Engineering CRISPR/Cas9-Loaded Nanocarriers for Targeted Editing of Biofilm-Regulatory Genes in Resistant Food Industry Strains with Advanced Quantitative RNA-Seq Transcriptomic Analysis

Behzad beizaei[1,*],Morteza jamshid eini[2]
[1] Master's student in Food Science and Engineering, Islamic Azad University, Tehran Science and Research Branch
[2] PhD in Food Technology, Islamic Azad University, North Tehran Branch

ABSTRACT

This comprehensive study elucidates the de novo synthesis and physicochemical fine-tuning of hierarchically engineered multilaminar nanocarrier assemblies encapsulating CRISPR/Cas9 ribonucleoprotein (RNP) complexes, devised for ultra-precise, locus-specific genomic perturbation of essential biofilm-regulatory operons within recalcitrant bacterial biofilm consortia prevalent in agro-industrial food processing ecosystems. Fabrication employed iterative layer-by-layer (LbL) polyelectrolyte self-assembly integrated with surface stealthing via polyethylene glycol (PEG) conjugation and targeting ligand functionalization to optimize endocytic uptake kinetics, circumvent intracellular endonuclease-mediated degradation, and mediate temporospatial endosomal escape culminating in efficacious cytoplasmic RNP bioavailability. Highly specific, computationally optimized single-guide RNAs (sgRNAs) were strategically designed to target conserved genetic loci responsible for exopolysaccharide (EPS) biosynthesis pathways, quorum sensing hierarchical networks, c-di-GMP signaling cascades, and multidrug resistance (MDR) efflux systems, all critically orchestrating biofilm maturation, extracellular polymeric substance (EPS) matrix robustness, and phenotypic antibiotic tolerance. Post delivery, extensive transcriptomic rewiring was quantitatively profiled via ultra-deep, strand-specific RNA sequencing (RNA-Seq), employing stringent normalization and differential expression models complemented by integrative systems biology platforms including gene set enrichment analysis (GSEA), weighted gene co-expression network analysis (WGCNA), and regulatory motif discovery to elucidate disruption of transcriptional modules underpinning biofilm resilience and antimicrobial defense. Functional validation encompassing confocal laser scanning microscopy (CLSM) and high-throughput crystal violet biofilm biomass quantification assays confirmed significant attenuation of biofilm architectural integrity, EPS matrix deposition, and MDR phenotype suppression, thereby substantiating the genomic editing efficacy. The integrative theranostic nano-genomic platform described herein exemplifies a revolutionary modality for precision microbial biofilm modulation, enabling programmable, scalable, and biosafe biocontrol interventions in industrial food production lines, advancing microbial ecology engineering frontiers, enhancing product sterility, and mitigating entrenched biofilm-driven contamination risks with far-reaching implications for next-generation antimicrobial therapeutics and food safety technologies.

Keywords: Multilayer nanocarriers, CRISPR/Cas9, biofilm resistance, RNA-Seq, gene editing, quorum sensing, antimicrobial resistance

INTRODUCTION

The pervasive formation of recalcitrant bacterial biofilms within food processing environments represents a formidable challenge to food safety and industrial hygiene, primarily due to their heightened tolerance to conventional antimicrobials and capacity for horizontal gene transfer facilitating multidrug resistance (MDR) (Donlan & Costerton, 2002; Zhang et al., 2021). Biofilms are complex, surface-adherent microbial consortia enmeshed in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, extracellular DNA (eDNA), and lipids, which together confer structural robustness and biochemical resilience (Flemming et al., 2016). The EPS matrix, alongside quorum sensing (QS) regulated genetic circuits and cyclic-di-GMP intracellular signaling, orchestrates biofilm maturation, phenotypic heterogeneity, and antimicrobial recalcitrance (Römling et al., 2013; Hengzhuang et al., 2021). Given the inadequacy of traditional disinfectants to eradicate mature biofilms—resulting from impaired penetrance and phenotypic tolerance—novel, molecularly targeted strategies are imperative (Bridier et al., 2015).

The CRISPR-Cas9 system, an adaptive prokaryotic immune mechanism, has emerged as a revolutionary genome editing tool capable of precise, locus-specific double-strand breaks (DSBs) facilitated by programmable single-guide RNAs (sgRNAs), enabling targeted gene disruption (Jinek et al., 2012). Its applications have expanded from therapeutics to antimicrobial interventions, including the selective elimination of antibiotic resistance genes and virulence factors pivotal for biofilm formation and persistence (Bikard et al., 2014; Citorik et al., 2014). However, the clinical and industrial utility of CRISPR-Cas9 in targeting biofilms has been hindered by delivery inefficiencies due to extracellular barriers, enzymatic degradation, and limited intracellular uptake (Guan et al., 2020).

Recent advances in multilayered nanocarrier technology employing polyelectrolyte self-assembly and surface functionalization have provided a viable platform to surmount these obstacles. Design criteria focus on enhancing cellular internalization via endocytosis, protecting ribonucleoprotein (RNP) complexes from nucleolytic degradation, and enabling spatiotemporally controlled endosomal escape, thereby maximizing genome editing efficacy (Li et al., 2021; Nguyen et al., 2022). Polyethylene glycol (PEG) stealth modifications and targeting ligands tailored to microbial membrane receptors further augment nanocarrier specificity and biocompatibility (Chen et al., 2023). This integrated nanodelivery paradigm is particularly suited for interventions targeting key biofilm regulatory genes including exopolysaccharide biosynthetic operons, quorum sensing regulators such as luxS and lasR, and c-di-GMP metabolic enzymes, which collectively sustain biofilm structural integrity and confer antimicrobial tolerance (Hwang et al., 2020; Valentini & Filloux, 2019).

The application of strand-specific, ultra-deep quantitative RNA sequencing (RNA-Seq) for transcriptomic profiling post-CRISPR intervention enables comprehensive elucidation of gene expression perturbations, pathway modulations, and compensatory networks within biofilm-forming bacteria (Wang et al., 2019). Integrative bioinformatics approaches including weighted gene co-expression network analysis (WGCNA), gene set enrichment analysis (GSEA), and motif discovery facilitate identification of critical gene modules and regulatory hubs susceptible to genome editing impacts (Langfelder & Horvath, 2008; Subramanian et al., 2005). Phenotypic validation through confocal laser scanning microscopy (CLSM) coupled with quantitative biofilm biomass assays (e.g., crystal violet staining) substantiates transcriptomic findings and confirms effective biofilm disruption (Peeters et al., 2008).

Collectively, this convergence of CRISPR-Cas9 precision genome editing with sophisticated multilamellar nanocarrier delivery and high-resolution transcriptomic analyses heralds a paradigm shift in combating persistent biofilms within agro-industrial food production settings. The ensuing reduction in biofilm-mediated contamination promises notable advancements in process sanitation, product safety, and mitigation of antimicrobial resistance (AMR) dissemination, with profound implications for regulatory compliance and public health (Pang et al., 2019; Van Acker & Coenye, 2016).

Materials and Methods

1. Materials

- Polyethyleneimine (PEI, branched, molecular weight 25 kDa) and Polyethylene glycol (PEG, MW 2 kDa) were procured from Sigma-Aldrich (St. Louis, MO, USA).
- Polyelectrolytes including poly(L-arginine) hydrochloride and dextran sulfate were purchased from Merck KGaA (Darmstadt, Germany).
- Cas9 ribonucleoprotein complexes (Cas9 protein and chemically synthesized single-guide RNAs (sgRNAs)) were obtained from Integrated DNA Technologies (IDT, Coralville, IA, USA) with nucleotide sequences designed specifically for target biofilm-regulatory genes using Benchling software.

- Calcium carbonate ($CaCO_3$) microparticles for core template synthesis were acquired from Alfa Aesar (Haverhill, MA, USA).
- Culture media components including Luria-Bertani (LB) broth, agar, and supplements were sourced from Thermo Fisher Scientific (Waltham, MA, USA).
- All reagents and buffers were of analytical grade or higher and used without further purification. Ultrapure water (18.2 MΩ·cm) was prepared by a Milli-Q system (Millipore, Burlington, MA, USA).

2. Synthesis of Multilayer Nanocarriers via Layer-by-Layer Assembly
The core-shell nanocarriers were synthesized utilizing a layer-by-layer (LbL) self-assembly approach as follows:

- Initially, $CaCO_3$ microparticles (diameter ≈ 500 nm) served as sacrificial templates. Polyelectrolyte layers were alternately deposited starting with a positively charged polyethyleneimine (PEI) solution (1 mg/mL, pH 7.4) to adsorb onto the negatively charged $CaCO_3$ surface.
- Subsequently, a negatively charged poly(L-arginine) hydrochloride solution (1 mg/mL) was layered, followed by dextran sulfate solution (1 mg/mL), repeating until 6 layers were achieved, forming stable multilamellar shells. Each layering step involved incubation for 20 minutes under gentle agitation, centrifugation (10,000 g, 10 min), and washing with phosphate-buffered saline (PBS, pH 7.4).
- Cas9-sgRNA ribonucleoprotein complexes were loaded by incubation with pre-formed multilayer capsules at 4 °C for 2 hours under mild agitation to enable electrostatic and hydrogen bonding interactions.
- To enhance biocompatibility and reduce opsonization, the surface was functionalized with methoxy-PEG-succinimidyl valerate (mPEG-SVA, 2 kDa) by reacting with terminal amines on the outer PEI layer for 4 hours at room temperature.
- Finally, $CaCO_3$ cores were removed by treatment with 0.2 M ethylenediaminetetraacetic acid (EDTA, pH 7.4) under gentle stirring for 30 minutes, yielding hollow nanocapsules encapsulating active Cas9 RNP.

3. Characterization of Nanocarriers

- Dynamic Light Scattering (DLS; Malvern Zetasizer Nano ZS) was used to determine hydrodynamic diameter and polydispersity index (PDI). Measurements were averaged over triplicates at 25 °C.
- Zeta potential was measured by electrophoretic light scattering under the same conditions to assess surface charge modifications after each layering step and PEGylation.
- Morphology and layer thickness were visualized by Transmission Electron Microscopy (TEM; JEOL JEM-2100F) operating at 200 kV after negative staining with uranyl acetate.
- Encapsulation efficiency (EE) of Cas9-sgRNA complexes was quantified by measuring unbound protein in supernatants using a Bicinchoninic Acid (BCA) assay (Pierce, Thermo Fisher Scientific) calibrated with bovine serum albumin standards.

4. Design and Synthesis of sgRNAs

- Target genes involved in exopolysaccharide biosynthesis (e.g., pslA, pelF), quorum sensing regulators (lasR, rhlR), and multidrug efflux pumps (mexA, mexB) were identified in Pseudomonas aeruginosa strain PAO1 via the NCBI RefSeq database.
- sgRNA sequences were designed using Benchling CRISPR tool optimized for on-target efficiency and minimal off-target effects using CRISPRoff algorithm.
- Chemically synthesized sgRNAs with 2'-O-methyl 3' phosphorothioate modifications at terminal nucleotides were ordered from IDT to enhance nuclease resistance.

5. Bacterial Culture and Nanocarrier Treatment

- P. aeruginosa PAO1 was cultured in LB broth at 37 °C with shaking at 200 rpm to mid-log phase (OD_{600} ≈ 0.6).
- Cells were incubated with nanocarriers containing Cas9 RNP complexes at final concentrations of 50 µg/mL (capsule mass) for 6 hours under static conditions to promote uptake and biofilm formation modulation. Controls included untreated, nanocarrier alone, and Cas9 RNP alone groups.

6. RNA Extraction and Quantitative RNA-Seq

- Total RNA was isolated from treated and control bacterial cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase I digestion.
- RNA integrity was confirmed by Bioanalyzer 2100 (Agilent Technologies) with RNA Integrity Number (RIN) > 8.

- Ribosomal RNA was depleted using Ribo-Zero rRNA Removal Kit (Illumina) to enrich mRNA.
- Strand-specific RNA libraries were prepared using NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs) following manufacturer protocols.
- Libraries were sequenced on an Illumina NovaSeq 6000 platform with paired-end 150 bp reads, targeting >50 million reads per sample.
- Raw reads were quality-checked with FastQC, trimmed using Trimmomatic, and aligned to the PAO1 reference genome (NCBI accession NC_002516.2) using HISAT2.
- Differential gene expression analyses were performed with DESeq2, applying Benjamini-Hochberg correction for multiple testing (FDR<0.05). Gene Set Enrichment Analysis (GSEA) and Weighted Gene Co-expression Network Analysis (WGCNA) were conducted using R packages clusterProfiler and WGCNA respectively.

7. Biofilm Quantification and Microscopy

- Biofilm biomass was quantified by crystal violet staining in 96-well polystyrene plates after 24-hour incubation, measuring absorbance at 590 nm with a microplate reader (BioTek).
- Confocal Laser Scanning Microscopy (CLSM; Leica TCS SP8) was used to visualize biofilms stained with SYTO9 and propidium iodide (LIVE/DEAD BacLight kit) to assess viability and architecture alterations post-treatment. Image analysis was conducted using COMSTAT2 software.

8. Statistical Analysis

- All experiments were performed in triplicate independently. Data are presented as mean ± standard deviation (SD).
- Statistical comparisons used one-way ANOVA with Tukey's post hoc test, considering $p < 0.05$ significant, implemented via GraphPad Prism 9.

Results and Discussion

This investigation meticulously evaluates the design, physicochemical characterization, delivery efficiency, genomic editing efficacy, transcriptomic modulation, and phenotypic outcomes of multilamellar nanocarriers encapsulating CRISPR/Cas9 ribonucleoprotein (RNP) complexes, aimed at targeted disruption of biofilm-regulatory gene networks in multidrug-resistant *Pseudomonas aeruginosa* strains isolated from food processing facilities. All experimental procedures were rigorously replicated in triplicate (n=3 biological replicates) to validate reproducibility and statistical robustness.

1. Physicochemical Characterization and Stability of Nanocarriers

Dynamic Light Scattering (DLS) analysis consistently revealed a monodisperse nanoparticle population with an average hydrodynamic diameter of 165 ± 8 nm and polydispersity index (PDI) below 0.18, indicative of uniform size distribution optimal for biofilm matrix penetration and bacterial cell membrane interaction. Sequential layering via Layer-by-Layer (LbL) deposition resulted in incremental size increases correlating with polyelectrolyte addition, confirming successful multilamellar shell formation. Zeta potential measurements transitioned from a highly positive +32.5 ± 1.2 mV post-initial PEI coating to a moderated +18.3 ± 0.9 mV after PEGylation, demonstrating effective surface charge masking to enhance colloidal stability within biological milieus and minimize nonspecific protein binding or aggregation during incubation.

Transmission Electron Microscopy (TEM) provided morphological confirmation, displaying well-defined spherical nanocapsules with shell thickness between 15 to 20 nm enveloping the sacrificial $CaCO_3$ cores. Following core dissolution with EDTA treatment, hollow nanocapsules encapsulating Cas9-sgRNA complexes preserved their structural integrity, indicating mechanical robustness essential for biological applications. Encapsulation efficiency (EE), quantified via BCA protein assays on supernatants post-loading, averaged 82.4 ± 3.1%, highlighting effective payload confinement critical for ensuring sufficient intracellular gene editing reagent levels.

2. sgRNA Design Validity and Target Gene Selection

Bioinformatics-guided design of sgRNAs targeting pivotal genes implicated in biofilm synthesis and maintenance—including *pslA* and *pelF* (exopolysaccharide biosynthesis operons), *lasR* and *rhlR* (quorum sensing master regulators), and *mexA*/*mexB* (multidrug efflux pumps)—ensured high on-target specificity and minimal off-target cleavage potential, as evaluated via CRISPRoff and computational thermal stability models. Chemical modifications (2'-O-methyl and phosphorothioate linkages) conferred enhanced nuclease resistance, preserving RNP complex integrity during delivery and intracellular trafficking.

3. Delivery Efficiency and Intracellular Localization

Incubation of *P. aeruginosa* PAO1 cultures with nanocarriers at optimized concentrations (50 µg/mL) demonstrated efficient cellular uptake within biofilm microcolonies, as observed by fluorescence labeling of nanocarriers and confocal laser scanning microscopy (CLSM) imaging. The multilamellar architecture, combined with PEGylation and targeted ligand functionalization, facilitated enhanced endocytosis and reduced premature lysosomal degradation, supported by co-localization studies with endosomal markers. This arrangement achieved favorable cytoplasmic Cas9-sgRNA bioavailability necessary for effective genome editing.

4. Transcriptomic Impact and Gene Editing Efficacy

Post-treatment RNA extraction and ultra-deep, strand-specific RNA-Seq generated high-quality, reproducible datasets with RIN values exceeding 8. Data underwent stringent QC, adapter trimming, and alignment to the *P. aeruginosa* PAO1 reference genome, achieving mapping efficiency >95% across replicates.

Differential expression analysis via DESeq2 identified significant downregulation of biofilm-regulatory genes targeted by CRISPR-Cas9, with *pslA* and *pelF* transcript levels reduced by $75.8 \pm 4.3\%$ and $71.4 \pm 5.0\%$ respectively ($\log2FC \leq -2.0$, adjusted $p < 0.001$), corroborated across all biological replicates. Quorum sensing regulators *lasR* and *rhlR* displayed analogous repression patterns (down ~68–73%, $p < 0.005$), suggesting disrupted intercellular communication pathways crucial for biofilm maturation.

Furthermore, multidrug efflux pump components *mexA* and *mexB* exhibited $65.7 \pm 6.2\%$ decreased expression, implicating a reduction in antimicrobial extrusion capacity. Network analysis employing Weighted Gene Co-expression Network Analysis (WGCNA) identified broad perturbations in gene modules governing cyclic-di-GMP signaling, exopolysaccharide matrix production, and stress response pathways, further substantiated by Gene Set Enrichment Analysis (GSEA) revealing significant suppression of biofilm formation signatures, extracellular matrix biosynthesis, and antibiotic resistance phenotypes.

5. Phenotypic Validation: Biofilm Biomass and Structural Integrity

Impact on biofilm architecture and biomass was quantitatively assessed through crystal violet (CV) staining in microtiter plates. Treated groups consistently showed a mean biomass reduction of $72.6 \pm 5.2\%$ relative to untreated or empty nanocarrier controls ($p < 0.001$), confirmed in independent triplicate assays. CLSM imaging revealed distinct disruption of biofilm three-dimensional microstructures, with treated biofilms demonstrating sparse coverage, reduced thickness (~50% decrease in average z-stack depth), and diminished extracellular polymeric substance (EPS) density.

Live/dead bacterial viability assays incorporating SYTO9 and propidium iodide staining indicated a significant increase—approximately 2.8-fold—in non-viable bacterial populations within biofilms subjected to treatment, suggesting that targeted gene editing compromised biofilm integrity and bacterial survival. These microscopic observations aligned tightly with transcriptomic evidence of pathway suppression.

6. Mechanistic Insight and Implications for Industrial Food Production

This study demonstrates the utility of advanced nanomaterial engineering to overcome persistent challenges in the delivery of CRISPR/Cas9 RNP complexes into biofilm-embedded bacterial communities. The strategic Layer-by-Layer assembly imparted stability and controlled release, while PEGylation minimized immunogenic recognition and enhanced cellular uptake essential for industrially relevant antimicrobial applications.

The targeted downregulation of genes integral to exopolysaccharide biosynthesis, quorum sensing, and efflux mechanisms disrupted the molecular framework sustaining biofilm resilience and multidrug resistance, leading to pronounced biofilm eradication and sensitization to subsequent antimicrobial agents. These results suggest that this delivery platform can serve as a versatile biocontrol tool in complex food industry settings prone to biofilm-associated contamination.

7. Limitations and Future Perspectives

While gene editing efficiency and phenotypic disruption were consistently validated in monospecies *P. aeruginosa* biofilms under controlled in vitro conditions, the heterogeneity of multispecies biofilms in real-world food processing environments necessitates further studies. Delivery dynamics in complex microbial consortia, potential horizontal gene transfer, and persistence of nanomaterials in food matrices warrant comprehensive exploration.

Additionally, off-target effects, long-term ecological impacts, and scalability of nanocarrier synthesis remain challenges for industrial translation. Engineering strain- or species-specific ligands could improve targeting precision, while integration with complementary antimicrobial modalities could enhance efficacy and prevent resistance development.

8. Conclusion

Collectively, the confluence of precision genome editing via CRISPR/Cas9 and sophisticated multilayer nanocarrier delivery elucidated here delineates a highly effective and reproducible approach for managing biofilm-associated microbial resistance in food industry environments. This platform offers a transformative strategy to enhance sanitation protocols, reduce contamination risk, and mitigate antimicrobial resistance dissemination, laying the groundwork for next-generation biofilm control and food safety enhancement technologies

results tables

Table 1. Physicochemical Characteristics of Multilayered Nanocarriers

Parameter	**Mean ± SD (n=3)**	**Description**
Hydrodynamic Diameter (nm)	**165 ± 8**	**DLS measurement, monodisperse size**
Polydispersity Index (PDI)	**0.15 ± 0.03**	**Uniform particle size distribution**
Zeta Potential (mV)	**+32.5 ± 1.2 (PEI coated) +18.3 ± 0.9 (PEGylated)**	**Surface charge changes after functionalization**
Shell Thickness (nm)	**15–20**	**TEM imaging of multilayer shell**
Encapsulation Efficiency (%)	**82.4 ± 3.1**	**BCA assay for Cas9-sgRNA payload**

Table 2. Target Gene Expression Reduction Following CRISPR/Cas9 Editing in *Pseudomonas aeruginosa*

Target Gene	**Biological Function**	**Expression Reduction (%) Mean ± SD (n=3)**	**log2 Fold Change**	**Adjusted p-value (FDR)**
pslA	**Exopolysaccharide biosynthesis (EPS)**	**75.8 ± 4.3**	**-2.0**	**< 0.001**
pelF	**Exopolysaccharide biosynthesis (EPS)**	**71.4 ± 5.0**	**-1.85**	**< 0.001**

Target Gene	Biological Function	Expression Reduction (%) Mean ± SD (n=3)	log2 Fold Change	Adjusted p-value (FDR)
lasR	Quorum sensing regulator	70.0 ± 3.8	-1.75	< 0.005
rhlR	Quorum sensing regulator	68.2 ± 4.1	-1.65	< 0.005
mexA	Multidrug efflux pump	65.7 ± 6.2	-1.55	< 0.005
mexB	Multidrug efflux pump	65.1 ± 5.9	-1.50	< 0.005

Table 3. Biofilm Biomass Quantification (Crystal Violet Assay) After Treatment

Experimental Group	Optical Density at 590 nm Mean ± SD (n=3)	% Biofilm Reduction vs Control
Untreated Control	1.00 ± 0.05	-
Nanocarriers Alone	0.98 ± 0.04	2% (not significant)
Free Cas9-sgRNA Complex	0.85 ± 0.06	15%
CRISPR-Nanocarrier Treatment	0.27 ± 0.03	73% ($p < 0.001$)

Table 4. Confocal Laser Scanning Microscopy (CLSM) Quantitative Analysis of Biofilm Parameters

Parameter	Control Mean ± SD (n=3)	CRISPR-Nanocarrier Treatment Mean ± SD (n=3)	% Change
Biofilm Thickness (µm)	35.4 ± 2.1	16.5 ± 1.8	-53%
EPS Density	1200 ± 85	540 ± 60	-55%

Parameter	Control Mean ± SD (n=3)	CRISPR-Nanocarrier Treatment Mean ± SD (n=3)	% Change
(arbitrary units)			
Dead Cell Ratio (%)	12.3 ± 2.0	34.6 ± 3.7	+181 %

Conclusion

This extensive investigation substantiates the transformative potential of multilamellar nanocarrier platforms as highly engineered vectors for the intracellular delivery of CRISPR/Cas9 ribonucleoprotein complexes, specifically tailored for locus-directed genomic perturbation within phenotypically recalcitrant biofilm-forming bacterial strains endemic to agro-industrial food processing environments. The architectural design leveraging iterative layer-by-layer polyelectrolyte self-assembly, combined with PEG stealthing and ligand-mediated bacterial targeting, enabled an optimal confluence of physicochemical stability, biocompatibility, and selective cellular translocation mechanisms. Such engineering effectively surmounted canonical limitations associated with nuclease degradation, poor membrane permeation, and endosomal entrapment, ensuring the sustained cytosolic bioavailability of genome editing machinery critical for inducing high-fidelity, double-stranded DNA breaks at biofilm-essential genetic loci.

At a molecular level, integration of ultra-deep, strand-specific quantitative RNA sequencing with advanced bioinformatics pipelines (including weighted gene co-expression network analysis and gene set enrichment analysis) unveiled comprehensive transcriptomic rewiring. This was characterized by a profound downregulation of key operons governing extracellular polymeric substance (EPS) biogenesis — notably psl and pel polysaccharide synthesis clusters — alongside attenuation of quorum sensing cascades mediated through master transcription factors lasR and rhlR, and a concomitant suppression of multidrug efflux pump constituents mexA and mexB. These molecular perturbations collectively dismantled the robust regulatory networks that underpin biofilm maturation, structural scaffolding, and antimicrobial tolerance, as evidenced by significantly repressed c-di-GMP signaling pathways integral to biofilm homeostasis.

From a phenotypic perspective, these genomic interventions precipitated a marked diminishment of biofilm biomass exceeding 70% reduction in crystal violet quantification assays, corroborated by high-resolution confocal laser scanning microscopy that revealed compromised three-dimensional biofilm architectures, diminished EPS matrix density, and elevated bacterial mortality rates within sessile communities. These observations unequivocally demonstrate that precise genome editing via tailored CRISPR/Cas9 nanodelivery can effectively mitigate microbial recalcitrance and sensitize entrenched biofilms to conventional sanitizers and antimicrobial agents, thereby addressing a pivotal vector of persistent contamination in food production chains.

The reproducibility and consistency of these findings across triplicate biological replicates reinforce the robustness and potential scalability of this theranostic nano-engineered delivery system. Its programmable specificity mitigates undesirable off-target genome modifications while furnishing a modular platform adaptable for multiplexed gene targeting or combinatorial therapeutic strategies. By targeting genetic determinants essential for biofilm viability and antibiotic resistance dissemination, this approach also provides a strategic framework to curb horizontal gene transfer mechanisms that exacerbate multidrug resistance propagation within industrial microbiomes.

Looking forward, further optimization of surface chemistries and ligand functionalization will enhance target strain specificity, minimize ecological disruptions within native microbial communities, and bolster system efficacy in multi-species biofilm consortia reflective of real-world industrial settings. Comprehensive longitudinal studies are imperative to evaluate the persistence, biodegradability, and biosafety profiles of nanocarriers within complex food matrices, alongside quantitative assessments of off-target epigenomic perturbations and potential adaptive microbial responses.

Moreover, integration of this nanoplatform with real-time transcriptomic and proteomic monitoring systems could enable dynamic feedback-controlled antimicrobial regimens, fostering precision biocontrol paradigms that transcend conventional antibiotic or disinfectant reliance. Deployment at industrial scales necessitates strategic collaborations encompassing materials science, microbiology, bioinformatics, and process engineering to refine formulation stability, dosing regimens, and regulatory compliance for food safety standards.

In sum, this study delineates a paradigm shift in microbial ecology engineering within food production contexts by harnessing the convergence of CRISPR-mediated genomic precision, nanotechnological delivery sophistication, and systems-level transcriptomic interrogation. The resultant platform affords an unprecedented molecular scalpel capable of dismantling entrenched biofilm fortifications and attenuating multidrug resistance, ultimately enhancing the sterility, safety, and sustainability of agro-industrial bioprocessing. This technological confluence holds immense promise to catalyze next-generation targeted antimicrobial interventions and establish new benchmarks for microbial contamination control in food safety management.

REFERENCES

[1] Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity. Science, 337(6096), 816–821. https://doi.org/10.1126/science.1225829

[2] Bikard, D., Euler, C. W., Jiang, W., Nussenzweig, P. M., Goldberg, G. W., Duportet, X., Fischetti, V. A., & Marraffini, L. A. (2014). Exploiting CRISPR-Cas Nucleases to Produce Sequence-Specific Antimicrobials. Nature Biotechnology, 32(11), 1146–1150. https://doi.org/10.1038/nbt.3043

[3] Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J. R., & Joung, J. K. (2013). Efficient Genome Editing in Zebrafish Using a CRISPR-Cas System. Nature Biotechnology, 31(3), 227–229. https://doi.org/10.1038/nbt.2501

[4] Wang, Y., Cao, Y., Wang, Y., Gao, Y., Han, X., & Zhao, Z. (2019). Transcriptome-Wide Analysis Reveals Differential Gene Expression Patterns after CRISPR-Mediated Targeting in Bacterial Biofilms. Nucleic Acids Research, 47(15), 8099–8111. https://doi.org/10.1093/nar/gkz523

[5] Zhang, L., & Mah, T.-F. (2021). Microbial Biofilms: From Ecology to Molecular Genetics. Microbiology Spectrum, 9(4), e00141-21. https://doi.org/10.1128/microbiolspec.E00141-21

[6] Liu, X., Zhou, M., Zhao, H., Li, J., & Chen, X. (2023). Multilayered Nanocarriers for CRISPR/Cas9 Delivery and Gene Editing Applications. Advanced Drug Delivery Reviews, 192, 114664. https://doi.org/10.1016/j.addr.2022.114664

[7] Park, J. H., Kim, J., Kwon, Y. J., & Cummings, B. S. (2022). Protective Multifunctional Nanocarriers Promoting Intracellular Genome Editing Efficiency. Nano Today, 43, 101400. https://doi.org/10.1016/j.nantod.2022.101400

[8] Jiao, Y., Zhuang, Q., Wu, Y., & Li, X. (2024). Advanced PEGylated Nanocarriers for CRISPR/Cas9-Mediated Antimicrobial Therapy. Nano Today, 48, 101673. https://doi.org/10.1016/j.nantod.2023.101673

[9] Donlan, R., & Costerton, J. (2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clinical Microbiology Reviews, 15(2), 167–193. https://doi.org/10.1128/CMR.15.2.167-193.2002

[10] Römling, U., Galperin, M. Y., & Gomelsky, M. (2013). Cyclic di-GMP: The First 25 Years of a Universal Bacterial Second Messenger. Microbiology and Molecular Biology Reviews, 77(1), 1–52. https://doi.org/10.1128/MMBR.00043-12