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Research Article

Effect of Conjugated siRNA-Gold Nanoparticles on antibiotic-resistance Integron class I Gene

"Reduce antibiotic resistance by siRNA"

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ABSTRACT

Background: This study explores the application of gold nanoparticles (AuNPs) as a delivery tool for nucleic acids, specifically small interfering RNA (siRNA), in genetic engineering to achieve gene silencing. **Methods:** This study act on utilizes commercially available AuNPs, characterizing them through techniques such as Field Emission Scanning Electron Microscopy and X-ray diffraction. The study focused on the impact of these AuNPs on delivering siRNA to bacterial cells, particularly *Pseudomonas aeruginosa*, without the need for additional conjugation compounds. **Results:** The results show the AuNPs were homogeneously shaped with smaller-sized particles exhibiting a nearly spherical form, and their size increased from 5nm to larger sizes. Various concentrations of AuNPs did not affect the viability of *P. aeruginosa*. The efficiency of conjugation was confirmed using gel documentation, and the silencing of the antibiotic resistance integron gene (intI) in *P. aeruginosa* was assessed through a plating method. The results demonstrated a high

transformation frequency, with a significant percentage (100%) of colonies showing complete transformation from resistance to sensitivity. **Conclusion:** The findings indicated the potential of using AuNPs as a delivery system a promising approach for gene silencing in genetic engineering, particularly for combating antibiotic resistance in bacteria.

Keywords

gold nanoparticles; siRNA; gene-editing; gene silencing; antibiotic resistance; integron class I gene

INTRODUCTION

The concept of "gene silencing delivery systems" refers to the techniques employed for transporting short-interference RNA (siRNA) to specific locations to achieve efficient and targeted suppression of gene expression. Various delivery techniques can be employed to achieve gene silencing, including viral vectors, non-viral vectors, antibody-based delivery, dual targeting systems, nanotechnology-based systems, particular ligands, and combination therapies. These methods maximize siRNA delivery to target cells and protect siRNA from degradation. Nevertheless, there exist some obstacles related to the use of siRNA, including transport mechanisms, stability concerns, off-target effects, specificity issues, and potency limitations [1]. One of the main challenges in the field of RNAi-based therapy lies in the development of a delivery method that accomplishes all the requirements for clinical applicability [2]. The important role of delivery systems in facilitating gene silencing in bacteria lies in their ability to provide efficient and accurate delivery of siRNA molecules to the targeted bacterial cells. The utilization of nanoparticle-conjugated siRNA

is a highly promising strategy for inducing gene silencing, due to its numerous advantages, including simplicity of manufacture and chemical characterization, large packaging capacity, lack of immunogenicity, and potential for tissue selectivity. Nanoparticles have garnered considerable interest as non-viral gene transfer vectors. This has positioned them as a viable alternative to the widely utilized viral vectors [3]. Nanoparticles can protect siRNA from degradation and enhance its transportation to specific cellular targets [4]. Various types of nanoparticles are employed for the delivery of siRNA, encompassing lipid-based nanoparticles, polymer-based nanoparticles, gold nanoparticles, and iron oxide nanoparticles. The conjugation of nanoparticles with siRNA can be achieved through either covalent bonding or electrostatic interactions. The utilization of nanoparticles for the transport of siRNA has demonstrated encouraging outcomes in the field of cancer therapy, with several siRNA-based pharmaceuticals having passed evaluation in clinical studies [5, 6]. There are numerous potential biological and biomedical uses for metal-based nanoparticles, including gold, silver, platinum, iron oxide, quantum dots, etc. They are presently being thoroughly studied to take advantage of the qualities that make them a viable option for future clinical applications [7, 8]. Gold nanoparticles possess distinctive optical properties, as well as simple production and surface modification capabilities. They may be selectively and cooperatively coated with nucleic acids using either covalent or non-covalent conjugation methods [9, 10]. The covalent attachment of nucleic acid strands to gold nanoparticle cores, commonly between 13 and 15 nm in size, is achieved through the utilization of thiol moieties [11, 12]. This method is applied to DNA and siRNA, which can be attached directly to gold cores or to gold cores that have been polymerized. It has been

demonstrated that coating nanoparticles with hydrophilic molecules like polyethylene glycol (PEG) reduces immunological activation and extends circulation time as a common anti-fouling technique [13,14]. Ligand density, hydrophobicity, avidity, and length must all be considered and tuned for effective nanoparticle targeting [15, 16]. Another important feature of nanoparticles is their size; researchers have discovered that they work best when they are between 100 and 250 nm in diameter [13]. Therefore, this study aims to study the main character and conjugation efficiency of gold nanoparticles to siRNA be used as delivery system in genetic experiment.

MATERIAL AND METHODS

Characterization of gold nanoparticles (AuNPs)

Gold Colloidal nanoparticles with a size range: 5-20 nm diameter, weight concentration 100 ppm with red color and spherical morphology were supplied from Via Carbon Nano Materials (VCN) Co. Ltd. (Iran). It was used as a delivery system for siRNA to bacterial cells. To test the structural, optical characteristics, and conjugation ability of AuNPs, A variety of tests were applied, as outlined below:

High-Resolution Scanning Electron Microscopy using Field Emission (FESEM)

The FESEM technique was employed to investigate the morphological features and microstructure characterization of the AuNPs. In the FESEM technique, the beam of electrons is focused on the material/s under test for image generation through surface scanning. The atoms of the material/s interact with the electron beam, which results in a

single electron generation, which leads to obtaining some composition information and surface morphology [17].

X-Ray Diffraction (XRD)

The XRD analysis technique was used in this study to evaluate the physical properties, composition, crystal structure, and phase recognition of the AuNPs used. XRD uses the remarkable interaction between monochromatic X-rays and crystalline samples. These monochromatic X-rays are created by a cathode ray tube and filtered. The radiation is then precisely focused on the sample. The interaction between the sample and incident rays generates positive interference (and a diffracted ray) under appropriate conditions. Line broadening in the XRD pattern indicates nanoparticles [18].

Monitoring of Cell Viability after treatment with AuNPs by oxidation-reduction assay

Minimum inhibitory concentration of gold nanoparticles against bacterial cultures was analyzed by using Resazurin dye in 96-well plates (7-hydroxy-3H-phenoxazine-3-one 10-oxide). When resazurin is chemically decreased by aerobic respiration generated by cell growth, it transforms from blue and non-fluorescent to pink and highly fluorescent, signifying cell viability. The *P. aeruginosa* cultures were diluted to 5x105 CFU/ml overnight. In successive columns of a microtiter plate, the gold nanoparticles were diluted 1:2 in LB broth from a starting concentration of 100 ppm to an end concentration of 3.125 ppm. In each well, 100 µL of the diluted bacteria and 100 µL of the gold nanoparticle solution were thoroughly mixed with seven replicates of each AuNP concentration (100, 50, 25, 12.5, 6.25, and 3.125 ppm). The experiment includes two control groups: 100 µL of LB broth

without cells and $5x10^5$ CFU/ml heat-killed cells. The plates were then incubated at 37°C for 24 hours. After incubation, a 0.015% resazurin solution was added to each well at a volume of 20 µL., and further incubated for 2–4 h for the observation of color change. On completion of the reduction of resazurin to resorufin, indicate the presence of a live cell [19].

Conjugation of Gold nanoparticles to siRNA modified method

Conjugated gold nanoparticles with designed siRNA were employed to induce silence to the targeted gene (ex, integron gene intI) of *Pseudomonas aeruginosa*. The conjugation efficiency was optimized by preparing different concentrations of sodium chloride (NaCl) (3, 2, 1.5, 1, and 0.5 M).

The experimental procedure involved the following steps:

1. Preparation of Gold Nanoparticles**:**
    - 3 ml of gold nanoparticles were mixed with 2 ml of 3M NaCl in a 10 ml tube.
    - The mixture was incubated in a shaker incubator for 30 minutes at 60°C.
2. Addition of siRNA:
    - 20 µL of siRNA, prepared by dissolving in nuclease-free water to achieve a final concentration of 100 picomol/µL, was added.
    - The tube was returned to the shaker incubator for an additional 10 minutes.
3. Incremental Addition of NaCl:
    - 2000 µL of residual NaCl concentrations were added incrementally under the same incubation conditions:
        - 500 µL of 0.5 M NaCl (added in 250 µL portions every 30 minutes).
        - 500 µL of 1 M NaCl (added in 250 µL portions every 30 minutes).

- 500 µL of 1.5 M NaCl (added in 250 µLportions every 30 minutes).
- 500 µL of 2 M NaCl (added in 250 µL portions every 30 minutes).

4. Detection of Conjugation by Gel Electrophoresis:
    - Gold-siRNA conjugates were subjected to electrophoresis on 1.5% agarose gels.
    - Electrophoresis was conducted for one hour at 100 V.
    - Gels with conjugated gold nanoparticles, unconjugated gold nanoparticles, and free DNA of different lengths were run.
    - Visualization of siRNA conjugated to gold nanoparticles was achieved using a gel imaging system, with the position of loading the conjugated gold nanoparticles reversed from anode to cathode poles.

This approach allowed for the assessment of the efficiency of gold nanoparticle-siRNA conjugation. The use of gel electrophoresis facilitated the visualization and confirmation of successful conjugation [20].

Silencing of the intI gene by conjugated siRNA

The experiment involves silencing the intI gene in *P. aeruginosa* isolates using conjugated siRNA. The process begins by culturing the bacterial isolates on cetrimide agar plates and incubating them overnight at 37°C. Bacterial suspensions are then prepared in Brain Heart Infusion Broth (BHIB) from the plate by taking a loopful. After a 24-hour incubation at 37°C, 200 µL of bacterial suspension adjusted to 0.5 McFarland is inoculated into 7020 µL of a conjugated salt solution. This solution was prepared in advance, and the

entire mixture was incubated in a shaker incubator for 24 hours at 37°C, as determined by previous experiments to be the optimal conditions for gene silencing.

Replica plate method to Study of effect of gene silencing on antibiotic resistance

The process involves culturing a loopful of a silencing suspension on a cetrimide agar plate to obtain single colonies through the streaking plate method, followed by an 18-hour incubation at 37°C. Identification of these cells is achieved by replica-plating colonies to three MH agar plates: one master plate without antibiotics and others with varying types and concentrations of antibiotics. The selected antibiotics are added according to CLSI 2021 guidelines [21], considering the antibiotic resistance of isolates before silencing. A grid petri dish is used for MH agar plates, and single colonies are transferred using a sterile toothpick, spotted in the center of squares on both master and antibiotic plates, then incubated at 37°C until colonies form. Silencing frequency is determined by counting colonies inhibited on an antibiotic-containing MH agar plate.

Antibiotic susceptibility test by Disk diffusion method

The disk diffusion method (modified Kirby–Bauer) was used for further susceptibility testing on Mueller–Hinton agar. Inocula were prepared in sterile saline and adjusted to 0.5 McFarland (≈1.5 × 10^8 CFU/ml). Plates were uniformly swabbed and antibiotic disks were applied using sterile forceps. After incubation at 37°C for 18–24 hours, inhibition zone diameters were measured in millimeters. Results were interpreted according to CLSI (2021) guidelines.

Statistical Analysis.

The statistical software SPSS v. 24.0 was used to program the current data. The Pearson-Chi-square test was performed to identify significant variations within the parameters, which were observed as frequencies and percentages. It was determined that $P \leq 0.05$ was significant.

RESULTS AND DISCUSSION

Characterization of gold nanoparticles (AuNPs)

Gold nanoparticle (AuNPs) was identified in the Chemistry Analysis Center (CAC), Baghdad, Iraq, by using the following tests as a confirmatory step to verify the size, shape, and purity of the used nanoparticles.

High-Resolution Scanning Electron Microscopy using Field Emission (FESEM)

On the basis of the surface investigation, topographical analysis has been carried out and FESEM pictures were taken. The gold nanoparticles were homogenous, as seen by the FESEM pictures (Fig. 1). Other than that, it was found that particles are nearly spherical in shape at lesser sizes and get larger as they get closer to 5 nm. AuNP morphology is thought to be a crucial feature that influences cellular uptake; research has shown that spherical GNPs have a higher rate and extent of cellular uptake than rod-shaped ones. Size also plays a significant role in determining the GNPs' half-life. [9].

X-Ray Diffraction (XRD)

The XRD technique is used for determining the crystalline structure and also the grain size of nanoparticles. The diffraction patterns acquired for the AuNPs as shown in Fig.2. The XRD results show the peak crystallinity of gold nanoparticles, which is typical at 2 thetas between 10^0 -80^0. Diffraction angles of 31.67^0 and 45.41^0 is a form of face-centered cubic (FCC) crystal which has a value of hkl (111) and (200). This data corresponds to JCPDS No. 01-089-3697

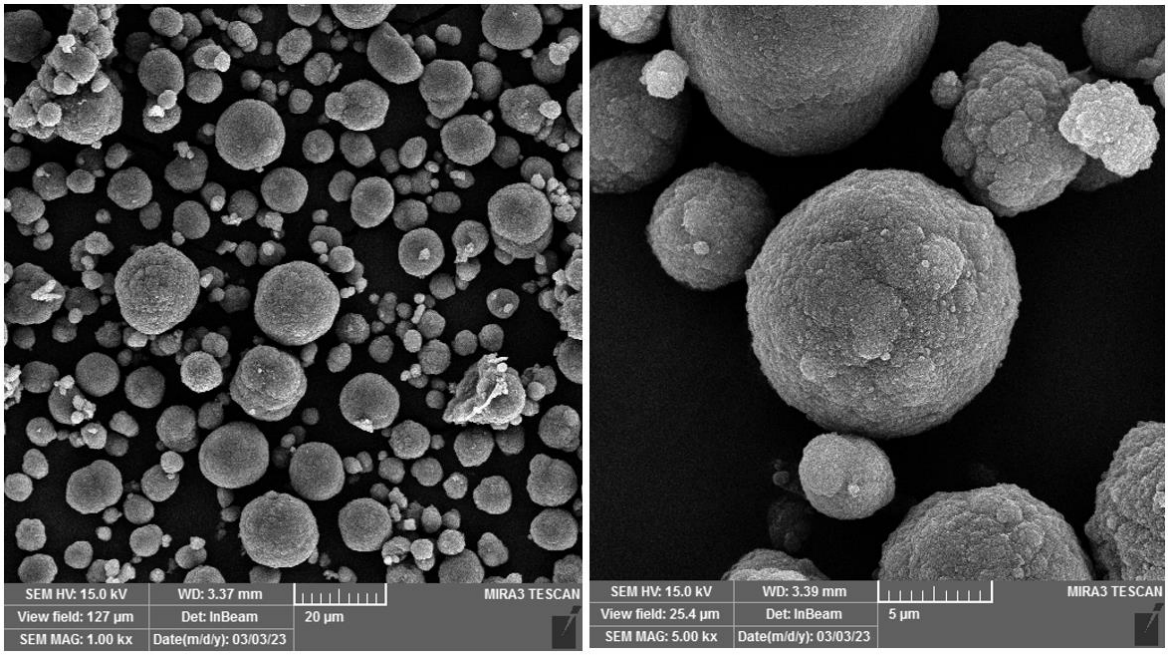

Fig.1. FE-SEM Image of gold Nanoparticles

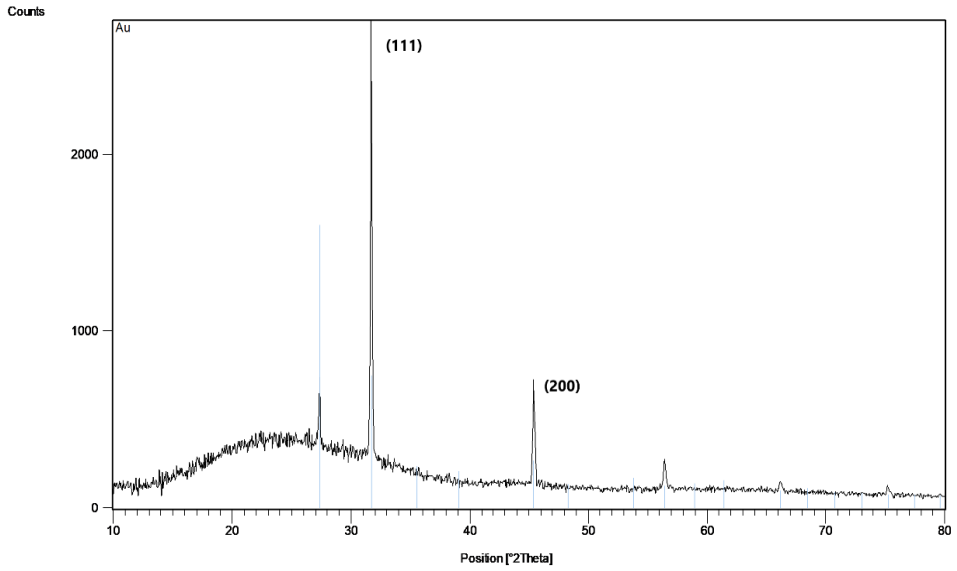

Fig.2. The gold nanoparticles' X-ray diffraction pattern.

Cell Viability after treatment with gold nanoparticles

An oxidation-reduction experiment was used to assess the viability of *P. aeruginosa* exposed to different AuNP treatments. Cell viability was indicated using the redox-sensitive dye resazurin. The non-fluorescent blue resazurin is converted to fluorescent red resorufin by metabolically active cells. Resazurin cannot be reduced by non-living cells, which is a sign of cell death. The cells are viable based on this obvious change in color and fluorescence. AuNPs were added to freshly cultured and diluted bacterial cultures in LB broth (about 5x105 CFU/ml) at concentrations between 100 and 3.125 ppm. The outcome shows that the vitality of *P. aeruginosa* is unaffected by changing AuNP concentrations, with seven replicates for each isolate (Fig. 3).

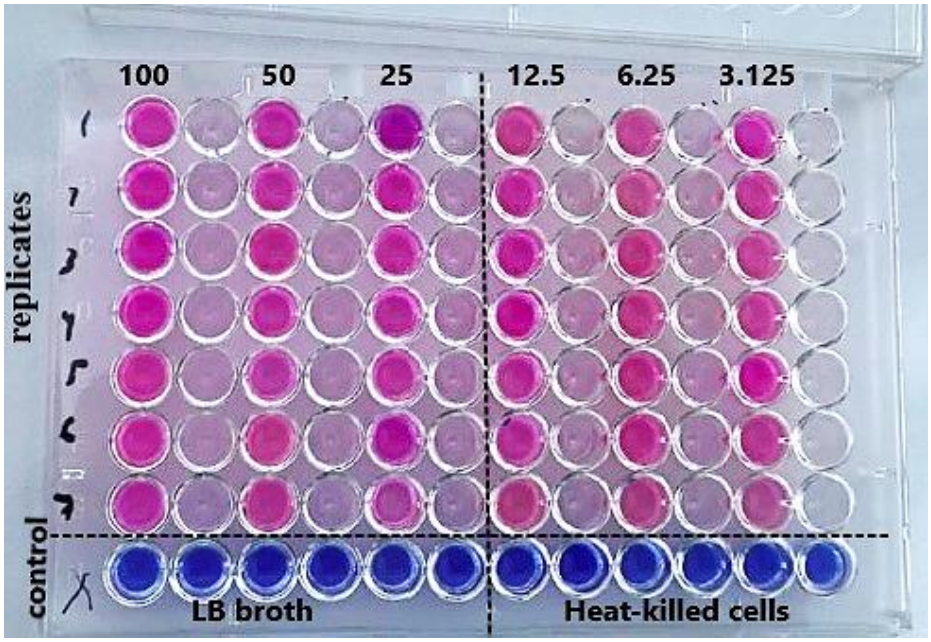

Fig.3. Effect of AuNPs on *P. aeruginosa* isolates viability.

It is not recognized that AuNPs possess intrinsic antibacterial properties. Au is an extremely uncommon, inactive, and non-toxic metal to microorganisms [22, 23]. Gold is a versatile metal that may bind a wide variety of ligands. The antibacterial activity of nanoparticles (NPs) depends on their size. The specific antibacterial mechanism of action of larger NPs has not been fully understood; an increase in bacterial cell membrane tension results from the adsorption of large NPs, which causes the membrane to mechanically change, ultimately causing cell rupture and death [24, 25].

Detection of the conjugation of gold nanoparticles to siRNA by gel electrophoresis

Binding siRNA to gold nanoparticles offers several advantages, including enhanced cellular uptake, protection of siRNA from degradation, and the potential for targeted delivery [26]. However, the process requires careful design and optimization to ensure effective

binding, delivery, and gene silencing outcomes. Employing a gel electrophoresis experiment, siRNA-gold nanoparticle conjugates were carefully investigated. Gels with conjugated siRNA-AuNPs appeared as a fluorescent band in gel wells, unconjugated gold nanoparticles migrated in the opposite direction to normal DNA migration, while free siRNA with a very small size cannot be recognized. The red safe stained bands of the siRNA-AuNPs were directly visualized by gel imaging system, Fig.4. A vast array of biomolecules, such as DNA, RNA, proteins, peptides, medicines, genes, and other molecules of therapeutic importance, can be targeted, intracellularly trafficked, and delivered by gold nanoparticles, which are remarkable molecular transporters. Because of their physicochemical characteristics, they do not significantly cause cytotoxicity [27, 12]. Several research groups have developed ways for functionalizing gold and other nanoparticles utilizing oligonucleotides, either alone or with modifications [20].

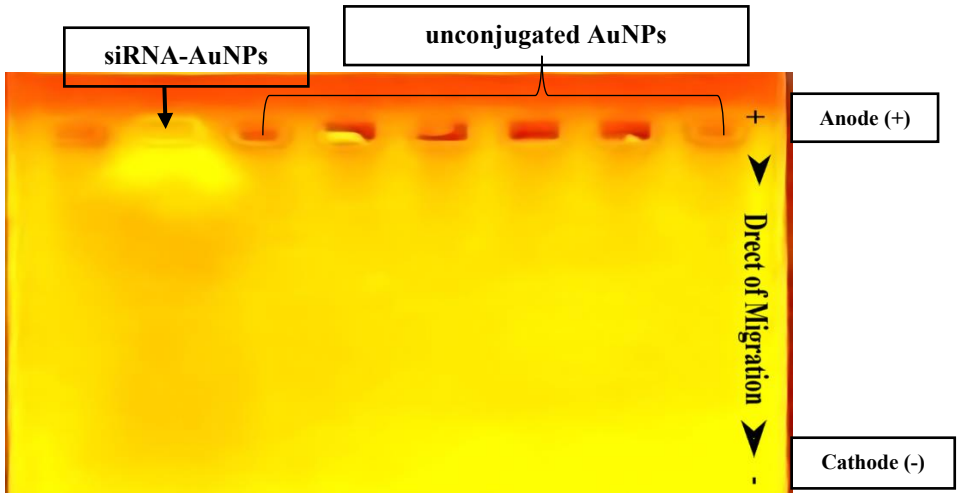

Fig.4. Agarose gel electrophoresis of siRNA-AuNPs conjugates on 1.5 % agarose gels stained with red safe stain and run for one hour at 100 V. The conjugated siRNA-AuNPs mostly stayed in the gel wells and showed clear fluorescent signals, marked by arrows. This reflects the larger size and slower movement of the nanoparticle-siRNA complexes through the gel. In contrast, the unconjugated gold nanoparticles moved in the opposite direction compared to standard DNA migration because of their surface charge.

Frequency of the transformed *Pseudomonas aeruginosa* silenced by siRNA-AuNPs

The process involves attaching siRNA molecules to the surface of AuNPs, which act as carriers to protect and transport the siRNA to the target cells. Micro gold nanoparticles can enter cells by attaching to pore proteins and entering through the porin. The siRNA-AuNP conjugates are introduced to target cells, and the AuNPs protect the siRNA from deterioration, promote endosomal escape of the siRNA into the cytoplasm, and promote in cellular uptake through endocytosis. Targeting of the intI gene of *P. aeruginosa* to determine whether the system can induce gene silencing efficiently. Very little research has gone on such an approach up to this point. The replica plate method was used to determine if variant colonies growing within the antibiotic, which showed high resistance to it before treatment with siRNA-AuNPs. This technique helps us in selecting bacterial cells that are subjected to silencing and determining the frequency of transformation. In this technique, we selected five isolates for testing the efficiency of siRNA to inhibit the intI gene (P.A6, P.A11, P.A24, P.A32, P.A61), to study the effect of silencing phenotypically, the original layout of bacterial colonies is preserved when they are moved from one plate to another.

The results of silencing the intI gene showed the disappearance of the colony when incubated in the presence of antibiotics (AK and CE) (Fig. 5). In contrast to this, the same colony was present on the master plate, which was free from antibiotics. The transformation frequency was higher with amikacin when the result showed that colonies of isolates (P.A 6, P.A 32, P.A 61) were completely (100%) transformed from resistance to sensitivity, while isolate P. A 24% showed (98%) of the colonies transformed to sensitive in contrast, 100% of the colonies in isolate P.A11 remained resistant to amikacin. Plating method also showed

that the frequency of transformed colony on the plate with Ceftazidime was less than what appeared with the Amikacin plate, and the transformation frequency ranged from (2%-10%) as illustrated in Table 1. This phenotype alteration was transient, and the silencing was forfeited upon subculturing. Results of this study showed there is a very highly significant difference ($P<0.001$***) between transformed (resistance) and non-transformed colony (resistance). Also, we note that Pre-treatment with siRNA dramatically reduced the load of bacteria, where all the colonies showed poor growth on antibiotic plates. This can be attributed to the effect of siRNA on bacterial cells. These results are similar to those obtained before by [28]. This can be a spotlight on using siRNA to prevent or eliminate bacterial infections. The rise in antibiotic-resistant bacterial pathogens not only makes it more difficult to treat infectious diseases and places a financial strain on the healthcare system, but it also makes medical procedures that rely on infection prevention more difficult, like surgery, cancer treatment, organ transplants, dental work, and caring for premature children [29]. In order to keep up with the rapid increase in antibiotic resistance, new approaches are being explored to continue producing new medicines. Antimicrobial oligonucleotides might theoretically offer the greatest variety of efficacy while having the lowest chance of unrecoverable resistance [30]. Class 1 integrons carry many gene cassettes that confer resistance to beta-lactam and aminoglycoside antibiotics; therefore, the majority of integron-positive isolates may develop resistance to these antibacterial agents [31, 32]. Class 1 integrons harbor many genes for antibiotic resistance and play a significant role in the spread of antimicrobial resistance in medical environments [33, 34].

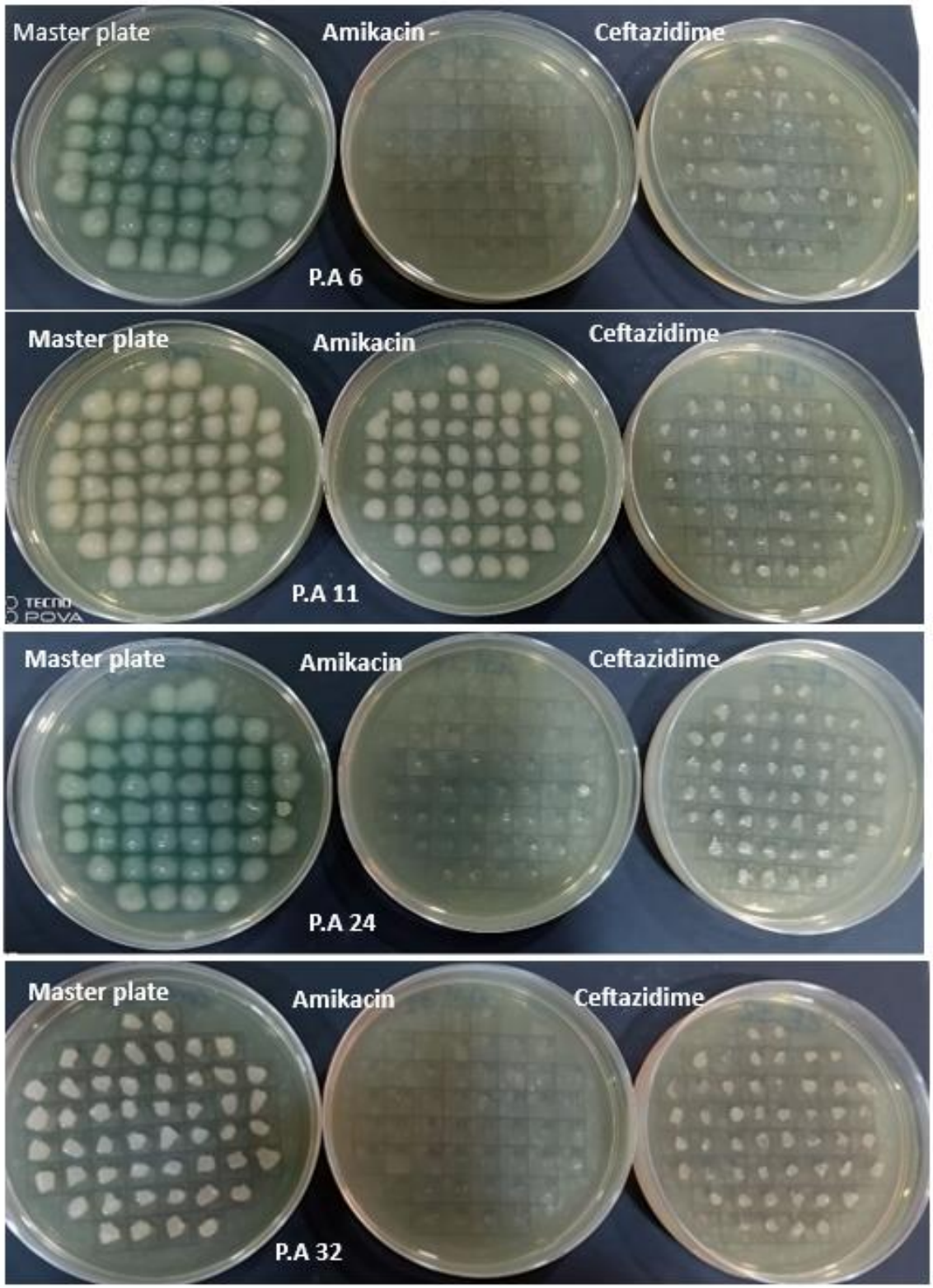

Fig.5. Replica plate method of *P. aeruginosa* isolates after silencing intI gene.

Table (1): Frequency of transformation on Replica plate after silencing intI gene

Isolate No.	Master plate	Plate with Amikacin			Plate with Ceftazidime		
	Colonies	Resistance	Sensitive	Frequency of transformation	Resistance	Sensitive	Frequency of transformation
P.A 6	50	0	50	100%	45	5	10%
P.A 11	50	50	0	0%	45	5	10%
P.A 24	50	1	49	98%	49	1	2%
P.A 32	50	0	50	100%	48	2	4%
P.A 61	50	0	50	100%	49	1	2%
P value		*P<0.001***			*P<0.001***		

Phenotypic changes associated with intI gene silencing.

Depending on the isolates that were subjected to silencing by design siRNAI, only the transformed colonies were selected from the master plate in the replica plate method. These colonies have been investigated for antibiotic sensitivity by disk agar diffusion method according to CLSI recommendation (CLSI, 2021). The antimicrobial effect of gene silencing on *P. aeruginosa* was evaluated using the agar disk diffusion method. A clear difference in susceptibility patterns was observed before and after silencing (Table 2). Post-silencing, all isolates demonstrated a marked shift from drug resistance to increased susceptibility. Specifically, the isolates, which were previously resistant to Piperacillin and showed intermediate resistance to Aztreonam, became sensitive to both antibiotics after treatment.

Additionally, their response to Amikacin improved, shifting from resistant to intermediate susceptibility (Fig 6).

Table (2): Antibiotic susceptibility before and after silencing

ISOLATE No.	Sensitivity before silencing	CAZ	FEP	TOP	CN	AK	CIP	LEV	MEM	IPM	CL	ATM	FO	P
P.A 6	**MDR**	R	R	R	R	R	S	R	S	R	S	I	R	R
P.11	PDR	R	R	R	R	R	R	R	R	R	R	I	R	R
P.A.24	**MDR**	R	R	S	R	I	S	S	R	S	S	S	R	S
P.A 32	**XDR**	R	R	S	R	R	S	R	S	R	S	R	R	R
P.A 61	**XDR**	R	R	R	R	R	S	I	S	S	R	S	R	R
ISOLATE No.	Sensitivity after silencing	CAZ	FEP	TOP	CN	AK	CIP	LEV	MEM	IPM	CL	ATM	FO	P
P.A 6	**Sensitive**	R		S	S	R	S	S	S	S	S	I	R	R
P.11	XDR	R	R	S	S	R	R	R	R	S	R	I	R	R
P.A.24	**Sensitive**	R	R	S	S	I	S	S	S	S	S	S	R	S
P.A 32	MDR	R	R	S	S	R	S	S	S	S	S	I	R	R
P.A 61	MDR	R	R	R	R	R	S	I	S	S	R	S	R	R

*CAZ: Ceftazidime, FEP: Cefepime, TOP: Tobramycin, CN: Gentamicin, AK: Amikacin, CIP: Ciprofloxacin, LEV: Levofloxacin, MEM: Meropenem, IPM: Imipenem, CL: Colistin, ATM: Aztreonam, FO: Fosfomycin, P: Piperacillin

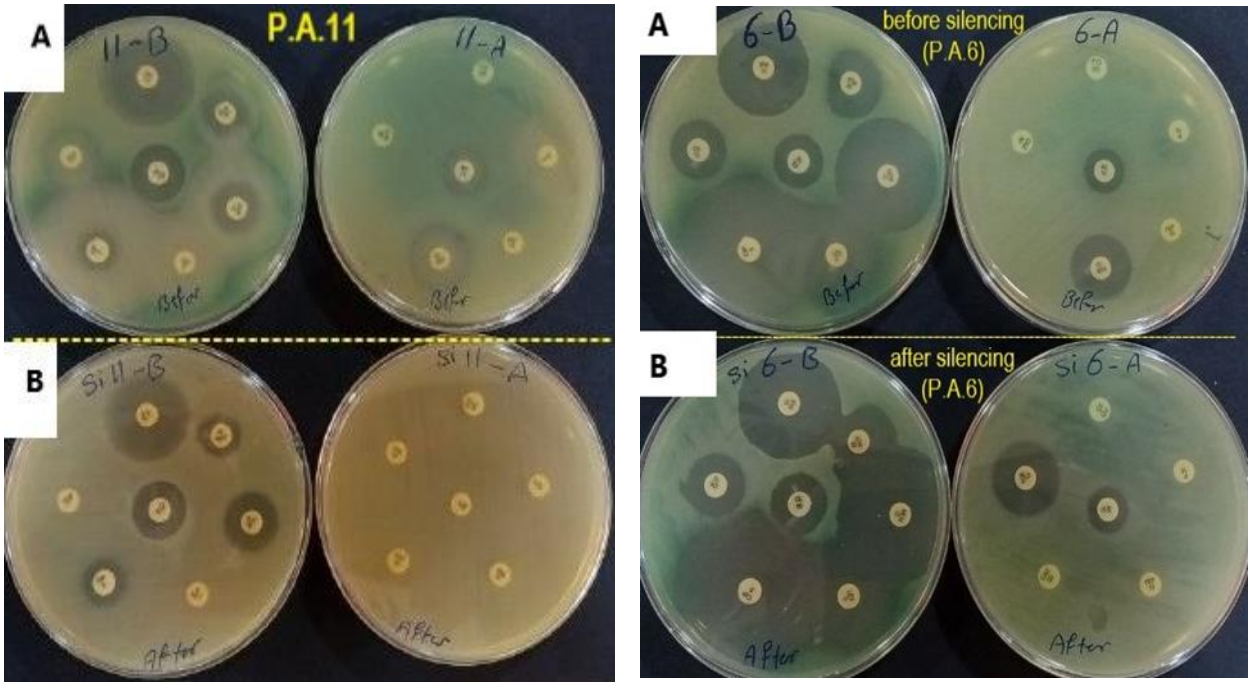

Fig.6: Phenotypic alteration by disc diffusion methods for Antibiotic susceptibility before and after silencing (A: before silencing; B: after silencing), Top two plates for isolates before silencing, and down two plates for isolates after treated with siRNA.

STUDY LIMITATIONS

The importance of including suitable controls, like gold nanoparticles alone, is recognized. In this study, cell viability assays was conducted after treating with AuNPs, and did not observe significant cytotoxicity or growth inhibition. This supports the biocompatibility of AuNPs in these experimental conditions. However, while these results suggest that the phenotypic changes are probably not due to general cellular toxicity, Integron gene expression was not directly assessed. Accordingly, an AuNP-only control for integron expression was not included, which is acknowledged as a limitation. Therefore, while the increase in antibiotic sensitivity aligns most closely with siRNA-mediated effects,

Accordingly, the potential role of AuNPs alone in integron regulation cannot be completely ruled out.

CONCLUSIONS

In conclusion, the results highlight the importance and efficiency of non-viral particles as effective carriers for transporting nucleic acids into bacterial cells while preserving cell viability. The findings suggest the promising capability of delivering siRNA particles to bacterial cells. The transformation from antibiotic-resistant to antibiotic-sensitive colonies underscores the crucial role of particles as reliable carriers for nucleic acid molecules, offering protection against enzymatic degradation.

List of Abbreviations

Abbreviation	Definition
°C	Degrees Celsius
µl	Microliter
AK	Amikacin
AuNPs	Gold nanoparticles
BHIB	Brain Heart Infusion Broth
CAC	Chemistry Analysis Center
CE	Ceftazidime
CFU	Colony-forming unit
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic acid
FCC	face-centered cubic
FESEM	Field Emission Scanning Electron Microscopy
GNB	Gram-negative bacteria
GNPs	Gold nanoparticles
IntI	Integron class 1
JCPDS	Joint Committee on Powder Diffraction Standards
LB	Luria-Bertani broth
M	Molar
MDR	multi-drug resistance
MH	Muller Hinton

MTP	Microtiter Plate Method
NaCl	sodium chloride
NPs	nanoparticles
PEG	Polyethylene glycol
ppm	Part per million
RNA	Ribonucleic Acid
SDs	Standard deviations
siRNA	small interference RNA
SPSS	Statistical Package for the Social Sciences
V	Volt
VCN	Via Carbon Nano Materials
XRD	X-Ray Diffraction

Author Contributions

All aspects of this study, including conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, visualization, writing—original draft preparation, writing, review and editing, supervision, and project administration, were carried out solely by the author. The author has read and approved the final version of the manuscript and take full responsibility for all aspects of the work.

Ethics Committee Approval

All experimental procedures and research activities related to this study were conducted in accordance with institutional and national guidelines. This research was approved by the Ministry of Health – Baghdad Al-Karkh Health Directorate, Iraq, under official approval number 211, dated 5/7/2022. The research project was registered with project number 2022293, Baghdad Al-Karkh. I confirm that all necessary ethical approvals were obtained before the commencement of the study.

Availability of Data and Materials

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Funding

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Conflicts of Interest

The author declares no conflicts of interest regarding this manuscript.

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The authors declare that ChatGPT and Grammarly were used solely for language editing (grammar and spelling) to improve the clarity of the manuscript. No AI tools were used for data analysis, interpretation, or generation of scientific content.

An online tool was used solely to improve image clarity and resolution for presentation purposes, without any modification to the scientific content of the figures. The online tool ImgUpscaler (https://imgupscaler.ai/ar/sharpen-image/) was utilized exclusively for this purpose.

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