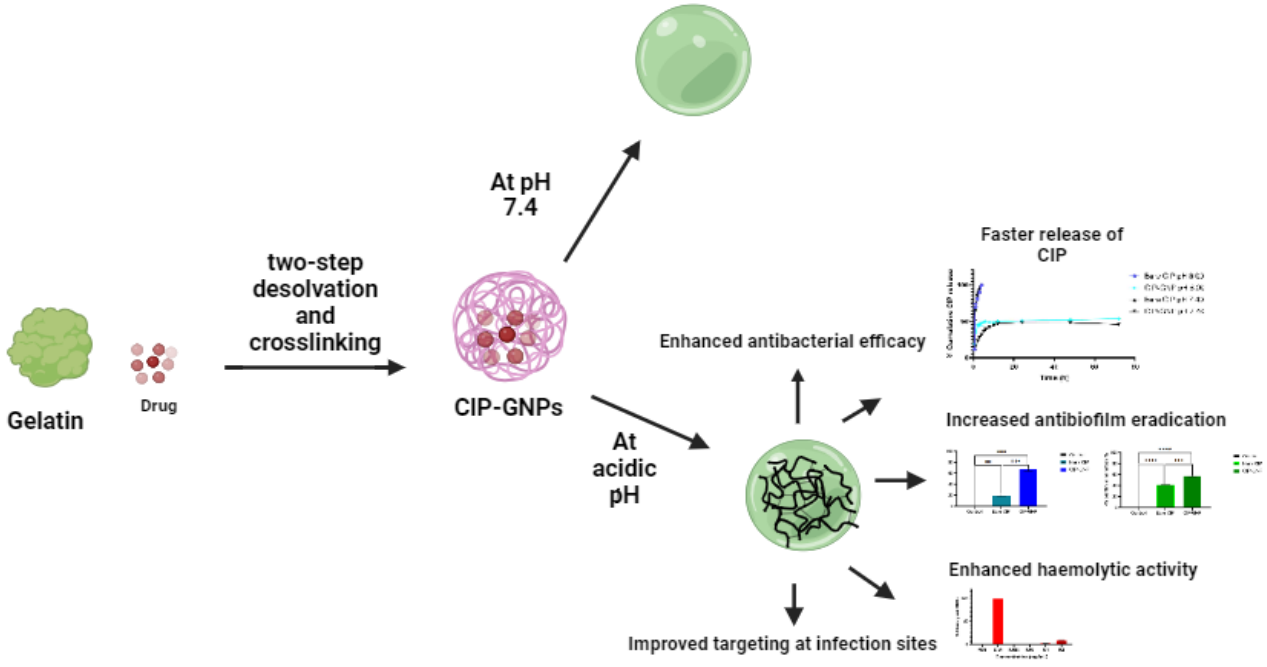
Abstract

Despite the advances in infectious disease management, antimicrobial resistance remains a worldwide concern. Consequently, the development of new treatment approaches is an immediate necessity. Herein, we report pH-responsive gelatin nanoparticles loaded with ciprofloxacin (CIP-GNPs) to enhance the antibacterial effectiveness of ciprofloxacin against multidrug-resistant bacteria. *In vitro,* biosafety testing identified CIP-GNPs as non-hemolytic. CIP-GNPs had a particle size, polydispersity index, zeta potential, and entrapment efficiency of 212.3 ± 1.739, 0.259 ± 0.023, + 4.58± 0.148 mV and 38.1 ± 3.85%, respectively. The CIP-GNPs demonstrated pH responsiveness with an increase in particle size from 204.1 ± 0.100 to 226.4 ± 0.451 nm and a charge switch on the zeta potential from -3.59 ± 0.428 to 1.06 ± 0.271 mV, followed by a significantly faster release of CIP at pH 6.0 compared to 7.4. The *in vitro* antibacterial activity of CIP-GNPs showed 2-fold lower minimum inhibitory concentration values compared to bare ciprofloxacin against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). Moreover, the bacterial-killing kinetic test showed 100%

elimination of MRSA and *P. aeruginosa* within eight and one hour(s) of treatment with CIP-GNPs, respectively. In contrast, 100% elimination of MRSA and *P. aeruginosa* was observed within 24 and 12 hours of treatment with bare ciprofloxacin, respectively. CIP-GNPs eliminated 3.75-fold MRSA biofilm compared to bare ciprofloxacin, whereas 1.4-fold *Pseudomonas aeruginosa* biofilms were eliminated. As a result, CIP-GNPs could function as an effective approach for increasing antimicrobial efficacy and bypassing mechanisms of resistance against bacterial infections.

Keywords: pH-responsive, Gelatin nanoparticles, ciprofloxacin, bacterial infections

Graphical abstract



1. Introduction

Bacterial infections have been recognized as a major threat amongst infectious disorders, due to their elevated likelihood of inflicting premature death (1). According to recent reports, bacterial infections were responsible for 7.7 million fatalities worldwide (2). Since the middle of the 20th century, antibiotics have been employed to treat bacterial infections (3). However, this success was promptly followed by the emergence of antimicrobial resistance (AMR), which accounted for 4.95 million deaths, rendering it one of the greatest contributors to mortality (4)**.** As a result of the rapidly developing antibiotic resistance challenge, most formerly accessible antimicrobial therapeutics for treating bacterial infections have become outdated (5). Furthermore, the development of multi-drug resistant bacterial strains due to unregulated and widespread usage of antibiotics has reduced the reservoir of medically effective antibiotics (6). The spreading of resistant genes across and within bacterial species has been ascribed to an upsurge in multidrug-resistant (MDR) pathogens like methicillinresistant *Staphylococcus aureus* (MRSA) (7). Following a Lancet report, MRSA infections resulted in nearly 100,000 fatalities in 2019 (8). As a result, novel ways for regaining antibiotic potency for enhanced therapeutic results in these illnesses are essential (9).

Conventional dosage forms of antibiotics have served as the sole method of administration for antibiotics since their first discovery in the 1940s (10). However, their drawbacks include noncontrolled and non-targeted release leading to low drug levels at infection sites (11). This leads to sub-optimal drug distribution and activity, enhanced side effects, cell or tissue toxic effects and poor adherence by patients, all of which worsen treatment outcomes and accelerates the emergence of antibiotic resistance (12). To overcome these limitations, nano-drug delivery systems (NDDS) are designed to improve the delivery and efficacy of the existing medications to specified target sites (13). Furthermore, they preserve optimum drug levels while concurrently decreasing undesirable effects (14). To address the mounting threat of AMR, different NDDS are being explored.

Biopolymeric nanoparticles composed of biodegradable polymers such as proteins can serve as effective drug delivery systems for sustained, regulated, and targeted release to enhance therapeutic outcomes while also reducing the adverse effects of manufactured drugs (15). Protein-based gelatin nanoparticles (GNPs) are particularly promising since they are non-toxic (16), biocompatible, biodegradable (17), and relatively simple to produce using methods such as nanoprecipitation, desolvation, and coacervation (18). For the dissolution of GNPs which

leads to the continuous and long-term release of the drug, they must be cross-linked using glutaraldehyde, aldehyde groups or transglutaminase (19). In recent years, several researchers have explored the delivery of antibiotics using GNPs, antibacterial drugs such as vancomycin (20), and erythromycin to enhance bacterial therapy (21). Ciprofloxacin (CIP) is a fluoroquinolone antibiotic that has been employed to treat bacterial illnesses like pneumonia and urinary tract infections (22). Furthermore, CIP is an effective therapy for patients with multiple infections or predisposition variables for gram-negative infections (23). However, changes in specific CIP domains have been linked to quinolone resistance in clinical strains of bacteria. As a result, novel strategies and mechanisms are necessary to circumvent these constraints and improve the efficacy of CIP (24). Additionally, infected sites create a unique microenvironment with an acidic pH caused by the underlying disease development. This can operate as a physiological trigger, causing medications to be released from nanosystems carrying pH-sensitive moieties. This method has been extensively investigated and has demonstrated potential in targeting the administration of several treatments against bacterial infections (25). Therefore, in comparison to traditional methods of administration, the aforementioned advantages make GNPs an excellent nanosystem for enhanced antibiotic delivery of CIP. Although GNPs have been used for CIP delivery (26), this is the first study to report pH-responsive GNPs for CIP delivery to target bacterial infections.

The drawbacks of GNPs, such as early drug release and insufficient infection site targeting persist irrespective of their benefits (27). pH-responsive nanosystems have thus been suggested by research as a potential solution to further maximize drug targeting and efficiency (28). pHresponsive nanoparticles undergo physicochemical alterations to their surface properties and material structure in response to pH-stimuli. These may involve surface charge switching, dissociation, or swelling in a way that prioritizes drug release at the targeted area (29). However, this approach is still in its initial stages, and only a few systems have been documented, primarily in cancer (30). Hence, more research studies remain necessary to further investigate the concept while exploring the possible application of pH-responsive nanosystems against other disorders including bacterial infections.

Herein, this study reports the formulation of pH-responsive GNPs loaded with CIP to target and improve the treatment of bacterial infections. The study aims to maximize therapeutic results by reducing the optimal dose and side effects of bare CIP. This nanosystem will be formulated using the two-step desolvation method which results in the production of smaller, and evenly

distributed nanoparticles, and glutaraldehyde will be added to cross-link the nanoparticles due to its efficiency in stabilizing biomaterials. To our knowledge, this is the first study to report pH-responsive CIP-GNPs for the treatment of bacterial infections.

2. Materials and methods

2.1 Materials

Acetone, Oleic acid, & Ciprofloxacin [1- cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1piperazinyl)-3-quinoline carboxylic acid)] hydrochloride (CIP) were purchased from SigmaAldrich (United States of America). Glutaraldehyde solution was purchased from SigmaAldrich (Germany). Gelatin powder was obtained from Associated Chemical Enterprises. Milli Q water was acquired from the water purification system Elix® from Millipore Corp (USA). Mueller Hinton Broth (MHB) and Mueller Hinton Agar (MHA) were acquired from BioLab Inc (South Africa). The sheep blood was obtained from United Scientific SA cc (South Africa). The bacterial strains used Methicillin-resistant *Staphylococcus aureus* (ATCC 700699), *Escherichia coli* (27853), *Pseudomonas aeruginosa* (ATCC 35218), and *Staphylococcus aureus* (ATCC 25923) were obtained from DLD Scientific (South Africa). Analytical-grade reagents and solvents were used for all techniques in this study.

2.2 Preparation of CIP-GNPs

The CIP-GNPs were prepared using the two-step desolvation method (31). Briefly, 200 mg of gelatin was dissolved in 10 mL of Milli-Q water with the application of heat (70 ºC). Thereafter, 10 mL of acetone was added to precipitate the high molecular gelatin weight fraction followed by the removal of the low molecular weight fraction by decantation. The high molecular weight gelatin fraction was re-dissolved in 10 mL of Milli Q water and 5 mg of CIP was added. For the second desolvation, 5 mL of acetone that had been mixed with 185 µL of oleic acid.100 µL of glutaraldehyde was added to cross-link the nanoparticles and to stabilize the formulation. Thereafter, the product was incubated for 30 minutes, and acetone was removed in a vacuum using a Rotary evaporator for 2 hours at 100 rpm and 67 ºC. The product was sonicated for 12 minutes at a sonication power of 30 amps.

2.3 Characterization of GNPs

2.3.1 Determination of particle size, polydispersity index (PDI), zeta potential (ZP) and morphology

The average size, PDI, and ZP of the CIP-GNPs were measured by the dynamic light scattering method using the Zetasizer Nano ZS90 (Malvern Instruments, UK). Before the measurements, CIP-GNPs were diluted with phosphate-buffered saline (PBS) (pH 7.4, 6, and 4.5). This was done in triplicate at 25 ºC. The surface morphology of CIP-GNPs was studied using a Transmission Electron Microscope (TEM, JEOL 2100). For the TEM imaging, the samples were placed in a TEM grid, stained using 1% uranyl acetate, and viewed after drying. The TEM was operated at 100 kV voltage.

2.3.2 Entrapment efficiency (EE%) study

The ultrafiltration method was used for determining the EE% (32). CIP-GNPs were centrifuged for 30 min at 3000 rpm in Amicon ®centrifuge tubes. CIP was quantified utilizing a High-Pressure Liquid Chromatography (HPLC), (a Shimadzu, Japan) model that used a UV detector wavelength of 277 nm. At a flow rate of 1 mL/min, a mobile phase of acetic acid (2.5 % v/v) in water and acetonitrile: methanol (1:1) was pumped down in a Nucleosil 100-5 C18 (250 mm × 4.6 mm). The calibration equation was y = 62803x + 61606 and R^2 = 0.9972. The EE% of CIP-GNPs was determined using the formula: (33)

$$EE\% = \frac{Weight\ of\ CIP\ in\ nanoparticles}{Weight\ of\ CIP\ added} \times 100$$

2.3 *In vitro* biosafety of CIP-GNPs (Haemocompatibility).

The haemolytic activity of CIP-GNPs was determined via a method previously reported in the literature (34). PBS (pH 7.4) was used to wash the fresh sheep blood to extract the red blood cells three times, followed by centrifugation for 10 minutes at 3000 rpm. Different concentrations of CIP-GNPs ranging between 0.05 to 0.2 mg/mL were obtained through serial dilutions using PBS (pH 7.4) and incubated for 30 minutes at 37 ºC. Thereafter, to remove any remaining blood cells, the samples were centrifuged at 10 000 rpm for 10 minutes at 4ºC. 200 µL of the supernatant was then carefully collected and transferred into 96-well plates. This was followed by the analysis of haemoglobin released by the lysed cells at 540 nm using a microplate spectrophotometer (Spectrostar Nano, Germany). The controls namely, negative,

and positive were prepared by the addition of 0.2 mL of red blood cell suspension to 1.8 mL PBS (7.4) and distilled water, respectively. Haemolysis percentage was calculated using the following equation (30):

$$\text{Haemolysis\%} = \frac{ABS - ABS_0}{ABS_{100} - ABS_0} \times 100$$

2.4 *In vitro* drug release study

A dialysis release method was used to conduct *in vitro* drug release tests of the encapsulated CIP from the developed GNPs at pH 7.4 and 6.0 (35). Briefly, a dialysis bag (pore size cut off: 14 000 Da) comprising 2 mL of bare CIP, and CIP-GNPs was immersed in 40 mL of the release mediums (PBS pH 7.4 and 6.0) and stored at 37 ºC in a shaking incubator at 100 rpm. At predefined periods 0.5, 1,2,3,4,5,8,10,12,24, 48, and 72 hours, 2 mL of PBS was taken and replaced by 2 mL of PBS to keep the sink conditions constant. The cumulative quantity of CIP released was determined using the HPLC method following the protocol stated in 2.3.

2.5 *In vitro* antibacterial activity

2.5.1 Determination of Minimum Inhibitory Concentrations

Blank-GNP, bare CIP and CIP-GNP's antibacterial activity were tested against *E. coli*, *S. aureus*, *P. aeruginosa* and MRS*A* using the micro broth dilution method (36). Bacterial cultures were grown in nutrient broth and maintained at 37 ºC in a shaking incubator with a speed of 100 rpm. After 16 hours, the bacterial concentration was adjusted to 0.5 McFarland's standard. Bacterial suspensions were diluted with Muller-Hinton broth (MHB) (pH 7.4 and 6.0) to 5×10^5 colony-forming units (CFU)/ mL. CIP, blank-GNPs, and CIP-GNPs were diluted in MHB from 50 to 0.025 µg/mL in 96-well plates. Bacterial suspensions were added to the plates and put in a shaker incubator at 37 ºC incubator at 100 rpm. For determining the minimum inhibitory concentration (MIC), the samples were spotted in triplicate on Muller-Hinton agar plates (MHA) and incubated at 37 ºC for 24 hours, following 24, 48 and 72 hours of incubation.

2.5.2 Determination of Fractional Inhibitory Concentration

To gain additional insight into the combined antibacterial activity of CIP and blank GNPs on CIP-GNPs, the Fractional Inhibitory Concentration (FIC) index was calculated. Using the

following formulas, the ΣFIC was computed and examined based on the MIC values determined from the *in vitro* antibacterial analysis.

$$\text{FIC (CIP)} = \frac{(the\ MIC\ of\ CIP\ in\ concentration\ with\ blank\ GNPs)}{(the\ MIC\ of\ CIP} \quad (37)$$

$$\text{FIC (blank-GNPs)} = \frac{(the\ MIC\ of\ blank{-}GNPs\ in\ combination\ with\ bare\ CIP)}{(the\ MIC\ of\ blank{-}GNPs)} \quad (38)$$

$$\Sigma\text{FIC} = \text{FIC (CIP)} + \text{(blank-GNPs)}$$

Table 1: Interpretation of ΣFIC Index values

FIC Index	Interpretation
ΣFIC ≤ 0.5	Synergistic action
0.5 <ΣFIC ≤ 1	Additive action
1 <ΣFIC ≤2	Indifference
ΣFIC ≥2	Antagonistic action

2.5.3 Bacterial killing kinetics

A colony count method was used to analyse the bacterial killing kinetics of CIP-GNPs and bare CIP against MRSA and *P. aeruginosa* (39)**.** To prepare MRSA cultures and *P. aeruginosa,* an overnight culture was diluted with nutrient broth and incubated for 16 hours. Then, by diluting bacterial cultures with PBS (pH 7.4 and 6.0), a 5×10^5 CFU/mL was obtained. Five times the MIC of the bacterial suspension of bare-CIP and CIP-GNPs were added to the bacterial suspensions, and the mixtures were placed on a shaking incubator at 37 ºC with 100 rpm. Following this, 100 µL of every sample was collected, swabbed, and plated in triplicate on MHA plates at predefined intervals of 0, 1, 2, 4, 6 ,12 and 24 hours. Colonies were counted following a 24-hour incubation period at 37 ºC.

2.5.4 Biofilm eradication

The eradication of biofilm development of CIP-GNPs against MRSA and *P. aeruginosa* was assessed using a microtiter plate test (40). Before growing in MHB (pH 7.4 and pH 6.0), the bacterial cultures were modified to 0.5 McFarland's standard. 200 µL of the culture was added to 96-well plates. The plates were kept at 37 ºC for 14 days of incubation to ensure the growth of mature biofilm. To get rid of non-adherent bacteria, plates were rinsed with PBS pH 7.4 after incubation. Following the rinsing step, biofilms were treated with 100 µL of CIP-GNPs and

bare CIP are five times MIC and incubated at 37 ºC for 24 hours. PBS (pH 7.4) was then used to eliminate treatments and non-adherent bacteria and the plates were dried for 15 minutes at 25 ºC. Following the staining with 0.1 % (w/v) crystal violet solution, the wells were rinsed with PBS (pH 7.4). Thereafter, 30 % acetic acid was used to solubilize the stain. At 550 nm, the absorbance was measured with a Spectrostar Nano Plate reader (SpectraMax M2, USA). The following formula was used to calculate the percentage of biofilm eradication:

$$\text{Biofilm eradication \%} = \frac{100 - ABS\ 500nm\ treated\ cells}{ABS\ 550nm\ untreated\ cells} \times 100 \quad (41)$$

2.6 Statistical analysis

The findings in this study have been analysed using the software Microsoft Excel and GraphPad and expressed as the mean ± standard deviation (SD), with all experiments conducted in triplicate. Particle size, PDI, ZP, EE %, and *in vitro* drug release were statistically analysed using one-way analysis of variance (ANOVA), with differences deemed significant at a P value of < 0.05.

3. Results and Discussion

3.1 Preparation, characterization, and optimization of CIP-GNPs

3.1.1 Determination of particle size, PDI, ZP and entrapment efficiency

Initially, studies were conducted to determine the optimal formulation characteristics to prepare unloaded GNPs. Gelatin, glutaraldehyde, and oleic acid were combined in varying ratios to produce CIP-GNPs with suitable PS, PDI, ZP, and drug content. As shown in **Table 2**, the 2:1:2 gelatin: glutaraldehyde: oleic acid ratio displayed a higher particle size and PDI of 315.1 ± 3.012 nm and 0.361 ± 0.072, respectively. Whilst the 2:1:0.5 ratio displayed lower particle size and PDI of 212.3 ± 1.739 nm and 0.259 ± 0.023, respectively. This could be attributable to that the size of the nanoparticles is proportional to the concentration of oleic acid in the mixture, with higher concentrations leading to larger particles (42). Therefore, a 2:1:0.5 ratio was used for subsequent optimization due to its favorable particle size and PDI.

Table 2: The effect of Oleic acid ratio on CIP-GNPs. Results in the table are presented as mean ± SD (n =3)

Gelatin: Glutaraldehyde: Oleic acid	Particle size (nm)	PDI	ZP (mV)
2:1:2	315.1 ± 3.012	0.361 ± 0.072	1.98 ± 0.152
2:1:1	239 ± 2.987	0.208 ± 0.017	2.24 ± 0.269
2:1:0.5	212.3 ± 1.739	0.259 ± 0.023	4.58± 0.148

Thereafter, the effect of sonication time was determined by adjusting the duration of sonication from 5 to 12 min, while maintaining constant power. **Table 3** demonstrates that the particle size and EE% of CIP-GNPs were steadily reduced with increasing sonication time, going from 315.2 ± 1.739 nm and 29.2 ± 2.67% to 212.3 ± 1.739 and 38.1 ± 3.85% after 5 and 12 min, respectively. The CIP-GNPs displayed a low EE% of less than 40% in all the sonication time intervals, this could be because of the water-solubility properties of CIP (43). Meanwhile, the PDI decreased from 0.369 ± 0.043 to 0.259 ± 0.023 after 5 and 12 min, respectively. According to Asadi *et al*., longer sonication time generates more single nanoparticles than clusters because the process of sonication disintegrates large nanoparticle clusters into smaller ones or even shreds them up into individual particles (44). Greater sonication activity occurred with prolonged sonication times, which caused CIP-GNPs to disperse and, as a result, reduced particle size and PDI. These results are consistent with a previous report on Mannan-coated GNPs by Kaur *et al* (45). Moreover, these results led to the selection of a 12 min sonication time for the subsequent preparation, intending to achieve a substantially small particle size and restrained dispersion of the nanoparticles as well as a high EE%. Therefore the 12 min sonication time was selected as a final formulation for further characterization, with particle size, PDI, ZP, and EE% of 212.3 ± 1.739, 0.259 ± 0.023, + 4.58± 0.148 mV and 38.1 ± 3.85% respectively.

Table 3: The effect of sonication time on particle size, PDI, and ZP. Results in the table are presented as mean ± SD (n=3)

Sonication time (min)	Particle size (nm)	PDI	ZP (mV)	EE%
5	315.2 ± 1.739	0.369 ± 0.043	2.14 ± 0.325	29.2 ± 2.67
10	241.7 ± 3.108	0.260 ± 0.022	2.66 ± 0.287	31.6 ± 3.01
12	212.3 ± 1.739	0.259 ± 0.023	4.58± 0.148	38.1 ± 3.85

3.1.2 Morphology of CIP-GNPs

TEM imaging was used to evaluate the morphology and verify particle size of the CIP-GNPs, as illustrated in **Figure 1**. It was observed that the GNPs were homogenous and spherical (46). The morphological studies found the particle size to be 200nm (SD) which correlates with 212.3 nm (SD) from the Dynamic Scattering Light method.

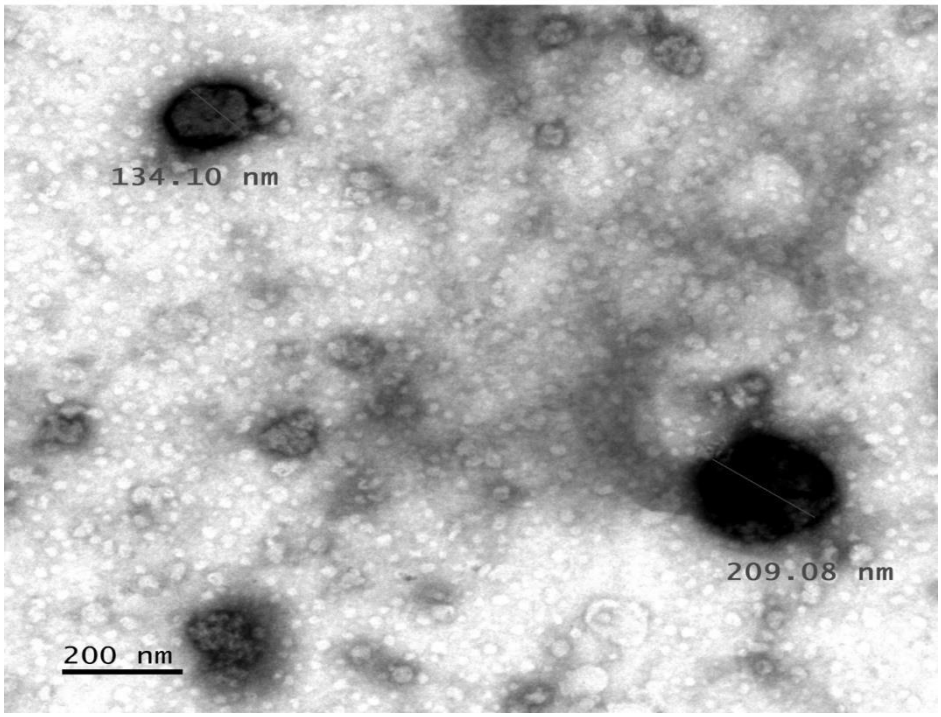


Figure 1: TEM image of CIP-GNPs (200 nm)

3.2 *In vitro* Hemocompatibility studies

Preclinical testing to determine CIP-GNPs' biocompatibility with blood components is essential, because of their distinct physical and chemical properties and small size, which may interfere with red blood cells more than conventional drugs (34). In this work, CIP-GNPs demonstrated non-hemolytic activity in red blood cells at concentrations varying from 0.025 mg/mL to 0.1 mg/mL, as shown in **Figure 2.** These findings suggest that CIP-GNPs could be appropriate for intravenous delivery.

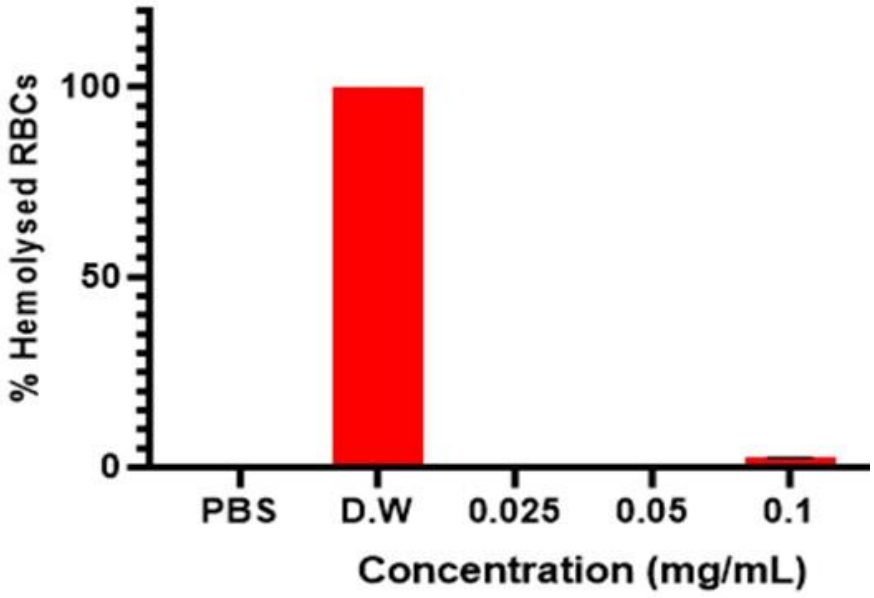


Figure 2: Hemolytic activity of CIP-GNPs, distilled water (negative control), and PBS (positive control) at different concentrations against red blood cells. Data in the graphs are presented as mean ± SD (n = 3).

3.3 *In vitro* pH-responsiveness

3.3.1 Particle size, PDI, Zeta potential

The optimized formulation was utilized to confirm the pH responsiveness of the system, the impact on the particle size, PDI and ZP of the CIP-GNPs was investigated by using DLS by incubation of the formulation at various pH conditions (pH 7.0, 6.0, and 4.5). The swelling behavior and size dispersion range of gelatin depends solely on whether it is functioning as an

acid or base at specific pH values (44). As shown in **Table 4**, pH responsiveness was confirmed by the increase in particle size at different pH conditions. The particle size at pH 7.4, 6.0, and 4.5 was 204.1 ± 0.100, 206.3 ± 0.360, and 226.4 ± 0.451 nm respectively, exhibiting stability at physiological pH 7.4 with a PDI of 0.361 ± 0.016. There was an increase in particle size from 204.1 ± 0.100 to 206.3 ± 0.360 with changes in pH from 7.4 to 6.0, and this can be advantageous for fast drug release at target sites since pH 6.0 represents acidic conditions at bacterial-infected sites (45). The increase in size could result from the swelling of gelatin in response to pH changes. Changing pH values from physiological to acidic causes considerable swelling in gelatin nanoparticles due to enhanced ionization (46). Thus, this change in size can augment the fast release of CIP at the target site at a level lethal for the effective elimination of bacterial infections (47).

The side chains of gelatin include both carboxylic (-COOH) and amino groups ($-NH_2$), meaning it is poly-amphoteric (47). When the pH was adjusted to acidic, a surface charge switch occurred, causing the zeta potential to shift from -3.59 ± 0.428 to 1.06 ± 0.271. This is due to the protonation of -COO- and NH_2 in gelatin chains into -COOH and NH_3^+, respectively. This charge shifting could also be attributable to counter chloride ions from the HCL solution used to modify pH (48). Thus, Coester *et al*, reported that gelatin chains are electrostatically stable at high surface charges due to stronger repulsive forces (49).

Table 4: The effect of pH on particle size, PDI, and Zeta potential. Results in the table are presented as mean ± SD (n=3)

pH	Particle size (nm)	PDI	Zeta potential (mV)
7.4	204.1 ± 0.100	0.361 ± 0.016	-3.59 ± 0.428
6.0	206.3 ± 0.360	0.168 ± 0.006	1.06 ± 0.271
4.5	226.4 ± 0.451	0.163 ± 0.010	2.67 0.362

3.3.2 *In vitro* drug release study

The dialysis bag diffusion method was used for comparing the drug release profiles of CIP-GNPs to those of bare CIP for 72 hours at 37 ºC, pH 7.4 and 6.0, as shown in **Figure 4**. Bare CIP was completely released within 8 hours at both pH 7.4 and 6.0. It was observed that the CIP released from GNPs was pH and time-dependent, with a quicker release at pH 6.0 than at

pH 7.4 for up to 72 hours. The faster release can be attributed to the protonation of gelatin's amine functional groups in an acidic medium. This produces electrostatic attraction among positively charged ions, thereby relaxing the gelatin layer (50). After 72 hours, 53.46 % and 45.90 % of CIP was released at pH 6.0 and pH 7.4, respectively. The smooth surface and compacted structure of gelatin may have contributed to the comparatively low release percentage (51). This demonstrated that pH-triggered release can preserve and prevent drug degradation at physiological pH, while also enhancing targeted release and drug localization and accessibility at the acidic site of infection, potentially augmenting antibacterial efficacy.

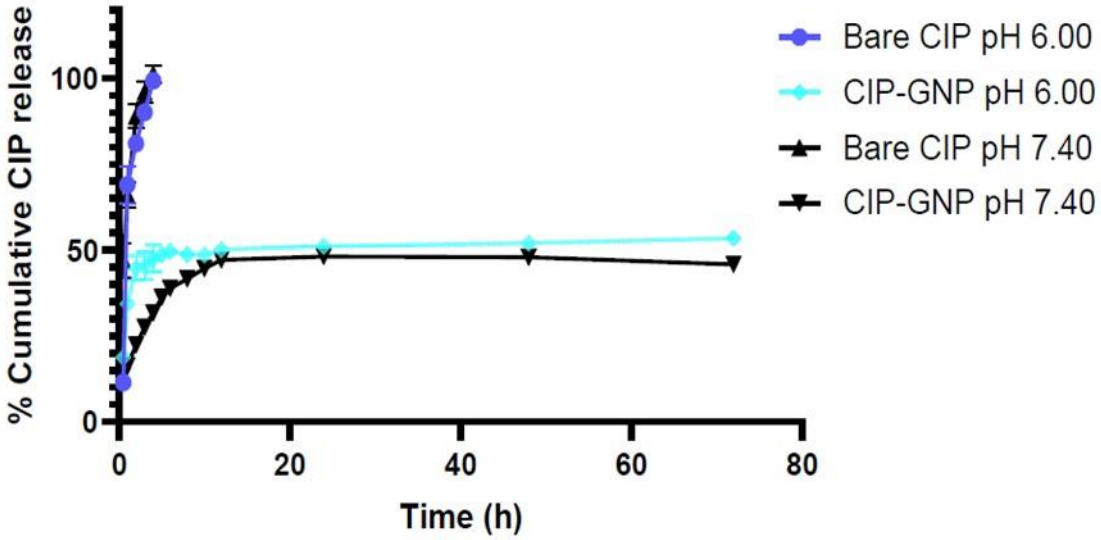


Figure 3: *In vitro* drug release of bare CIP and CIP-GNPs at pH 7.4 and 6.0 for 72 hours. Data in the graph are presented as mean ± SD (n = 3).

The pH 7.4 and 6.0 showed statistically significant differences among the different pH dissolution media with 2.28 and 3.13 mean dissolution time, and 44.35 and 71.56% dissolution efficiency, respectively. The fit factors can be expressed by two approaches as shown in Table 5, *f1* (the difference factor) and *f2* (the similarity factor) (52). *f1* was (>15), 28.37 which attributes that the two profiles are significantly different. Whereas *f2* was less than (<)50, 46.1 denoting that the similarity between the two profiles is low.

Table 5: The dissolution profiles for Mean Dissolution Time (MDT), Dissolution Efficiency (DE), Difference factor (*f1*), and Similarity factor (*f2*).

Dissolution media	MDT	% DE	*f1*	*f2*
pH 7.4	2.28	44.35	28.37	46.1
pH 6.0	3.13	71.56		

f1 = Difference factor ($0 < f1 < 15$), *f2* = Similarity factor ($50 < f2 < 100$)

3.4 *In vitro* antibacterial studies

3.4.1 Minimum Inhibitory Concentration

The antibacterial activity of bare CIP and CIP-GNPs tested against gram-positive bacteria MRSA and *S. aureus* and gram-positive bacteria *P. aeruginosa* and *E. coli* was determined using the broth dilution method at pH 7.4 and pH 6.0. As displayed in Table 6, the MIC values of CIP-GNPs against *S. aureus* and MRSA at pH 7.4 were 1.56 µg/ml and 12.5 µg/ml respectively. Whereas the MIC values for bare CIP against *S. aureus* and MRSA were 1.56 µg/ml and 50 µg/ml, respectively at both pH conditions. The antibacterial activity of CIP-GNPs showed a four-fold reduction of MRSA compared to the bare CIP, whereas CIP-GNPs and CIP demonstrated comparable antibacterial activity. Moreover, the MIC values of CIP-GNPs against *S. aureus* and MRSA at pH 6.0 were 0.78 µg/ml and 6.25 µg/ml, respectively, which indicates an eight-fold reduction when compared to bare CIP. The enhanced extended antibacterial activity of CIP-GNPs, in comparison to bare CIP, could be attributable to its subcellular size with a large surface area and the presence of oleic acid which is known for its antibacterial properties (53). The antibacterial activity of CIP-GNPs extended for up to 72 hours at both pH 7.4 and 6.0. This could be related to the encapsulation of CIP into pH responsive GNPs, granting defence against any type of deterioration while minimizing the depletion of CIP prior to reaching the site of infection via targeted delivery, thus prolonging its half-life, and preserving its efficacy against bacterial infections (54).

The antibacterial activity of CIP-GNPs against *P. aeruginosa* as displayed in Table 7 showed a two, and four-fold reduction of bacteria at pH 7.4 and 6.0, respectively compared to bare CIP. After 24 hours, the MIC values for CIP-GNPs against *E. coli* increased at both pH conditions, this antibacterial effect may have been due to the rapid release of CIP from the GNPs prior to

the sustained drug release. CIP-GNP's sustained release profile is appropriate for intravenous delivery, as it has been shown to avert possible co-infections. The CIP-GNPs' antibacterial action could be attributed to structural differences in targeted bacteria's cell walls (gram-positive or gram negative). In gram-positive bacteria, the thick peptidoglycan in the cell wall forms a barrier to breaking molecules and reaching the cell membranes (55).

The MIC values for bare CIP against *P. aeruginosa* and *E. coli* were 1.56 µg/ml after 24 hours at both pH 7.4 and 6.0. There was total killing for bare CIP against *E. coli* at both pH conditions after 48 hours because CIP exerts powerful antibacterial activity against gram-positive bacteria by attaching to bacterial enzymes such DNA gyrase and topoisomerase IV (56). This interaction causes the development of a quinolone-enzyme-DNA complex. Following binding, the enzyme's shape changes. The enzyme degrades DNA, and the drug blocks the binding of the fragmented DNA strands, hence stopping DNA replication. Eventually, this causes impairment of DNA in bacteria which results in cell death (57).

Table 6: The *in vitro* antibacterial activity of bare CIP, blank GNP, and CIP-GNP showing MIC (µg/mL) result against gram-positive bacteria (*S. aureus* and MRSA) at pH 7.4 and 6. Data in the table are presented as mean ± SD (n=3).

pH 7.4	***S. aureus* (MIC µg/mL)**			**MRSA (MIC µg/mL)**		
Time (hrs.)	**24**	**48**	**72**	**24**	**48**	**72**
CIP	1.56	1.56	1.56	50	50	50
Blank GNP	>500	>500	>500	>500	>500	>500
CIP-GNP	1.56	1.56	3.125	12.5	25	50
pH 6.0						
Time (hrs.)	**24**	**48**	**72**	**24**	**48**	**72**
CIP	1.56	1.56	1.56	50	50	50
Blank GNP	>500	>500	>500	>500	>500	>500
CIP-GNP	0.78	0.39	0.39	6.25	12.5	12.5

Table 7: The *in vitro* antibacterial activity of bare CIP, Blank GNP, and CIP-GNP showing MIC (µg/mL) result against gram-negative bacteria (*P. aeruginosa* and *E. coli*) at pH 7.4 and 6. Data in the table are presented as mean ± SD (n=3).

pH 7.4	***P. aeruginosa* (MIC µg/mL)**			***E. coli* (MIC µg/mL)**		
Time (hrs.)	**24**	**48**	**72**	**24**	**48**	**72**
CIP	1.56	3.125	6.25	0.098	TK	TK
Blank GNPs	>500	NA	NA	>500	>500	>500
CIP-GNPs	0.78	0.78	0.78	0.049	0.098	0.195
pH 6.0						
Time (hrs.)	**24**	**48**	**72**	**24**	**48**	**72**
CIP	1.56	0.78	0.78	0.195	TK	TK
Blank GNPs	>500	NA	NA	>500	NA	NA
CIP-GNPs	0.39	0.78	0.78	0.095	0.39	0.78

TK= Total killing, NA= No activity (n=3)

3.4.2 Determination of Fractional Inhibitory Concentration

ΣFIC index interpretations at each time interval which were used to comprehend the combination of the antibacterial impact of Blank-GNPs and CIP in the CIP-GNPs against MRSA, *S. aureus*, *P. aeruginosa,* and *E. coli*. The results are interpreted as indicated in **Tables 8 and 9.**

Table 8: ΣFIC of CIP-GNPs against gram-positive bacteria (*S. aureus* and MRSA) at pH 7.4 and 6.0.

pH 7.4	***S. aureus***		**MRSA**	
Time (hrs.)	**ΣFIC**	**Interpretation**	**ΣFIC**	**Interpretation**
24	1.003	Indifference	0.275	Synergism
48	1.003	Indifference	0.550	Additive
72	2.006	Antagonistic	0.100	Indifference
pH 6.0				
Time (hrs.)	**ΣFIC**	**Interpretation**	**ΣFIC**	**Interpretation**
24	0.502	Additive	0.138	Synergism
48	0.251	Synergism	0.275	Synergism
72	0.259	Synergism	0.275	Synergism

Table 9: ΣFIC of CIP-GNPs against gram-negative bacteria (*P. aeruginosa* and *E. coli*) at pH 7.4 and 6.0.

pH 7.4	***P. aeruginosa***		***E. coli***	
Time (hrs.)	**ΣFIC**	**Interpretation**	**ΣFIC**	**Interpretation**
24	0.502	Additive	0.500	Synergism
48	0.250	Synergism	-	-
72	0.125	Synergism	-	-

pH 6.0

Time (hrs.)	ΣFIC	Interpretation	ΣFIC	Interpretation
24	0.250	Synergism	0.487	Synergism
48	1.000	Additive	-	-
72	1.000	Additive	-	-

3.4.3 Bacterial killing kinetics

To investigate antibacterial activity further, the rate at which CIP-GNPs were able to eradicate *P. aeruginosa* and MRSA was examined. **Figure 4A** shows the findings of the time-killing study comparing CIP-GNPs and bare CIP against MRSA at a concentration five times greater than the MIC. Following 8 hours of incubation, bare CIP eliminated more than log 4 CFU/mL of MRSA, whereas CIP-GNPs eliminated 100 % of bacteria (approximately 5 log reduction). Following the incubation for 24 hours, bare CIP demonstrated approximately 5 log reduction. Therefore, CIP-GNPs could be administered to eradicate MRSA infections more effectively and quicker than conventional CIP. This substantial boost in activity may be attributed to the synergy provided by CIP-GNPs, which elevated CIP's activity against insensitive bacteria.

Moreover, **Figure 4B** illustrates the rate at which bare CIP and CIP-GNPs inhibit *P. aeruginosa* bacterial growth. 100% of *P. aeruginosa* colonies were eradicated by CIP-GNPs after 6 hours of incubation, roughly 6-log reduction. However, following 24 hours incubation, bare CIP eliminated 100% of bacteria (around a 6-log reduction). Consequently, *P. aeruginosa*- related infections could be efficiently treated with CIP-GNPs.

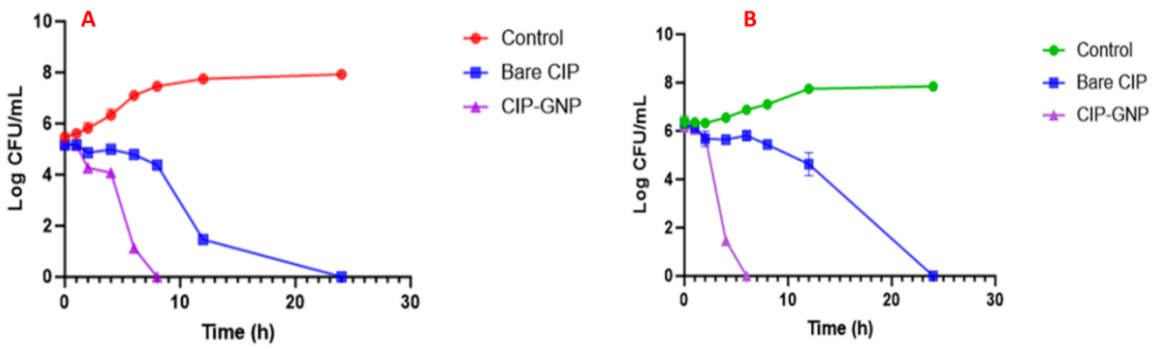


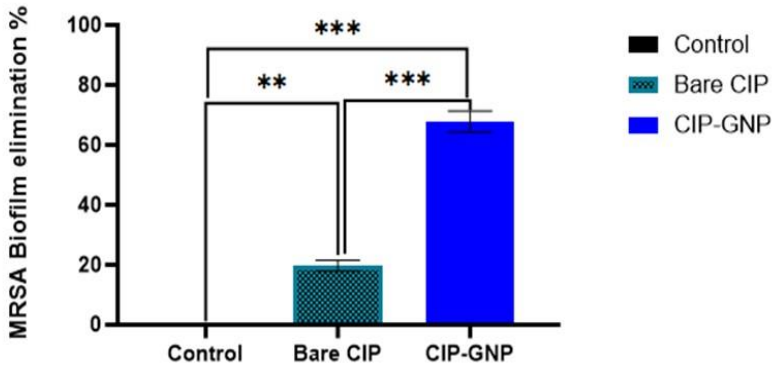
Figure 4: Killing kinetics of MRSA (A) and *P. aeruginosa* (B) subjected to bare CIP and CIP-GNPs at 5X MIC and PBS pH 7.4 (control). Data is presented as mean ± SD (n = 3) in the graphs.

3.4.4 Biofilm eradication

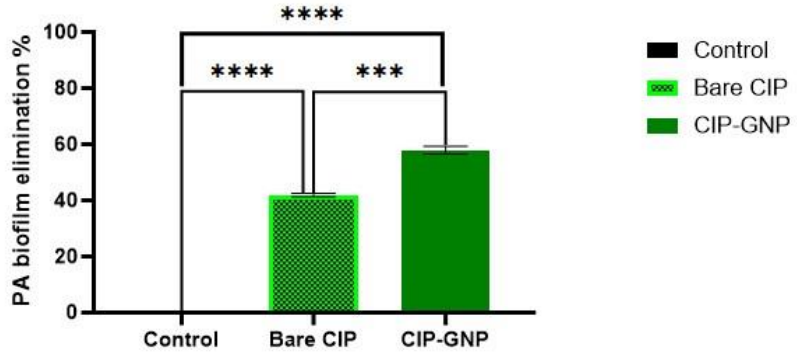
As biofilms shield bacteria from host defences and antibiotic therapy, the propensity of bacteria to develop biofilms is a significant factor in the development of antibiotic resistance (58). Larger and more regular dosages might be required to effectively eradicate biofilm formation using traditional dosage forms of antibiotics, which would raise the prospect of dose-dependent harm (59). The efficiency of CIP-GNPs in reducing biofilm formation was evaluated against MRSA and *P. aeruginosa* using crystal violet concentrations ten times greater than their corresponding MICs. The extracellular polysaccharides of biofilms are effectively penetrated by antibiotic drug carriers which are nanosized. **Figure 5A** demonstrates that CIP-GNPs eliminated 73% of MRSA biofilms, which is about 3.75 greater than bare CIP's antibiofilm activity (P-value <0.00142).

CIP-GNPs effectively removed 57% of *P. aeruginosa* biofilms, a 1.4-fold enhancement over bare CIP (P-value <0.0196) as shown in **Figure 5B**. However, their antibiofilm effectiveness against MRSA was more significant. These findings show that conventional CIP has greater activity in the eradication of *P. aeruginosa* biofilm. CIP-GNPs' improved antibiofilm activity could be attributed to gelatin swelling caused by increased ionization, resulting in elevated release of CIP from the nanosytem at concentrations suitable for biofilm removal. The nanosytem was able to attach itself to biofilm bacteria and the extracellular polymeric substances (EPS) in the biofilm milieu due to the positively charged surface of the CIP-GNPs (60). This allowed for improved penetration, retention, and potential disruption of the EPS. Additionally, the acidic biofilm microenvironment caused gelatin swelling, which increased the amount of accessibility that gelatinases had to the CIP-GNP core (61). Effective eradication

was achieved through the gelatinase-triggered degradation of the GNP, which increased the release of CIP.



A



B

Figure 5: MRSA and *P. aeruginosa* percentage of biofilms after treatment with bare CIP, CIP-GNPs, and PBS pH 7.4 (control). CIP-GNPs demonstrated considerable enhancement in the inhibition against both strains (P- values * >0.05, ** >0.01, and *** >0.0001). Data in the graphs are presented as mean ± SD (n = 3).

4. Conclusion

To maximize the therapeutic effect of currently available antimicrobial agents, the issues related to conventional dosage forms that contribute to the emergence of bacterial resistance necessitate the use of alternative delivery methods and biocompatible materials. To address these issues, pH-responsive CIP-GNPs were successfully developed using a two-step desolvation method for the targeted delivery of CIP against MRSA infections. The particle size, PDI, and surface charge of CIP-GNPs changed in response to a pH adjustment from 7.4 and 6.0, demonstrating its pH responsiveness. *In vitro* hemocompatibility testing confirmed the biosafety of CIP-GNPs. The nanosystem also demonstrated a prolonged and sustained CIP release profile, which corresponded to a longer *in vitro* antibacterial activity when compared to bare CIP. Furthermore, the nanosystem demonstrated a considerable reduction in bacterial cell (MRSA) count after treatment when contrasted with bare CIP. The crystal violet assay confirmed CIP-GNPs' antibacterial ability by demonstrating a substantially greater percentage of biofilm elimination as opposed to bare CIP. The results obtained indicate that novel CIP-GNPs may be successful in drug delivery to target regions of bacterial infection with low pH while also preventing the development of antimicrobial resistance. Furthermore, the proposed nanosystem may be employed to deliver different antimicrobial agents, thereby investigating the antibacterial potential against several types of bacteria.

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CHAPTER 4: CONCLUSION

4.1 Introduction

4.2 Significance of the findings

4.3 Recommendations

4.1 Introduction

Infectious diseases caused by bacterial infections are among the most frequent causes of death and morbidity. In recent years, conventional antibiotic dosage forms have been used successfully to treat bacterial infections. Nevertheless, the widespread use, combined with the widely known limitations of these dosage forms such as inadequate and non-targeted release, has resulted in a developing antibiotic resistance challenge. To mitigate the danger of antibiotic resistance, conventional antibiotic dosage forms must be modified to produce sustainable therapy alternatives. Nanotechnology is widely recognized to increase the efficiency of antibiotics for treating bacterial infections. This, consequently, serves as a safeguard against the emergence of resistance. Nano-antibiotic systems can be enhanced to respond to stimuli, leading to increased antibiotic administration in acidic infection sites (e.g., pH-responsive systems). Gelatin nanoparticles (GNPs) are effective nanosystems for antibiotic delivery along with enhanced antibiotic treatment. The current study was aimed at examining the potential of ciprofloxacin-loaded gelatin nanoparticles (CIP-GNPs), which were prepared to improve antibacterial activity and overcome resistance mechanisms against MRSA and *P. aeruginosa*. The main outcomes derived from the study data are summarized below:

- Novel pH-responsive CIP-GNPs were successfully formulated and optimized. The CIP-GNPs displayed a particle size, polydispersity index (PDI), zeta potential (ZP), and entrapment efficiency of 212.3 ± 1.739 nm, 0.136 ± 0.01, + 6.75 ± 0.15 mV, and 38.1 ± 3.85%, respectively. The morphology of the optimized CIP-GNPs was found to be homogenous, and spherical.
- *In vitro* biosafety testing identified CIP-GNPs as non-haemolytic at low concentrations.
- The CIP-GNPs demonstrated pH responsiveness with an increase in particle size from 204.1 ± 0.100 to 226.4 ± 0.451 nm, a charge switch on the zeta potential from -3.59 ± 0.428 to 1.06 ± 0.271 mV and a faster ciprofloxacin release at pH 6.0, as compared to pH 7.4.
- The *in vitro* antibacterial activity of CIP-GNPs showed 2-fold lower minimum inhibitory concentration values compared to bare ciprofloxacin against Methicillinresistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). Moreover, the bacterial-killing kinetic test showed 100% elimination of MRSA and *P. aeruginosa* within eight and one hour(s) of treatment with CIP-GNPs, respectively. In

contrast, 100% elimination of MRSA and *P. aeruginosa* was observed within 24 and 12 hours of treatment with bare ciprofloxacin, respectively. CIP-GNPs eliminated 3,75-fold MRSA biofilm compared to bare ciprofloxacin, whereas 1.4-fold *Pseudomonas aeruginosa* biofilms were eliminated

4.2 Significance of the findings

The present study establishes the groundwork for further research into stimuli-responsive nanosystems for treating bacterial infections. The relevance of these observations is described below:

New pharmaceuticals products

CIP-GNPs have a likelihood to become commercially viable medications after clinical trials. Academic researchers and the pharmaceutical industry can collaborate to create more efficient and secure medications. This may lead to greater job possibilities and improvement of the local community.

Improved patient therapy and disease treatment

The *in vitro* antibacterial activity investigations of the newly developed pH-responsive CIP-GNPs revealed enhanced efficacy and prolonged CIP release in acidic environments. This nanosystem has the capacity to enhance bacterial infection treatment by preserving the antibiotic throughout the systemic circulation, delivering appropriate antibiotic concentrations to infection sites, reducing the consumption of antibiotics in healthy locations, and increasing bacterial antibiotic absorption. These advantages may result in reduced antibiotic dose frequency, adverse medication responses, and toxicity. This may contribute to increased adherence from patients, improved antibacterial therapy, along decreased resistance to antimicrobial drugs.

Stimulation of new research

The effective formulation of CIP-GNPs and the findings of the various research studies may generate innovative research ideas, such as:

- The development of this pH-responsive multimodal GNP, which contains loaded CIP to increase the antibacterial effects and efficacy of the antibiotic, provides a basis for

further study on associated concepts. The efficacious usage of pH-responsiveness in this nanosystem may spur research into the integration of pH-responsiveness in dual stimuli-responsive nanosystems, which might include dual pH- and enzyme-responsive nanosystems. This study may also motivate further investigation of this nanosystem, which is CIP-GNPs with additional active components and combinations, as well as the quest for additional excipients based on the intended target, hence, enhancing disease treatment and extending the lifespan.

Enhanced treatment of bacterial infections

The *in vitro* antibacterial effect studies of the novel developed pH-responsive CIP-GNPs exhibited greater efficacy and sustained CIP release under acidic conditions. This innovative nanosystem has the promise to improve therapy for bacterial infections by improving the active drug level within bacterial cells and strengthening the entirety of the therapeutic results of the medicine. This potential strategy would increase the accessibility of potent antibacterial drugs, enabling to treatment of bacterial illnesses, preserving lives, and improving people's quality of life.

4.3 Recommendations

This research displayed the beneficial effects of using nanocarriers to prevent bacteria's additional persistence approaches. Moreover, *in vitro* tests indicated that CIP-GNPs surpassed bare CIP in the treatment of bacterial infections. Nevertheless, more research remains essential to reinforce the novelty while enhancing the delivery of CIP via CIP-GNPs.

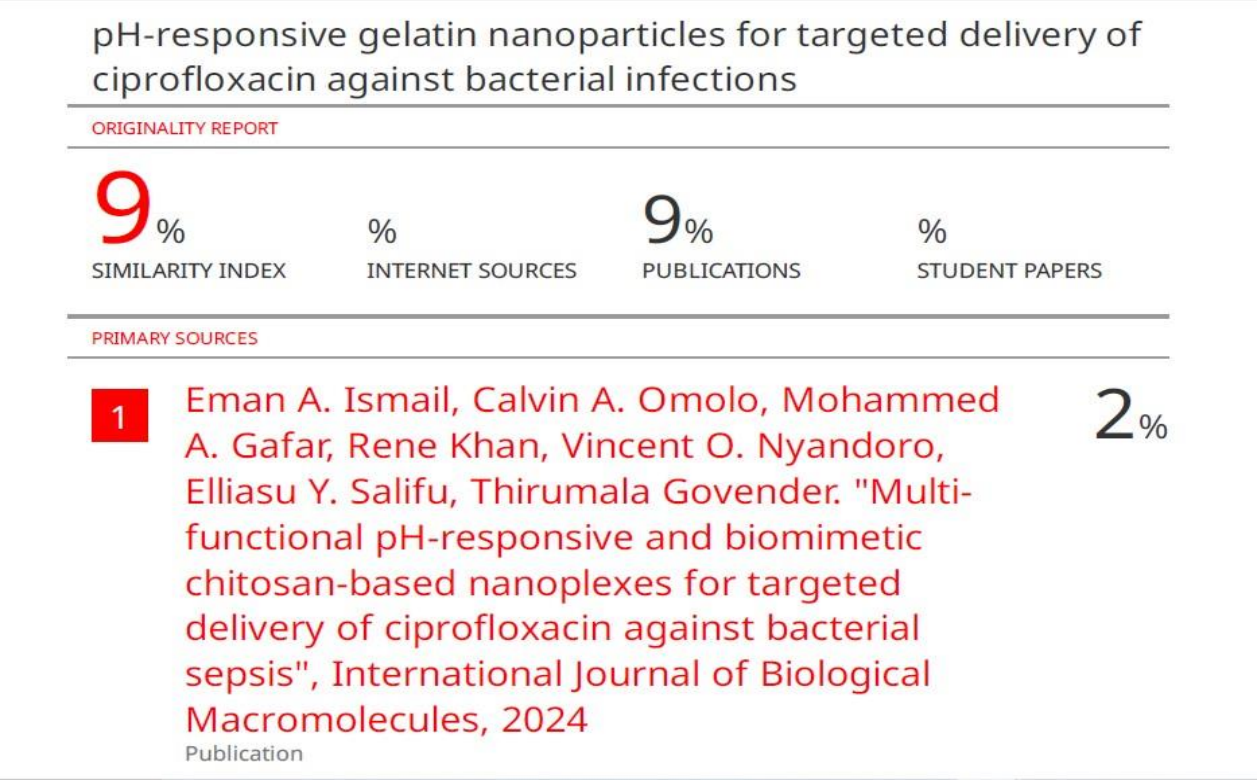
The subsequent studies are thus recommended:

- To demonstrate the biosafety of CIP-GNPs to normal body cells, cytotoxicity evaluations should be carried out.
- Long-term stability studies of CIP-GNPs can evaluate their physical as well as chemical stability.
- *In vivo* investigations are required to evaluate the antibacterial activity of CIP-GNPs and establish their suitability for clinical usage.
- To maximize therapeutic actions, the GNPs can be used to administer multiple antibiotics with diverse modes of action concurrently.

- In silico modelling and simulation studies are necessary for improved comprehension of the molecular interactions of CIP-GNPs contrary to MRSA and S. *aureus* bacteria.
- *In vivo* pharmacokinetic profiles may be employed to gain further knowledge about pHresponsive tailored drug administration, bioavailability, and biodistribution profiles.
- Additionally, the discovery of an approved procedure for scaling up the CIP-GNPs would simplify their incorporation into a producible product.

4.4 Conclusion

The outcomes of this research demonstrated the promise of pH-responsive CIP-GNPs to enhance antibacterial activity, combat antibiotic resistance, and ultimately lead to more effective bacterial infection therapy. This study assisted in enhancing nanocarrier methods of overcoming the limitations of traditional antibiotic dose forms and antibiotic resistance.



pH-responsive gelatin nanoparticles for targeted delivery of ciprofloxacin against bacterial infections

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

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2	Osama A. Madkhali. "Drug Delivery of Gelatin Nanoparticles as a Biodegradable Polymer for the Treatment of Infectious Diseases: Perspectives and Challenges", Polymers, 2023 Publication	2%
3	Sifiso S. Makhathini, Calvin A. Omolo, Ramesh Gannimani, Chunderika Mocktar, Thirumala Govender. "pH-Responsive Micelles from an Oleic Acid Tail and Propionic Acid Heads Dendritic Amphiphile for the Delivery of Antibiotics", Journal of Pharmaceutical Sciences, 2020	1%
4	Yongxu Mu, Lidong Gong, Tianjiao Peng, Jun Yao, Zhiqiang Lin. "Advances in pH-responsive drug delivery systems", OpenNano, 2021 Publication	<1%
5	Yajna Jaglal, Nawras Osman, Calvin A. Omolo, Chunderika Mocktar, Nikita Devnarain, Thirumala Govender. "Formulation of pH-responsive lipid-polymer hybrid nanoparticles for co-delivery and enhancement of the antibacterial activity of vancomycin and 18β-glycyrrhetinic acid", Journal of Drug Delivery Science and Technology, 2021 Publication	<1%
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