NANOPARTICLES FOR BACTERIAL TRANSFORMATION

M.Tech. Thesis

By

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NANOPARTICLES FOR BACTERIAL TRANSFORMATION

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **NANOPARTICLES FOR BACTERIAL TRANSFORMATION** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF TECHNOLOGY** and submitted in the **Department of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore**, is an authentic record of my work carried out during the period from August 2023 to May 2025 under the supervision of Dr. Sharad Gupta, Professor, Department of Biosciences and Biomedical Engineering.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

23/5/25

(ADVAIT SOHANI)

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

Thesis Supervisor 23/05/2023

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ADVAIT SOHANI has successfully given his M.Tech. Oral Examination held on **May 23, 2025.**

Signature of Supervisor of M.Tech. thesis

Date: May 23, 2025

Convener, DPGC

Date: May 23, 2025

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Advait

DEDICATION

This thesis is dedicated to my family, friends, and my seniors, each of whom has played a meaningful role in shaping this journey with their support, guidance, and encouragement.

Abstract

Gene delivery is a cornerstone of molecular biology, genetic engineering, and regenerative medicine, enabling the controlled introduction of foreign genetic material into target cells to induce desired biological responses. Traditional gene delivery systems, including viral vectors and synthetic carriers, suffer from limitations such as immunogenicity, cytotoxicity, instability, and lack of targeted delivery, making them less ideal for widespread therapeutic applications. To address these challenges, nanoparticle-mediated gene delivery has emerged as a promising alternative due to its biocompatibility, stability, and ability to provide a protective environment for genetic material. This study focuses on the formation of nanoparticles as gene delivery carriers and their role in genetic transformation. The nanoparticles were synthesized using a green chemistry approach, ensuring an environmentally sustainable and efficient fabrication process. The properties of these nanoparticles facilitated the formation of stable, uniform structures capable of encapsulating and delivering genetic material with high efficiency. The physicochemical characterization of these nanoparticles confirmed their desirable properties, including controlled size distribution and high encapsulation efficiency, making them viable carriers for gene delivery. The interaction of these nanoparticles with cells was assessed to determine their transformation efficiency, with results indicating that the nanoparticles successfully delivered genetic material into the target cells while maintaining structural integrity and bioactivity. The application of these nanoparticles for gene delivery provides numerous advantages over conventional systems, including enhanced transfection efficiency, reduced toxicity, and the potential for controlled and targeted release. Additionally, the integration of green chemistry principles in nanoparticle synthesis ensures minimal environmental impact while maintaining high biocompatibility and stability. The findings of this study highlight the importance of these nanocarriers in advancing gene delivery

strategies and pave the way for their potential applications in therapeutic gene delivery, vaccine development, and genetic modifications. Future research should focus on optimizing nanoparticle formulations, improving targeting capabilities, and exploring their applicability in large-scale gene delivery and clinical gene therapy. The scalability and reproducibility of nanoparticle synthesis must also be studied to establish their feasibility for industrial applications. Further investigations should explore the use of different nanoparticle compositions to enhance delivery efficiency, reduce off-target effects, and enable programmable release mechanisms. With continuous advancements in nanotechnology and biomaterials, nanoparticle-mediated gene delivery is expected to revolutionize the fields of genetic engineering and medicine by providing a safe, efficient, and sustainable alternative to conventional gene delivery systems.

Keywords: Nanoparticles, Plasmid, Bacteria, Green Chemistry, Genetic Transformation

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NOMENCLATURE

µm	Micrometre
AMR	Antimicrobal Resistance
B. megaterium	Bacillus megaterium
bp	Base pairs
Bt	Bacillus thuringiensis
°C	Degree Celcius
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium Chloride
Cas 9	CRISPR-associated Protein-9
CNTs	Carbon Nanotubes
ComEC	Competence operon E-associated protein C
ComGC	Competence operon G-associated protein C
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DLS	Dynamic Light Scattering
DNA	Deoxy Ribonucleic Acid
dsDNA	Double-stranded DNA
E. coli	Escherichia coli
fHbp	Factor H-binding Protein
HAp	Hydroxyapetite
LB	Luria-Bertani
LTA	Lipoteichoic Acids
MDR	Multi-Drug Resistant
nm	Nanometre
NP	Nanoparticle

PBS	Phosphate Buffered Saline
pDNA	Plasmid DNA
pDNA-NPs	Plasmid DNA-fused Nanoparticles
psy	Phytoene synthase
rDT	Recombinant DNA Technology
RM	Restriction Modification
S. aureus	Staphylococcus aureus
S. pneumoniae	Streptococcus pneumoniae
SEM	Scanning Electron Microscopy
siRNA	Silencing Ribonucleic Acid
SOC	Super-Optimal with Catabolite repression
SWCNTs	SingleWalled Carbon Nanotubes
T4P	Type-4 Pili
Ti Plasmid	Tumor-inducing Plasmid
UV-Vis	Ultraviolet-Visible
WTA	Wall Teichoic Acids
ZP	Zeta Potential

Chapter 1

Introduction

Incorporating nucleic acids into cells is a fundamental technique in molecular biology and plays a key role in real-world applications like gene therapy, vaccine development, and drug discovery. For example, delivering plasmid DNA allows cells to produce specific proteins while introducing siRNA, which can silence targeted genes. Over the past four decades, scientists have developed various delivery methods, broadly classified into viral and non-viral approaches. Non-viral methods can be further divided into mechanical, physical, and chemical techniques [1].

Introducing genes into cells is crucial in scientific research and gene therapy. However, cells don't naturally absorb foreign nucleic acids because their phospholipid bilayer acts as a protective barrier, blocking most water-soluble molecules like DNA and RNA. Scientists often need to control their expression levels inside cells to study how genes and proteins function and are regulated. Over the past 40 years, various chemical, physical, and biological methods have been developed to help nucleic acids cross this barrier and reach the cell's interior [2].

Gene therapies predominantly aim at somatic (non-germline) cells to address diseases while avoiding heritable genetic modifications. The targeted cell types vary based on the condition being treated and commonly include lung epithelial cells, hepatocytes (liver cells), and muscle cells. Hematopoietic stem cells (HSCs) are often selected as targets in gene therapies addressing blood-related disorders such as beta-thalassemia and sickle cell disease. By introducing corrective genes into HSCs, the genetic anomalies can be rectified, enabling the modified stem cells to give rise to a range of functional blood cell lineages [3].

1.1 Bacterial Transformation

The natural capacity of certain bacteria to take up external DNA, known as natural competence, enables them to acquire new genetic characteristics. This ability helps them adapt to shifting environmental conditions, including evading vaccine responses or developing resistance to antibiotics [4]. The foundational work by Mandel and Higa first demonstrated that Escherichia coli could take up foreign DNA. They found that incubating E. coli with bacteriophage λ DNA in a calcium chloride ($CaCl_2$) solution at 0°C resulted in successful transformation [5]. Moreover, a short heat shock at 42°C followed by rapid cooling on ice and dilution with a nutrient medium substantially improved the efficiency of transfection.

Among various chemically mediated transformation strategies, Hanahan's method stands out as particularly effective. It was developed through a series of optimizations following Cohen's original discovery that Ca^{2+} ions could facilitate DNA uptake in E. coli [5,6]. Dower and colleagues later introduced the concept of using high-voltage electroporation to transform E. coli cells [6]. This technique involves subjecting a mixture of cells and DNA to brief, high-intensity electric pulses with exponential decay characteristics, resulting in remarkably high transformation efficiencies [7]. The efficacy of this method hinges primarily on two critical factors: the strength of the electric field and the duration of the pulse [8].

In a parallel development, Shark et al. pioneered a novel physical transformation technique for prokaryotes using a biolistic method. In their approach, DNA-coated microprojectiles were shot at high velocity into Bacillus megaterium cells using a biolistic delivery

system. This technique successfully achieved transformation across all the tested strains [9–11].

1.2 Applications of Bacterial Transformation

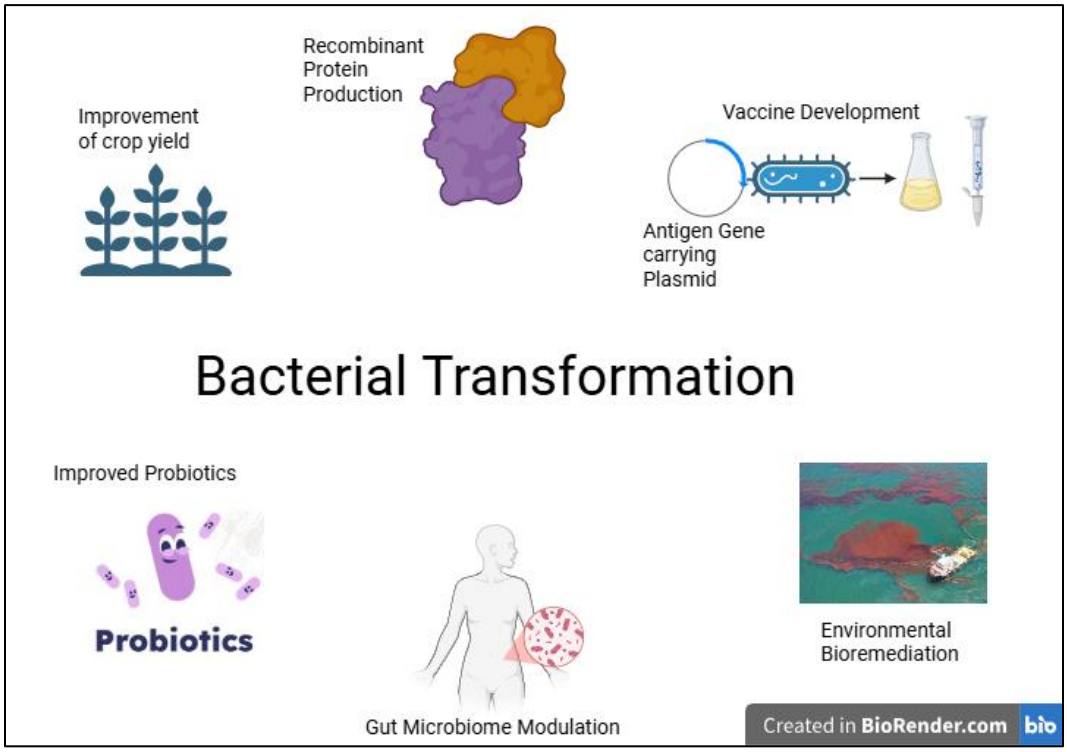


Figure 1: Applications of Bacterial Transformation

1.2.1 Improvement in crop yield

Bacterial Transformation has improved the process of transferring genetic material across species. A notable example is the development and widespread adoption of Bt cotton in India, where its integration into agriculture has reached nearly 100%, significantly influencing the cotton industry. Likewise, the adoption of Roundup Ready soybeans (Glycine max) in the United States has also approached full implementation. Golden rice (*Oryza sativa*),

a widely recognized genetically modified crop, is biofortified with beta-carotene, a precursor of vitamin A. This enhancement is achieved by introducing the *phytoene synthase (psy)* gene from daffodil (*Narcissus pseudonarcissus*) and the *phytoene desaturase (crtI)* gene from *Erwinia uredovora* [7].

A cornerstone of contemporary plant biotechnology, Agrobacterium-mediated transformation facilitates targeted genetic modifications aimed at improving crop traits like pest resistance, yield, and environmental resilience. This technique exploits the innate DNA transfer system of the soil bacterium Agrobacterium tumefaciens, which naturally inserts T-DNA from its tumor-inducing (Ti) plasmid into plant cells [8].

1.2.2 Recombinant protein production

The advancement of deconstructed viral vectors has revitalized the field of recombinant protein (RP) production using plants, offering a highly efficient platform characterized by enhanced protein yield, rapid production, scalability, versatility, safety, and cost-effectiveness. A significant breakthrough in plant glycoengineering has further expanded the potential of this approach by enabling the synthesis of RPs with customized N-glycans, thereby improving their functionality and safety. One of the key obstacles to the widespread commercial adoption of plant-based transient expression systems was the absence of a scalable method for transgene delivery into cells. However, as highlighted in this review, this challenge has been

effectively addressed through various agroinfiltration technologies. Continued optimization of these technologies is expected to accelerate the acceptance of plant transient expression systems for large-scale RP production [9].

1.2.3 Vaccine Development

In vaccine development, bacterial transformation serves a crucial role by allowing the genetic engineering of bacteria to express antigens, synthesize therapeutic proteins, or generate plasmid DNA for emerging vaccine technologies. This strategy capitalizes on the inherent capacity of bacteria to uptake and express exogenous DNA. At the same time, modern genetic manipulation techniques are employed to further augment the safety and effectiveness of the resulting vaccine candidates. Advances in genomics, coupled with innovative methodologies such as reverse vaccinology, have redefined strategies for vaccine candidate identification and development. Genome mining, combined with unbiased selection of novel antigens, offers a new pathway to elucidate mechanisms underlying bacterial pathogenesis. Bacterial transformation serves as a pivotal tool in this process, enabling the insertion and expression of newly identified antigenic genes in suitable bacterial hosts. This facilitates the production and subsequent characterization of vaccine candidates. The study of Factor H Binding Protein (fHbp), a major virulence factor of *Neisseria meningitidis*, exemplifies this approach, where bacterial transformation was employed to express and analyze the antigen for vaccine development. Harnessing transformation techniques, alongside genomic analyses of both the pathogen and the

host, enhances our comprehension of bacterial biology and the induction of protective immunity. Consequently, bacterial transformation plays a critical role in informing and accelerating the rational design of next-generation vaccines [10].

1.3 Conventional Transformation Methods and Their Limitations

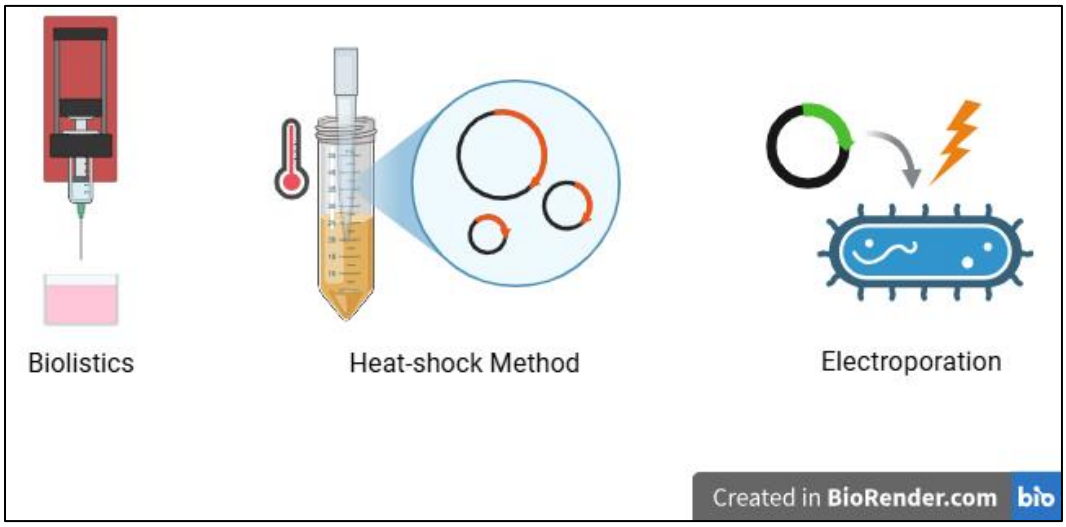


Figure 2: Conventional Transformation Methods

1.3.1 Particle Bombardment

Particle bombardment, also known as ballistic particle delivery, is a technique used to introduce nucleic acids into multiple cells simultaneously. In this method, microscopic gold or tungsten particles are coated with nucleic acids and propelled at high velocities, allowing them to penetrate cellular membranes. By adjusting parameters such as particle size and acceleration speed, this approach can be

optimized for the efficient transfection of adherent cell cultures [2]. This technique is extensively utilized in genetic vaccination, where localized expression of the delivered DNA is adequate to elicit a robust immune response [11].

1.3.2 Heat-shock Method

The heat shock method for plasmid DNA transformation into cells is a fundamental technique in molecular biology. It involves introducing a foreign plasmid or ligation product into bacterial cells. The process begins with a brief incubation on ice, followed by a heat shock at 42°C for 45 seconds before returning the cells to ice. Afterwards, SOC medium is added, and the transformed cells are incubated at 37°C for 30 minutes with agitation. To ensure colony isolation regardless of transformation efficiency, two different volumes of the transformed culture are plated [12]. Although this method enables the physical delivery of DNA into bacterial cells using high-velocity microprojectiles, it has several drawbacks. The equipment is expensive and often requires fine-tuning for each bacterial strain, making it less accessible and more time-consuming than other methods. There's also a risk of damaging cells due to the mechanical force, which can lead to reduced viability and inconsistent transformation results. Additionally, the efficiency tends to be lower in comparison to chemical or electrical methods, especially for smaller or more fragile bacterial species.

1.3.3 Electroporation

Electroporation, a method that utilizes high-voltage electric pulses to introduce DNA into cells, is applicable to a wide range of cell types. This highly efficient technique facilitates

both stable transformation and transient gene expression. Additionally, due to its relatively straightforward procedure with fewer steps, electroporation can often be a more convenient alternative to other transformation methods [13]. However, the technique can cause significant cell death if the voltage is too high or if impurities are present in the sample, such as salts that can trigger arcing. Preparing electrocompetent cells also requires careful handling and stringent conditions, which can be tedious. Moreover, some bacterial strains are naturally more resistant to electroporation, resulting in lower transformation rates despite repeated optimization attempts.

1.4 Internalization of DNA into Bacterial Cells

Except for Helicobacter pylori, transformable bacterial species are believed to utilize conserved mechanisms for the uptake and processing of foreign DNA. These processes depend on a core group of proteins encoded by genes that are co-expressed during the initiation of competence [14].

During transformation, exogenous double-stranded DNA (dsDNA) serves as the primary substrate; one strand undergoes degradation while the complementary strand is imported into the cell in a single-stranded form via the Competence operon E-associated protein C (ComEC) transmembrane channel, proceeding in the 3′ to 5′ direction [15]. Primarily informed by findings from Bacillus subtilis and S. pneumoniae, this model reflects the foundational understanding of transformation processes that have been thoroughly characterized in these two species.

Among Gram-positive bacteria, competence pseudopili are produced from genes located in the Competence G (ComG) operon, with Competence operon G-associated protein C (ComGC) serving as the primary pilin subunit responsible for pseudopilus formation [16]. The protrusion of competence pseudopili beyond the bacterial surface was initially thought to be minimal, based on the observation that a B. subtilis cell contains around 40–100 ComGC monomers. Given that each monomer is approximately 1 nm in length, this would result in pseudopili with an overall length of 40–100 nm, which is much smaller than the ~55 nm thickness of the periplasm and cell wall. However, a true Type-4 Pili (T4P), measuring 2–3 μm in length, was recently identified in S. pneumoniae cells, challenging previous assumptions [17].

1.5 Barriers to Bacterial Transformation

The process of bacterial transformation, wherein bacteria internalize exogenous DNA, encounters numerous barriers that constrain interspecies genetic exchange. Among these obstacles are molecular defense systems and structural impediments that hinder the successful integration of foreign DNA.

1.5.1 Internal Barriers

1.5.1.1 Restriction-Modification (RM) Systems

Among the various barriers, restriction-modification (RM) systems are the most prevalent, found in approximately 90% of bacterial genomes. These systems comprise a restriction endonuclease that targets and cleaves unmethylated DNA at specific recognition sites, along with a methyltransferase that safeguards the host genome by methylating these sequences [17]. For instance, Staphylococcus aureus employs type IV RM systems to degrade plasmids that lack its specific methylation pattern,

resulting in a dramatic reduction in transformation efficiency, up to 100,000-fold. To bypass RM systems, strategies often involve engineering DNA to evade recognition sequences (such as through "Syngenic DNA" tools) or employing methylation-mimicking techniques [15].

1.5.1.2 Mismatch Repair Systems

In Streptococcus species, the Hex system plays a critical role in the DNA mismatch repair (MMR) pathway by correcting replication errors and maintaining genomic stability. This system involves two main proteins, HexA and HexB. In Escherichia coli, the MMR pathway primarily depends on the coordinated activity of MutS and MutL. MutS detects base mismatches and insertion–deletion loops, and subsequently recruits MutL, which facilitates downstream repair events. Streptococcus lacks the endonuclease component found in Escherichia coli, and instead relies on a mechanism similar to the MutS–MutL-dependent pathway observed in eukaryotes. The HexA–HexB complex in Streptococcus compensates for this absence by initiating repair without a strand-specific nicking enzyme. This alternative MMR pathway is essential for replication fidelity. Disruption of Hex function leads to elevated mutation rates, increasing the risk of genomic instability and promoting potential pathogenic adaptations. The Hex system in Streptococcus and MutS, MutL in Escherichia coli serve as powerful barriers, rejecting heterologous DNA with sequence divergence greater than 0.6%. These systems identify and correct mismatches in donor-recipient DNA hybrids, leading to a significant reduction in recombination frequency as sequence divergence grows. In Bacillus, the impact of mismatch repair is less pronounced, with recombination primarily dependent on the presence of nearly identical homologous ends (≥20 bp) to initiate the process [18].

1.5.1.3 CRISPR-Cas Systems

CRISPR-Cas functions as a bacterial immune system, evolving by integrating foreign DNA into a CRISPR array. This DNA is subsequently transcribed into CRISPR guide RNAs, which direct the targeting and degradation of foreign DNA through nuclease activity. CRISPR-Cas systems are more prevalent in commensal enterococcal strains, where they help prevent the uptake of foreign DNA, such as drug resistance elements. In contrast, multidrug-resistant (MDR) or clinical enterococcal strains either lack functional defense systems or harbor non-functional variants, such as CRISPR2, which contains a spacer array but no cas genes. As a result, MDR strains exhibit greater genetic plasticity, which can enhance their survival and adaptability compared to strains with limited genetic diversity due to the absence of MDR genes [19]. In E. faecium, this is exemplified by clade B strains (commensal strains) examined, where CRISPR-Cas were sporadically present, with few MGE. In contrast, no CRISPR-Cas genes were identified in clades A1/A2, and the genome was littered with antimicrobial resistance (AMR) determinants [20].

1.5.2 Physical and Structural Barriers

Cell Wall Composition: Some bacteria possess a thick cell wall, approximately 40 nm in thickness, primarily composed of peptidoglycan. This structure is interspersed with wall polymers, including wall teichoic acid (WTA) and other polysaccharides. Lipoteichoic acids (LTA) extend from the top of the peptidoglycan layer, anchoring to the plasma membrane through covalent bonds [21].

Competence Machinery: Natural transformation necessitates the presence of species-specific competence proteins. For instance,

Streptococcus utilizes ComEC pore complexes for DNA uptake, which may selectively reject DNA with significant sequence divergence [22].

1.6 NP-mediated Bacterial Transformation

The application of nanoparticles in therapeutics has been well-established for drug delivery into animal cells. Bacterial transformation facilitated by nanoparticles is an emerging genetic engineering approach that employs nanomaterials to introduce foreign DNA into bacterial cells, surpassing traditional methods such as electroporation and heat shock. This technique not only simplifies experimental protocols and reduces costs but also enables efficient transformation across a broader spectrum of bacterial species. The effective introduction of plasmid DNA into microbial cells remains critical to the success of recombinant DNA technology. However, natural bacterial transformation is inherently restricted to specific species, primarily due to the electrostatic repulsion between the negatively charged DNA and bacterial membranes [23]. Recently, they have begun to attract considerable interest in microbiology as innovative carriers for DNA delivery. Nanoparticle-mediated transformation techniques demonstrate enhanced transformation efficiency and eliminate the need for competent cell preparation, thereby significantly shortening the overall transformation timeline.

In recent developments, transformation mediated by nanomaterials such as carbon nanotubes [24], sepiolite nanofibers [25], and chitosan nanoparticles [26] has been documented. Transformation methods employing these nanomaterials demonstrate enhanced efficiency and eliminate the need for competent cell preparation, thereby shortening the transformation timeline. Nevertheless, successful transformation

has thus far been achieved only in Gram-negative E. coli using these approaches [27].

1.7 Objectives

Objective 1: To synthesize and thoroughly characterize nanoparticles encapsulating genetic material, with the aim of evaluating their physicochemical properties and suitability for gene delivery applications.

Objective 2: To investigate the efficacy of plasmid-loaded nanoparticles in facilitating the transformation of bacterial cells, thereby assessing their potential as a non-viral gene delivery system.

1.8 Organization of the Thesis

Chapter 1 introduces the concept of using nanoparticles for Bacterial Transformation, explaining the rationale behind the study, and outlining its overall significance.

Chapter 2 delves into past research efforts related to nanoparticle applications in microbiology, identifies existing gaps, and clearly defines the specific problem this work aims to address.

Chapter 3 outlines the materials, experimental protocols, and analytical techniques used throughout the study, including the synthesis of nanoparticles, their characterization, and the approach taken for evaluating the transformation method.

Chapter 4 presents and interprets the experimental findings, offering a detailed discussion on how the results align with or diverge from

previous studies and what they imply in the context of nanoparticle-mediated bacterial transformation.

Chapter 5 concludes the thesis by summarizing the key conclusions drawn from the work and exploring potential avenues for further investigation and application in related areas of research.

Chapter 2

Review of Past Work and Problem Formulation

Nanoparticle (NP)-mediated gene delivery is a promising approach in genetic engineering, gene therapy, and biotechnology because it efficiently transports genetic material into target cells with minimal toxicity. Unlike traditional methods such as viral vectors, which pose risks of immunogenicity and insertional mutagenesis, NP-based systems offer enhanced biocompatibility, stability, and targeted delivery. Various types of nanoparticles, including lipid-based, polymeric, metallic, and inorganic nanocarriers, have been explored for gene delivery. These nanoparticles encapsulate or adsorb plasmid DNA (pDNA), small interfering RNA (siRNA), or messenger RNA (mRNA) and facilitate their cellular uptake through endocytosis or membrane fusion. Surface modifications, such as polyethylene glycol (PEG) coating or ligand conjugation, enhance circulation time and target specificity. Cationic polymers like polyethyleneimine (PEI) and poly(allylamine hydrochloride) (PAH) interact electrostatically with negatively charged nucleic acids, forming stable nano-complexes that protect genetic cargo from enzymatic degradation. Additionally, stimuli-responsive nanoparticles release their payload in response to environmental triggers such as pH, temperature, or redox conditions. In bacterial transformation, NP-mediated gene delivery can enhance plasmid uptake by improving cellular permeability and reducing shear stress compared to conventional methods like electroporation or heat shock. Moreover, metallic nanoparticles such as gold (AuNPs) and magnetic nanoparticles (MNPs) facilitate gene delivery through physical mechanisms like photothermal or magnetofection

approaches. The effectiveness of NP-mediated gene delivery depends on particle size, surface charge, and cellular interactions, which determine transfection efficiency and gene expression levels. Recent advances focus on developing non-toxic, biodegradable carriers with precise control over release kinetics, improving their translational potential in clinical and industrial applications. As research progresses, NP-mediated gene delivery is expected to revolutionize personalized medicine, vaccine development, and regenerative therapies by providing safer and more efficient alternatives for genetic modifications.

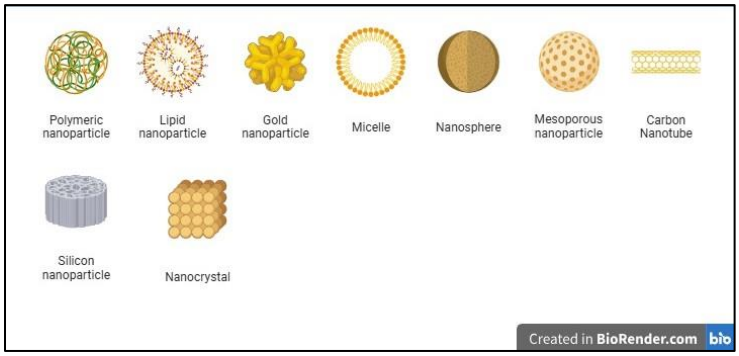


Figure 3: Some of the currently used NPs for Bacterial Transformation

Carbon nanoparticles have been reported to address several challenges associated with traditional gene delivery methods, such as host specificity, transformation complexity, and cellular or tissue damage caused by external forces. These nanomaterials are gaining attention due to their unique properties, including nanoscale size (1–100 nm), biocompatibility, and intrinsic autofluorescence, which allows for easy tracking within cells without the need for additional staining or fluorescent tagging [28]. Studies have suggested that functionalized single-walled carbon nanotubes can serve as carriers for delivering various biomolecules, such as plasmid DNA and siRNA, into cells [26, 27]. Single-walled carbon nanotubes (SWNTs) are cylindrical structures composed of a single layer of carbon arranged in

a hexagonal pattern. Functionalization with amino or carboxyl groups enables these nanotubes to form covalent or non-covalent interactions with biomolecules such as proteins, peptides, and nucleic acids. For the delivery of DNA oligonucleotides or siRNA, SWCNTs are typically designed with a tube diameter of 1–5 nm and a length ranging from 50 to 200 nm. Positively charged SWNTs have also been successfully utilized for in vivo siRNA delivery [32].

The use of chitosan nanoparticles for gene delivery has gained significant attention due to their ability to effectively encapsulate and protect plasmid DNA (pDNA) while enhancing transformation efficiency. Different molecular weights of chitosan have been explored for their ability to form stable nanoparticles with plasmid DNA, and their effectiveness in maintaining the supercoiled form of the plasmid has been analyzed using agarose gel electrophoresis [33].

One of the key challenges in gene delivery is protecting genetic material from enzymatic degradation. Studies have demonstrated that chitosan nanoparticles provide substantial protection against nuclease-mediated degradation, as evidenced by their resistance to DNase I digestion. Additionally, the efficiency of plasmid transformation mediated by these nanoparticles has been validated through assays measuring gene expression, showing significantly higher transformation rates compared to naked DNA or conventional polycation-based complexes.

The physicochemical stability of chitosan-based nanoparticles has also been investigated, revealing that their size and zeta potential remain stable over extended periods at physiological pH. However, changes in particle size were observed following lyophilization, particularly in the presence of cryoprotective agents. Despite these variations, chitosan nanoparticle formulations have consistently demonstrated superior transfection

efficiency in mammalian cells, significantly enhancing gene expression when compared to unprotected plasmid DNA [3].

Some findings suggest that high-molecular-weight chitosan formulations hold great promise as a non-viral gene delivery system, offering enhanced stability, protection, and transfection efficiency. Their potential application in animal studies positions them as a viable alternative to traditional viral vectors for gene therapy and biomedical research.

Hydroxyapatite (HAp), a calcium phosphate mineral, has garnered significant interest in biomedical engineering due to its excellent biocompatibility and bioactivity. In nanoparticle form, hydroxyapatite (HAp NPs) exhibits a strong affinity for nucleic acids, facilitating their transport across cell membranes. These nanoparticles have been successfully utilized as carriers for gene transformation in both animal and plant cells, demonstrating their potential in genetic engineering and therapeutic applications.

Studies have highlighted the potential of Arginine-Glucose functionalized hydroxyapatite nanoparticles (HAp NPs) as an effective plasmid delivery system for bacterial transformation. The functionalization of HAp with positively charged arginine significantly improved its binding affinity to plasmid DNA, achieving a binding efficiency of 90%. This modification led to higher transformation efficiency in both Gram-positive and Gram-negative bacteria compared to conventional methods such as calcium chloride treatment and electroporation [24].

An alternative strategy in nanoparticle-mediated transformation involves the use of nano-sized, needle-shaped materials like α-sepiolite and chrysotile asbestos as delivery agents. Rather than depending on electroporation or chemical permeabilization, this method capitalizes on the inherent physical characteristics of these nanomaterials. The nanoparticles

bind plasmid DNA and, under sliding friction against a hydrogel matrix in the presence of bacterial cells, aggregate into larger, sharper microstructures. These aggregates, propelled by mechanical friction, breach the bacterial membrane and transfer the DNA into the cytoplasm. This technique constitutes a unique, physically induced gene delivery mechanism in prokaryotes where nanoparticle geometry, friction-induced aggregation, and DNA adsorption capability collectively determine transformation success [25].

One of the key advantages of this nanovehicle-based transformation method is that it eliminates the need for preparing competent cells, making the process simpler and more accessible. Additionally, the procedure can be performed at room temperature, further streamlining the workflow. The non-toxic nature of HAp NPs adds to their appeal as a safe and efficient alternative for bacterial transformation. This innovative approach presents exciting possibilities for advancements in recombinant DNA technology, paving the way for improved gene delivery strategies in bacterial research and biotechnology [27].

The current research on nanoparticle (NP)-mediated bacterial transformation faces several limitations that must be addressed for its broader application. Functionalization of NPs is often required to enhance their interaction with genetic material and improve cellular uptake, adding complexity to their synthesis. Despite advancements, transformation efficiency remains limited, posing challenges for practical implementation. Additionally, concerns regarding potential toxicity and biocompatibility raise questions about the safety of NPs for clinical and environmental applications. The size and charge of NPs also impose constraints on DNA

loading capacity, potentially affecting the stability and efficiency of gene transfer. Furthermore, successful transformation is often dependent on additional external factors, such as buffer conditions, incubation time, and environmental parameters, which necessitate further optimization.

Chapter 3

Materials, Methods, and Instrumentation

3.1 Materials

The bacterial strain, E. coli DH5α, and the plasmid pET43 were kindly gifted by the Molecular Immunology Lab, led by Prof. Prashant Kodgire, IIT Indore. The LB Broth powder was purchased from HiMedia® Laboratories Pvt. Ltd.. The Plasmid Isolation Miniprep Kit, FavorPrep™ Plasmid Fast DNA Extraction (FAPD300) Kit was purchased from Favorgen Biotech Corp.

3.2 Instrumentation

3.2.1 UV-VIS Spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy is a fundamental analytical technique employed to quantify and characterize molecular species based on their light absorption properties. This method involves comparing the absorbance or transmittance of a sample against a reference blank, allowing the identification of specific wavelengths at which the sample absorbs ultraviolet or visible light. The underlying principle is based on the excitation of electrons to higher energy states upon photon absorption, with the specific wavelengths absorbed being dependent on the molecular structure and electronic configuration of the analyte.

UV-Vis spectroscopy operates in accordance with the Beer–Lambert law, which describes a linear relationship between the absorbance (A) of a solution and the product of the molar absorptivity coefficient (ε), the path length of the cuvette (l), and the concentration of the absorbing species (c). This relationship enables

accurate quantitative assessment of analyte concentrations, while the position and intensity of absorbance peaks provide valuable qualitative information regarding the molecular identity and environment. Owing to its simplicity, sensitivity, and non-destructive nature, UV-Vis spectroscopy is widely utilized in the analysis of nucleic acids, proteins, and other biomolecules [33].

3.2.2 Field-Emission Scanning Electron Microscopy (FESEM)

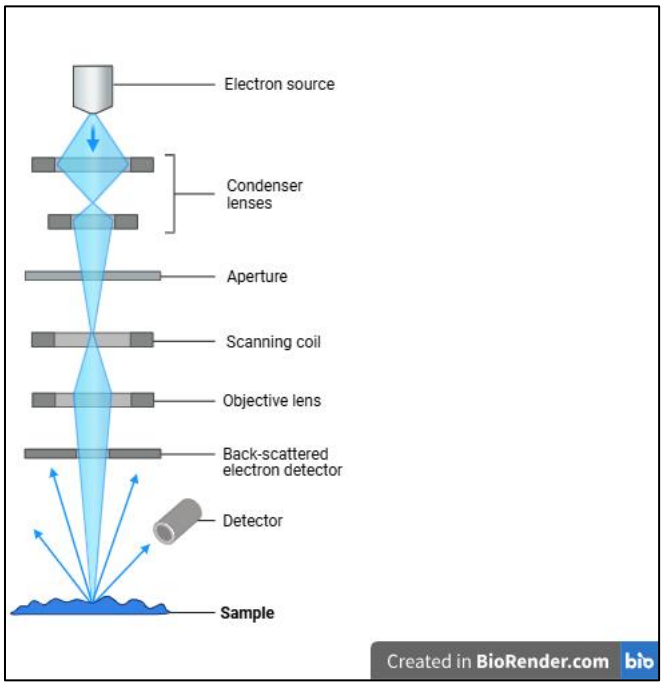


Figure 4: Schematic representation of a Field-Emission Scanning Electron Microscopy (FESEM) system illustrating the trajectory of the electron beam.

Field Emission Scanning Electron Microscopy (FESEM) is an advanced imaging technique that utilizes a field emission electron source to illuminate the specimen, as opposed to the conventional use of light in optical microscopy. This approach allows for high-resolution visualization of surface morphologies, including detailed

characterization of nanoparticle size, shape, and surface architecture.

FESEM provides both topographical and elemental insights with magnifications ranging from 10x to 300,000x, and offers an exceptionally high depth of focus, enabling the clear imaging of uneven surfaces. Compared to traditional Scanning Electron Microscopy (SEM), FESEM delivers superior image sharpness, minimal electrostatic distortion, and significantly enhanced spatial resolution reaching as fine as 1.5 nanometres, which is approximately three to six times higher than that of conventional SEM. This makes FESEM a powerful tool for nanoscale material characterization.

3.2.3 Dynamic Light Scattering (DLS) Spectroscopy

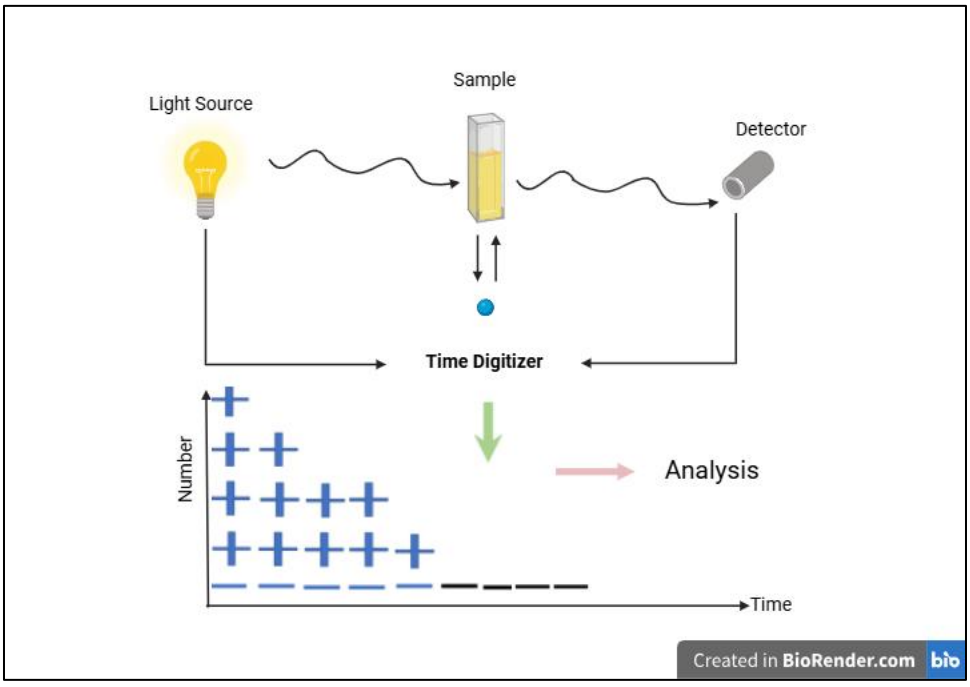


Figure 5: Schematic representation of a Dynamic Light Scattering Spectroscopy (DLS)

Among the most frequently employed techniques for assessing the size distribution of ultra-fine particles in polymer-based suspensions is dynamic light scattering (DLS), also referred to as quasi-elastic light scattering spectroscopy or photon correlation spectroscopy. This technique can detect particles as small as 1 nm. It is widely utilized for analyzing the sizes of proteins, nanoemulsions, micelles, colloids, nanoparticles, and polymers. Rather than measuring the exact diameter, DLS determines the hydrodynamic diameter of particles, which accounts for the particle's core and the solvation layer, thereby appearing slightly larger than the true physical size [34].

3.2.4 Zeta Potential (ZP) Measurement

When a colloidal particle moves within an electric field, the potential that arises at the shear or slipping plane is referred to as the Zeta potential (ZP), also known as the electrokinetic potential. ZP represents the electric potential difference between the stationary dispersion medium and the mobile part of the electric double layer (EDL) surrounding the particle. It essentially quantifies the energy required to bring a unit positive charge from infinity to the particle's surface. Zeta potential is commonly utilized to estimate the surface charge characteristics of nanoparticles. During electrophoresis, the direction in which particles migrate indicates whether the ZP is positive or negative. However, it is important to emphasize that ZP pertains solely to the electrostatic potential at the particle's surface; it does not measure actual charge or charge density. Consequently, only the absolute value of ZP is meaningful. Comparing its sign across different nanoparticle systems is unreliable and should be avoided, as ZP is not a direct indicator of surface charge magnitude or density [34].

3.3 Methods

3.3.1 Plasmid Isolation from Bacteria

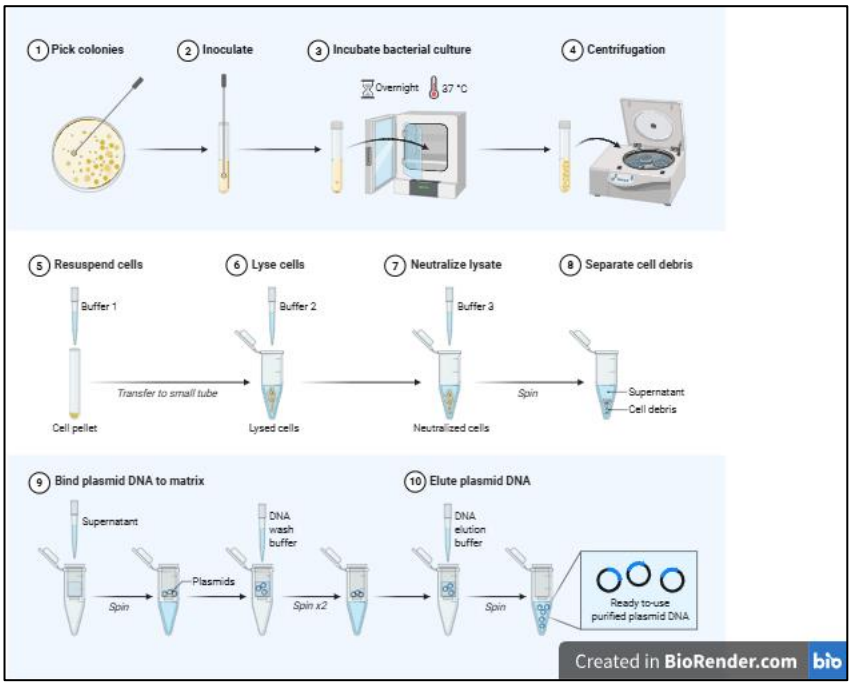


Figure 6: Plasmid Isolation Protocol

To begin the plasmid DNA isolation, 1–3 mL of a well-grown bacterial culture is transferred to a centrifuge tube. The sample is centrifuged at 11,000 ×g for one minute to pellet the cells, and the resulting supernatant is carefully discarded. The cell pellet is then thoroughly resuspended in 200 µL of FAPD1 Buffer, which must contain RNase A ensuring complete mixing with no visible pellet remaining. Following this, 200 µL of FAPD2 Buffer is added, and the tube is gently inverted 5 to 10 times. The lysate is incubated at room temperature for 2 to 5 minutes to facilitate cell lysis, ensuring not to exceed 5 minutes or use vortexing, as these actions could damage genomic DNA. To neutralize the lysate, 300 µL of FAPD3 Buffer is added, and the tube is immediately inverted 5 to 10 times to avoid uneven precipitation. The mixture is then centrifuged at

approximately 18,000 ×g for five minutes, during which a FAPD Column is placed in a Collection Tube. The cleared lysate (supernatant) is carefully loaded onto the column without disturbing the white precipitate, and centrifuged at 11,000 ×g for 30 seconds. The flow-through is discarded and the column returned to the same Collection Tube. Next, 400 µL of WP Buffer is added, followed by centrifugation at the same speed for 30 seconds, after which the flow-through is again discarded. This is followed by a wash with 700 µL of Wash Buffer pre-mixed with 96–100% ethanol under the same centrifugation conditions. The column is then centrifuged at full speed (~18,000 ×g) for three additional minutes to ensure complete drying, a critical step for removing residual ethanol. Subsequently, the FAPD Column is transferred to a clean 1.5 mL microcentrifuge tube. For elution, 50–100 µL of Elution Buffer or nuclease-free water is dispensed directly onto the center of the membrane, and the column is allowed to stand for one minute to facilitate absorption. Finally, the tube is centrifuged at full speed for one minute to elute the plasmid DNA, which is then stored at −20°C for future use [35].

3.3.2 Calculation of pDNA Encapsulation Efficiency in NPs

To evaluate the encapsulation efficiency of plasmid DNA within the synthesized nanoparticles, both empty NP and pDNA-loaded NP pellets were resuspended in 10% dimethyl sulfoxide (DMSO). The resulting suspensions were incubated at 37 °C to facilitate the release of any unbound or loosely associated DNA. Following incubation, the samples were centrifuged to separate the released DNA in the supernatant from the NP pellet. The concentration of plasmid DNA in the supernatant was quantified using a Nanodrop spectrophotometer, with the corresponding empty NP suspension as the blank control. These measurements were subsequently used to calculate the encapsulation efficiency.

The Encapsulation Efficiency Percentage was calculated using the formula:

$$\%EE = \frac{(pDNA-NP)f}{(pDNA-NP)i} \times 100$$

Where,

%EE = Percent encapsulation efficiency,

(pDNA-NP)i = the initial amount of pDNA-NP complex added to the reaction mixture,

(pDNA-NP)f = amount of pDNA-NP in the pellet

3.3.3 Scanning Electron Microscopy (SEM)

Morphological characterization of the formulated nanoparticles was performed using Scanning Electron Microscopy (SEM). Initially, glass slides were sectioned into smaller pieces with a diamond cutter and thoroughly cleaned with alcohol. Nanoparticle suspensions underwent sonication for 10–15 minutes to ensure uniform dispersion, after which a small volume of the sample was drop-cast onto the glass slides. These were subsequently placed in a desiccator and allowed to dry overnight. The dried slides were mounted on stubs using carbon adhesive tape, and to enhance surface conductivity, a thin 10 nm gold layer was sputter-coated over the samples for a duration of 2 minutes. Imaging was then carried out using an SEM at an accelerating voltage between 5–10 kV. Post-imaging, the nanoparticle size distribution was quantitatively assessed using ImageJ software.

3.3.4 Zeta Potential (ZP) Measurement

The Zeta potential of the synthesized nanoparticles was measured using the Litesizer 500 Dynamic Light Scattering (DLS) system. For this purpose, the NP pellets were carefully resuspended in 1 mL of phosphate-buffered saline (PBS), following which the suspension

was loaded into the instrument to determine the surface charge of the particles.

3.3.5 NP-mediated Bacterial Transformation

For nanoparticle-mediated bacterial transformation, Escherichia coli DH5α cells were cultured in Luria-Bertani (LB) broth until the optical density at 600 nm (OD600) reached approximately 0.5. The bacterial culture was then harvested by centrifugation and washed thoroughly using 1X phosphate-buffered saline (PBS) to remove residual media components. Plasmid DNA (pDNA) was encapsulated within the preformed nanoparticles (NPs), resulting in nanoplexes. These nanoplexes were then incubated with the washed bacterial cells under varying experimental conditions. Initially, transformation was assessed by incubating single bacterial samples with either pGIPZ-loaded NPs or pET43-loaded NPs for a duration of 6 hours. Following this, time-dependent transformation studies were conducted exclusively with pGIPZ-loaded NPs for durations ranging from 1 to 3 hours (hourly intervals), and subsequently from 12 to 60 minutes (at 12-minute intervals). Separately, transformation efficiency was evaluated as a function of pDNA concentration by varying the amount of encapsulated pGIPZ plasmid between 100 ng and 400 ng. Post-incubation, all bacterial samples were plated onto LB agar supplemented with ampicillin, as both pGIPZ and pET43 plasmids harbor ampicillin resistance genes. The plates were then incubated at 37 °C for 17 to 18 hours. As a negative control, untransformed bacteria were similarly plated on ampicillin-containing LB agar to confirm the absence of background growth.

Chapter 4

Results and Discussion

4.1 Plasmid Isolation (Agarose Gel Electrophoresis)

a) b)

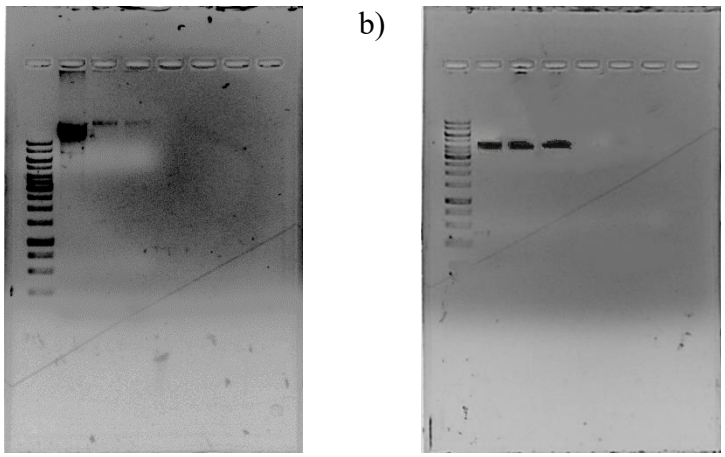
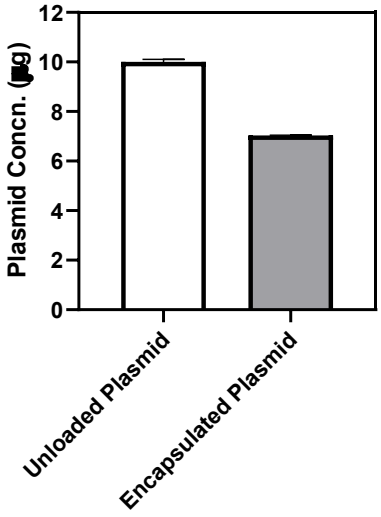


Figure 7: Agarose gel electrophoresis image showing the isolated plasmids a) pGIPZ and b)pET43. The distinct bands confirm successful extraction and integrity of the plasmid DNA.

The successful isolation of two plasmids, pGIPZ and pET43, was confirmed via Agarose Gel Electrophoresis (AGE), as shown in Figures 7 a) and 7 b) respectively. The gel corresponding to pGIPZ [Figure 7 a)] displays distinct, intact supercoiled bands in all lanes, with minimal smearing, indicating good quality and high-purity plasmid DNA. The gel representing pET43 plasmid [Figure 7 b)] shows well-resolved and consistently intense bands across all lanes, suggesting high plasmid yield and structural integrity. The absence of RNA contamination or degraded DNA in both samples affirms the efficiency of the isolation protocol. Additionally, comparison with the DNA ladder verifies that the bands correspond to the expected sizes for each plasmid, affirming the identity and integrity of the isolated constructs.

4.2 Calculation of Encapsulation Efficiency

Encapsulation Efficiency = ~70%



For 100µl,
7030 ng
= 7.03µg

Calculation of %EE :-

$$\%EE = \frac{7.03}{10} \times 100 = \sim\mathbf{70\%}$$

Figure 8*: *Bar graph showing plasmid DNA (pDNA) encapsulation efficiency. The first bar represents the initial amount of pDNA used before encapsulation (10 µg), while the second bar shows the amount successfully encapsulated after the process (7.03 µg). Error bars reflect standard deviation from three separate experiments (n = 3). The efficiency was calculated based on how much of the original pDNA was retained inside after encapsulation.

The results presented in Figure 8a) demonstrate the effective encapsulation of plasmid DNA within the prepared nanoparticles. Initially, 10 µg of plasmid DNA was used for the encapsulation process, as indicated by the first bar in the graph. Following nanoparticle formation, the amount of DNA successfully

encapsulated was quantified and found to be 7.03 µg, represented by the second bar. This corresponds to an encapsulation efficiency of approximately 70%, reflecting a substantial proportion of plasmid DNA retained within the nanoparticles. The Encapsulation Efficiency percentage was calculated by using the formula:

$$\%EE = \frac{(pDNA-NP)f}{(pDNA-NP)i} \times 100$$

Where,

%EE = Percent encapsulation efficiency,

(pDNA-NP)i = the initial amount of pDNA-NP complex added to the reaction mixture,

(pDNA-NP)f = amount of pDNA-NP in the pellet

The encapsulation efficiency percentage of the sample was calculated to be approx. 70%. Such efficient encapsulation is critical for ensuring adequate delivery of genetic material in downstream applications and confirms the suitability of the nanoparticle formulation method employed in this study. The substantial difference between the two groups (Unloaded plasmid and Encapsulated plasmid) confirms that the NPs efficiently loaded the genetic material. The error bars on both groups signify minimal variation in encapsulation efficiency among replicates.

4.3 SEM Imaging

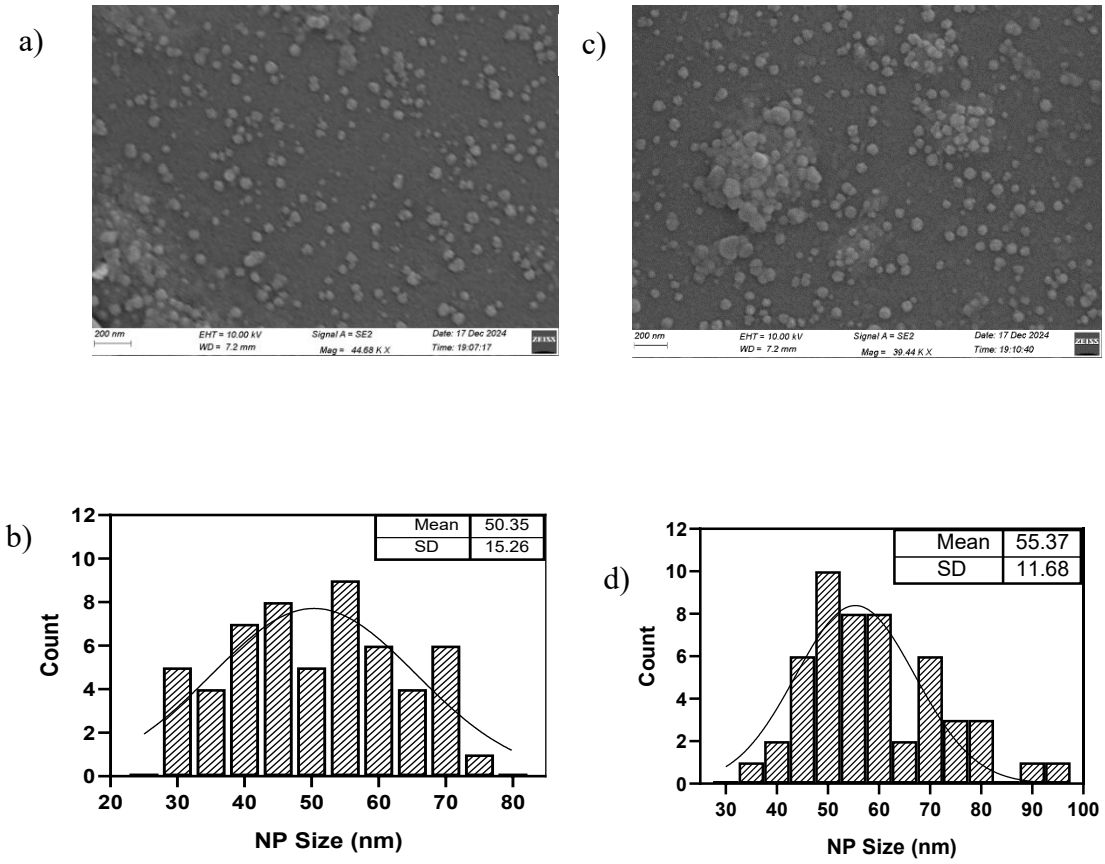


***Figure 9*:**

a) SEM Image of bare NPs at 44680 X magnification.

b) Size distribution of bare NPs

c) SEM image of pDNA-loaded NPs at 39440 X magnification

d) Size distribution of pDNA-loaded NPs

The size of the NPs in the SEM images was estimated using the ImageJ software and the statistical analysis was performed by using the Graphpad Prism software. SEM imaging of empty and plasmid-loaded NPs revealed an average particle size of around 50nm ± 15.26 nm and 55nm ± 11.68 nm, for empty and pDNA-loaded NPs respectively. The observed increase in size can likely be attributed to

the encapsulation of pDNA within the NPs. The observed increase in particle size after encapsulation suggests that the incorporation of additional material led to nanoparticle swelling or structural changes. The data confirm a consistent size increase post- encapsulation, supporting the successful loading of pDNA molecules within the NPs.

4.4 ζ-Potential Measurement

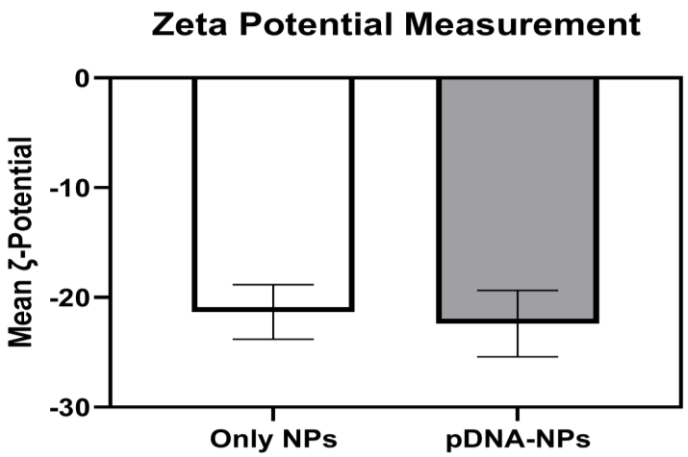


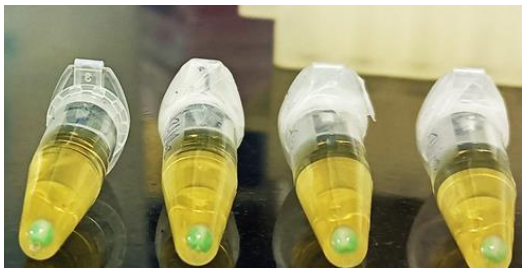
Figure 10*: *Comparison of zeta potential values between unloaded and plasmid DNA-loaded nanoparticles. The graph illustrates the surface charge of nanoparticles before and after encapsulation of plasmid DNA.

The data in Figure 10 indicates that the nanoparticles maintain a negative surface charge in both their unloaded and plasmid DNA-loaded forms. This negative charge likely contributes to their colloidal stability by facilitating electrostatic repulsion between particles, thereby minimizing the risk of aggregation. Upon encapsulation of plasmid DNA, a modest reduction in zeta potential is observed. This drop can be attributed to the interaction between

the negatively charged phosphate groups of the DNA and the nanoparticle surface, suggesting that the plasmid has been successfully associated with or internalized by the nanoparticles.

4.5 pDNA-loaded NP uptake by Bacteria

a)



b)

c)

d)

Figure 11*: *Visual assessment of bacterial uptake and transformation using plasmid-loaded nanoparticles.

(a) A distinct green-coloured bacterial pellet observed in microcentrifuge tubes, indicating successful uptake of nanoparticles by bacteria; the color is attributed to the reporter dye incorporated within the nanoparticles.

(b) LB-agar plate showing inoculation with untransformed bacteria, included as a negative control.

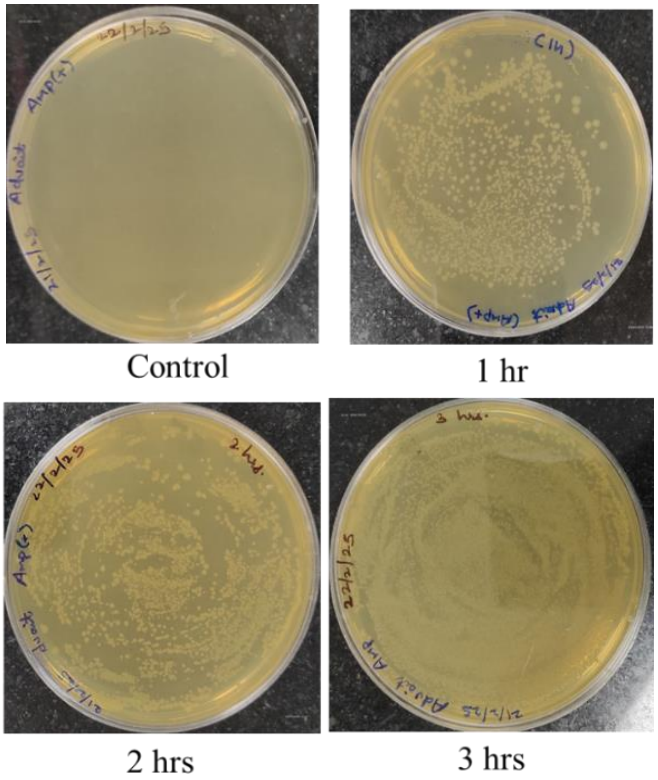
(c) Bacterial colonies on an LB-agar plate following transformation with nanoparticles loaded with plasmid pGIPZ.

(d) Bacterial colonies on an LB-agar plate following transformation with nanoparticles loaded with plasmid pET43.

The antibiotic selection plates clearly differ between the control and transformed bacterial cultures. The plate on the left, labeled with Amp(+), lacks bacterial growth, indicating that untransformed DH5α cells are unable to survive in the presence of ampicillin. In contrast, the plate on the right, where DH5α cells were transformed with the plasmid containing an ampicillin resistance gene, exhibits significant colony formation, confirming successful plasmid uptake and expression of antibiotic resistance. Numerous distinct colonies suggest efficient transformation and selection, validating the incorporation of the desired genetic material into the bacteria.

4.5.1 Time-dependent pDNA-NP mediated Bacterial Transformation

a)



b)

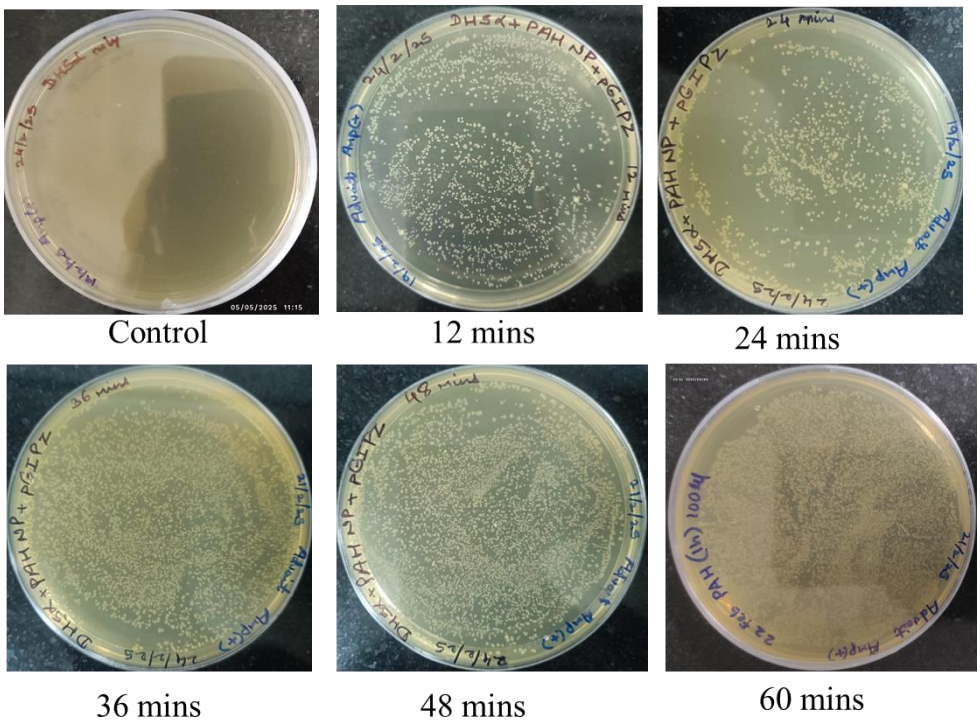


Figure 12*: *Bacterial uptake of plasmid DNA-bound nanoparticles assessed over time. (a) Following incubation with nanoparticles for 1, 2, and 3 hours, bacteria were plated on ampicillin agar to evaluate colony formation. (b) Minute-wise of pDNA-NPs by the bacteria at 12, 24, 36, 48, and 60 minutes. Untransformed bacteria served as the negative control, exhibiting no growth on selective plates in both the experiments.

To assess the time-dependent uptake of plasmid DNA (pDNA) facilitated by nanoparticle (NP) carriers, a comparative plating experiment was performed on ampicillin-containing LB agar plates. In this experiment, the plasmid pGIPZ, which carries an ampicillin resistance gene, was used. As depicted in Figure 12 a), the first plate acted as a negative control, where only untreated bacterial cells those lacking pDNA-bound NPs were spread. No colonies were observed on this plate, confirming that the bacterial strain employed was sensitive to ampicillin and did not have intrinsic resistance.

In contrast, the subsequent three plates represented bacterial samples treated with pDNA-loaded NPs for durations of 1 hour, 2 hours, and 3 hours, respectively, prior to plating, in an incubator-shaker, at 37°C, 220 rpm. A clear time-dependent trend in colony formation was evident, with the number of resistant colonies increasing progressively with longer incubation times. This observation indicates that the bacterial uptake of NP-bound pDNA is a gradual process, with higher transformation efficiency associated with longer exposure durations. The growing colony count over time highlights the efficiency of the NP-mediated delivery system in facilitating successful plasmid uptake and the expression of the antibiotic resistance gene.

To assess whether bacterial transformation could be achieved in less than one hour, a time-dependent uptake study of pDNA-NPs was conducted. While it was established that transformation occurs within one hour, this experiment aimed to determine the minimum incubation time required for successful plasmid uptake. A series of plates were prepared where bacterial cultures were incubated with pDNA-loaded NPs for varied durations of 12, 24, 36, 48, and 60 minutes. The first plate served as a negative control, containing bacteria without exposure to pDNA-loaded NPs, which resulted in no colony formation, confirming the bacterial strain's sensitivity to ampicillin.

For the other plates, bacterial samples were incubated in an incubator-shaker set at 37°C and 220 rpm, with a 12-minute interval between each time point [Figure 12b)]. A progressive increase in colony numbers was observed as the incubation time was extended. Remarkably, colonies were visible even after only 12 minutes of incubation, demonstrating that the NP-based delivery system facilitates rapid and efficient plasmid uptake. This finding highlights the potential of the method to achieve bacterial transformation well within one hour, thereby significantly reducing the time required for genetic modification. The plasmid used in this study, pGIPZ,

carries the ampicillin resistance gene, and the clear growth of colonies confirms successful transformation mediated by the nanoparticle delivery system.

4.5.2 pDNA concentration-dependent Bacterial Transformation

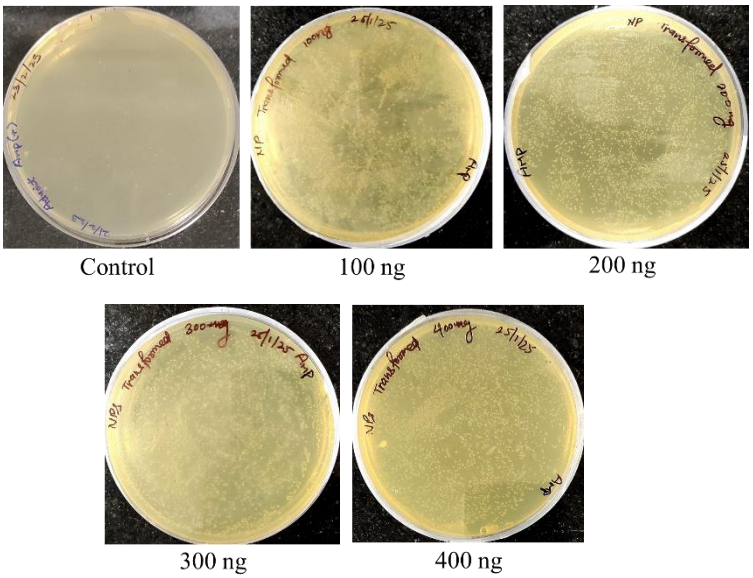


Figure 13: Colony formation of bacteria incubated with varying amounts (100–400 ng) of plasmid DNA. Bacteria were plated on selective agar after incubation with the pDNA-NPs. Untransformed bacteria were included as the negative control.

In another experiment, the impact of varying pDNA amounts on bacterial transformation efficiency was evaluated. The first plate again acted as the control, where only the bacteria were spread, without the addition of pDNA-fused NPs, resulting in no colony formation. The subsequent four plates contained bacteria incubated with pDNA-loaded NPs, with different amounts of pDNA (100, 200, 300, and 400 ng) used in each plate. Remarkably, even the lowest amount of pDNA, 100 ng, resulted in a considerable number of colonies, demonstrating the effectiveness of the

NP-mediated delivery system with minimal pDNA input. This experiment, performed using the plasmid pGIPZ, which carries the ampicillin-resistance gene, highlights the potential of this method to facilitate bacterial transformation with a relatively low dose of plasmid DNA.

Chapter 5

Conclusion and Scope of Future Work

5.1 Conclusion

The findings presented in this study collectively establish the potential of nanoparticle-mediated transformation as an efficient method for introducing plasmid DNA into bacterial systems. Beginning with the successful isolation of two plasmids, pGIPZ and pET43, the quality of the extracted DNA was verified through agarose gel electrophoresis. Both plasmids displayed distinct, well-defined bands, primarily in the supercoiled form, alongside minimal smearing or contamination, indicating high purity and structural integrity. The absence of degraded DNA or RNA further confirmed the reliability of the isolation protocol and the suitability of the plasmid samples for downstream applications.

Encapsulation of plasmid DNA into nanoparticles was achieved with notable efficiency. Using pGIPZ, a relatively large plasmid measuring 11.688 kb the encapsulation efficiency was calculated to be approximately 70%. This result highlights the ability of the nanoparticles to accommodate and stably load large genetic constructs, a feature often considered challenging in conventional delivery systems. The minimal variability observed between replicates underscores the consistency and reproducibility of the formulation process. Complementary SEM imaging revealed a measurable increase in average nanoparticle size following pDNA loading, shifting from roughly 50 nm (empty NPs) to 55 nm (pDNA-loaded NPs). This modest size increase is consistent with the successful internalization of plasmid DNA and suggests structural

accommodation within the polymeric network. Supporting this, zeta potential measurements showed a slight reduction in surface charge post-encapsulation, indicating effective electrostatic interaction between the negatively charged DNA and the nanoparticle matrix further contributing to nanoparticle stability.

The biological functionality of the pDNA-loaded nanoparticles was assessed through bacterial transformation assays using DH5α cells. Antibiotic selection on ampicillin-containing LB agar plates clearly distinguished transformed cells from controls. While untreated cells exhibited no growth, transformed cells formed numerous colonies, confirming not only uptake of the pGIPZ plasmid but also the successful expression of the encoded ampicillin-resistance gene. This outcome validated the core objective of the study, delivering functional plasmid DNA into bacterial cells using a nanoparticle-based approach.

Time-course experiments provided further insights into the kinetics of plasmid uptake. In a series of plating assays, bacterial cultures were incubated with pDNA-loaded nanoparticles for durations ranging from 12 minutes to 3 hours. A clear upward trend in colony formation was observed with increasing incubation time, indicating that the transformation efficiency improves progressively with longer exposure. Significantly, even the shortest incubation period of just 12 minutes yielded a substantial number of colonies, demonstrating the rapid and effective nature of the delivery system. A related experiment evaluated the impact of plasmid dosage, where bacteria were exposed to varying amounts of pDNA (100 to 400 ng) encapsulated in nanoparticles. Even at the lowest tested quantity (100 ng), the formation of distinct colonies was evident, reflecting the sensitivity and potency of the delivery method.

In conclusion, this work showcases a comprehensive strategy for nanoparticle-mediated plasmid delivery, covering plasmid preparation, nanoparticle formulation, physicochemical characterization, and biological validation. The ability to encapsulate and deliver large plasmids such as pGIPZ with high efficiency, combined with the system's rapid action and minimal DNA requirements, positions this method as a promising alternative to traditional transformation techniques. With further optimization and expansion, such nanoparticle-based systems may find broad application in microbial engineering, gene therapy research, and other fields requiring efficient and scalable gene delivery solutions.

5.2 Future Prospects

The present study has laid the groundwork for utilizing plasmid-encapsulated NPs as a promising alternative to conventional bacterial transformation techniques. While this approach has demonstrated encouraging potential in facilitating genetic material delivery into bacterial cells, several aspects remain unexplored and warrant further investigation. The future trajectory of this research can be shaped by addressing the following dimensions:

Firstly, optimization of particle characteristics such as surface chemistry, charge distribution, and size could significantly enhance the efficiency of gene transfer across a broader range of bacterial species. Tailoring these parameters to suit different cell types may improve the uptake and integration of plasmid DNA.

Secondly, a deeper mechanistic understanding of how these nanoparticles interact with bacterial membranes particularly during adhesion, internalization, and subsequent release of genetic cargo would provide valuable insights. Advanced imaging and molecular

tracking methods could help elucidate these interactions at the nanoscale.

Thirdly, the present study focused on a single plasmid system. Expanding this approach to include multi-plasmid delivery or co-delivery of other functional molecules such as small RNAs or regulatory proteins could open avenues for complex gene circuitry design within bacterial systems.

Fourth, the biocompatibility and long-term effects of these particles on host physiology and growth dynamics remain to be comprehensively studied. A systematic evaluation using model and non-model organisms under various environmental conditions would establish the safety profile of this approach.

Lastly, scaling up the synthesis process and testing its feasibility in large-volume bioreactors or field applications could bring these nanoparticles closer to real-world utility.

Beyond bacterial transformation, these nanoparticles hold immense promise in a variety of future applications:

They can serve as vehicles for delivering CRISPR-associated tools to perform precise gene editing in prokaryotic systems.

In microbial biotechnology, such particles may be used to introduce metabolic pathways for enhanced production of biofuels, pharmaceuticals, or specialty chemicals.

Their utility in synthetic biology could aid in constructing programmable bacterial biosensors that respond to environmental stimuli.

These nanoparticles might enable efficient transformation of environmental isolates, facilitating metagenomic studies and exploration of unculturable microbes.

In agriculture, they may be employed to engineer plant- associated bacteria for improved nitrogen fixation or biocontrol activities.

In conclusion, plasmid-loaded nanoparticles represent a versatile and evolving platform with the potential to reshape genetic manipulation strategies in microbiology. Continued research in this domain is essential for unlocking their full potential and translating laboratory findings into practical innovations.

REFERENCES

[1] P. Hahn and E. Scanlan, “Gene Delivery into Mammalian Cells: An Overview on Existing Approaches Employed In Vitro and In Vivo,” in *Nucleic Acid Transfection*, vol. 296, W. Bielke and C. Erbacher, Eds., in Topics in Current Chemistry, vol. 296. , Berlin, Heidelberg: Springer Berlin Heidelberg, 2010, pp. 1–13. doi: 10.1007/128_2010_71.

[2] S. J. Singer and G. L. Nicolson, “The Fluid Mosaic Model of the Structure of Cell Membranes: Cell membranes are viewed as two-dimensional solutions of oriented globular proteins and lipids.,” *Science*, vol. 175, no. 4023, pp. 720–731, Feb. 1972, doi: 10.1126/science.175.4023.720.

[3] N. J. Croucher *et al.*, “Rapid pneumococcal evolution in response to clinical interventions,” *Science*, vol. 331, no. 6016, pp. 430–434, Jan. 2011, doi: 10.1126/science.1198545.

[1] P. Hahn and E. Scanlan, “Gene Delivery into Mammalian Cells: An Overview on Existing Approaches Employed In Vitro and In Vivo,” in *Nucleic Acid Transfection*, vol. 296, W. Bielke and C. Erbacher, Eds., in Topics in Current Chemistry, vol. 296. , Berlin, Heidelberg: Springer Berlin Heidelberg, 2010, pp. 1–13. doi: 10.1007/128_2010_71.

[2] R. A. Butow and T. D. Fox, “Organelle transformation: Shoot first, ask questions later,” *Trends Biochem. Sci.*, vol. 15, no. 12, pp. 465–468, Dec. 1990, doi: 10.1016/0968-0004(90)90299-Q.

[3] A. Bozkir and O. M. Saka, “Chitosan–DNA Nanoparticles: Effect on DNA Integrity, Bacterial Transformation and Transfection Efficiency,” *J. Drug Target.*, vol. 12, no. 5, pp. 281–288, Jun. 2004, doi: 10.1080/10611860410001714162.

[6] M. Mandel and A. Higa, “Calcium-dependent bacteriophage DNA infection,” *J. Mol. Biol.*, vol. 53, no. 1, pp. 159–162, Oct. 1970, doi: 10.1016/0022-2836(70)90051-3.

[7] S. N. Cohen, A. C. Y. Chang, and L. Hsu, “Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of *Escherichia*

coli by R-Factor DNA," *Proc. Natl. Acad. Sci.*, vol. 69, no. 8, pp. 2110–2114, Aug. 1972, doi: 10.1073/pnas.69.8.2110.

[8] W. J. Dower, J. F. Miller, and C. W. Ragsdale, "High efficiency transformation of E.coli by high voltage electroporation," *Nucleic Acids Res.*, vol. 16, no. 13, pp. 6127–6145, Jul. 1988, doi: 10.1093/nar/16.13.6127.

[9] P. Schaub, S. Al-Babili, R. Drake, and P. Beyer, "Why Is Golden Rice Golden (Yellow) Instead of Red?," *Plant Physiol.*, vol. 138, no. 1, pp. 441–450, May 2005, doi: 10.1104/pp.104.057927.

[10] H.-H. Hwang, M. Yu, and E.-M. Lai, "*Agrobacterium* -Mediated Plant Transformation: Biology and Applications," *Arab. Book*, vol. 15, p. e0186, Jan. 2017, doi: 10.1199/tab.0186.

[11] Q. Chen and H. Lai, "Gene Delivery into Plant Cells for Recombinant Protein Production," *BioMed Res. Int.*, vol. 2015, pp. 1–10, 2015, doi: 10.1155/2015/932161.

[12] I. Delany, R. Rappuoli, and K. L. Seib, "Vaccines, reverse vaccinology, and bacterial pathogenesis," *Cold Spring Harb. Perspect. Med.*, vol. 3, no. 5, p. a012476, May 2013, doi: 10.1101/cshperspect.a012476.

[13] E. F. Fynan, R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson, "DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations.," *Proc. Natl. Acad. Sci.*, vol. 90, no. 24, pp. 11478–11482, Dec. 1993, doi: 10.1073/pnas.90.24.11478.

[14] A. Froger and J. E. Hall, "Transformation of plasmid DNA into E. coli using the heat shock method," *J. Vis. Exp. JoVE*, no. 6, p. 253, 2007, doi: 10.3791/253.

[15] H. Potter, “Transfection by electroporation,” *Curr. Protoc. Mol. Biol.*, vol. Chapter 9, p. Unit 9.3, May 2003, doi: 10.1002/0471142727.mb0903s62.

[16] C. Johnston, B. Martin, G. Fichant, P. Polard, and J.-P. Claverys, “Bacterial transformation: distribution, shared mechanisms and divergent control,” *Nat. Rev. Microbiol.*, vol. 12, no. 3, pp. 181–196, Mar. 2014, doi: 10.1038/nrmicro3199.

[17] I. Draskovic and D. Dubnau, “Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds,” *Mol. Microbiol.*, vol. 55, no. 3, pp. 881–896, Feb. 2005, doi: 10.1111/j.1365-2958.2004.04430.x.

[18] J. M. Mann, V. J. Carabetta, I. M. Cristea, and D. Dubnau, “Complex formation and processing of the minor transformation pilins of *Bacillus subtilis*,” *Mol. Microbiol.*, vol. 90, no. 6, pp. 1201–1215, Dec. 2013, doi: 10.1111/mmi.12425.

[19] R. Laurenceau *et al.*, “A Type IV Pilus Mediates DNA Binding during Natural Transformation in Streptococcus pneumoniae,” *PLoS Pathog.*, vol. 9, no. 6, p. e1003473, Jun. 2013, doi: 10.1371/journal.ppat.1003473.

[20] C. D. Johnston *et al.*, “Systematic evasion of the restriction-modification barrier in bacteria,” *Proc. Natl. Acad. Sci.*, vol. 116, no. 23, pp. 11454–11459, Jun. 2019, doi: 10.1073/pnas.1820256116.

[21] J. Majewski, P. Zawadzki, P. Pickerill, F. M. Cohan, and C. G. Dowson, “Barriers to genetic exchange between bacterial species: Streptococcus pneumoniae transformation,” *J. Bacteriol.*, vol. 182, no. 4, pp. 1016–1023, Feb. 2000, doi: 10.1128/JB.182.4.1016-1023.2000.

[22] C. N. Johnson, E. K. Sheriff, B. A. Duerkop, and A. Chatterjee, “Let Me Upgrade You: Impact of Mobile Genetic Elements on Enterococcal

Adaptation and Evolution," *J. Bacteriol.*, vol. 203, no. 21, pp. e00177-21, Oct. 2021, doi: 10.1128/JB.00177-21.

[23] F. Lebreton *et al.*, "Emergence of Epidemic Multidrug-Resistant Enterococcus faecium from Animal and Commensal Strains," *mBio*, vol. 4, no. 4, pp. e00534-13, Aug. 2013, doi: 10.1128/mBio.00534-13.

[24] M. S. Gilmore, D. B. Clewell, Y. Ike, and N. Shankar, Eds., *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. Boston: Massachusetts Eye and Ear Infirmary, 2014. Accessed: Apr. 28, 2025. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK190424/

[25] J. A. Baker, F. Simkovic, H. M. C. Taylor, and D. J. Rigden, "Potential DNA binding and nuclease functions of ComEC domains characterized *in silico*," *Proteins Struct. Funct. Bioinforma.*, vol. 84, no. 10, pp. 1431–1442, Oct. 2016, doi: 10.1002/prot.25088.

[26] A. Pandey, A. Devkota, A. Sigdel, Z. Yadegari, K. Dumenyo, and A. Taheri, "Citric acid/β-alanine carbon dots as a novel tool for delivery of plasmid DNA into E. coli cells," *Sci. Rep.*, vol. 11, no. 1, p. 23964, Dec. 2021, doi: 10.1038/s41598-021-03437-y.

[27] H. Tan, L. Fu, and M. Seno, "Optimization of Bacterial Plasmid Transformation Using Nanomaterials Based on the Yoshida Effect," *Int. J. Mol. Sci.*, vol. 11, no. 12, pp. 4962–4972, Dec. 2010, doi: 10.3390/ijms11124962.

[28] N. Yoshida, "Discovery and Application of the Yoshida Effect: Nano-Sized Acicular Materials Enable Penetration of Bacterial Cells by Sliding Friction Force," *Recent Pat. Biotechnol.*, vol. 1, no. 3, pp. 194–201, Nov. 2007, doi: 10.2174/187220807782330147.

[29] A. Bozkir and O. M. Saka, "Chitosan–DNA Nanoparticles: Effect on DNA Integrity, Bacterial Transformation and Transfection Efficiency,"

J. Drug Target., vol. 12, no. 5, pp. 281–288, Jun. 2004, doi: 10.1080/10611860410001714162.

[30] K. Deshmukh, S. R. Ramanan, and M. Kowshik, "Novel one step transformation method for Escherichia coli and Staphylococcus aureus using arginine-glucose functionalized hydroxyapatite nanoparticles," *Mater. Sci. Eng. C*, vol. 96, pp. 58–65, Mar. 2019, doi: 10.1016/j.msec.2018.10.088.

[31] X. Xu *et al.*, "Electrophoretic Analysis and Purification of Fluorescent Single-Walled Carbon Nanotube Fragments," *J. Am. Chem. Soc.*, vol. 126, no. 40, pp. 12736–12737, Oct. 2004, doi: 10.1021/ja040082h.

[32] N. W. S. Kam, Z. Liu, and H. Dai, "Functionalization of Carbon Nanotubes via Cleavable Disulfide Bonds for Efficient Intracellular Delivery of siRNA and Potent Gene Silencing," *J. Am. Chem. Soc.*, vol. 127, no. 36, pp. 12492–12493, Sep. 2005, doi: 10.1021/ja053962k.

[33] R. Krajcik, A. Jung, A. Hirsch, W. Neuhuber, and O. Zolk, "Functionalization of carbon nanotubes enables non-covalent binding and intracellular delivery of small interfering RNA for efficient knock-down of genes," *Biochem. Biophys. Res. Commun.*, vol. 369, no. 2, pp. 595–602, May 2008, doi: 10.1016/j.bbrc.2008.02.072.

[34] Z. Liu *et al.*, "Drug Delivery with Carbon Nanotubes for *In vivo* Cancer Treatment," *Cancer Res.*, vol. 68, no. 16, pp. 6652–6660, Aug. 2008, doi: 10.1158/0008-5472.CAN-08-1468.

[35] H.-H. Perkampus, *UV-VIS Spectroscopy and Its Applications*. in Springer Lab Manuals Ser. Berlin, Heidelberg: Springer Berlin / Heidelberg, 1992.

[36] J. Stetefeld, S. A. McKenna, and T. R. Patel, "Dynamic light scattering: a practical guide and applications in biomedical sciences,"

Biophys. Rev., vol. 8, no. 4, pp. 409–427, Dec. 2016, doi: 10.1007/s12551-016-0218-6.

[37] "FavorPrep™ Plasmid Fast DNA Extraction Kit," FAVORGEN BIOTECH CORP. Accessed: May 01, 2025. [Online]. Available: https://www.favorgen.com/en/product.php?act=view&cid=149&id=4

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