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Review Article

CRISPR-Enabled Nanocarrier Systems Against Multidrug-Resistant Bacteria: Emerging Potential of Bilosomes for Precision Antimicrobial Therapy

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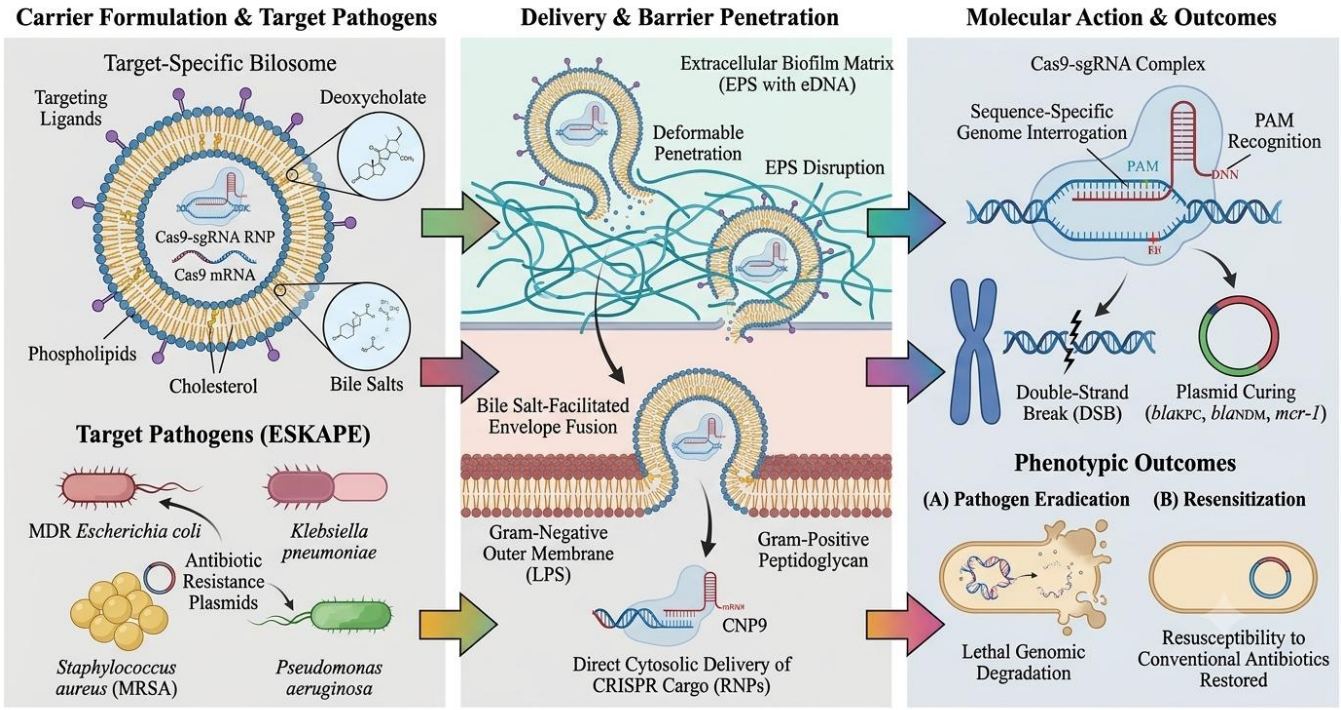
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Abstract

Objective(s): This review aims to evaluate the mechanistic convergence of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas genome-targeting technology with bile salt-stabilized vesicular nanocarriers (bilosomes) to circumvent physiological barriers and achieve sequence-specific eradication of multidrug-resistant (MDR) bacterial pathogens. **Data sources:** A systematic literature search was conducted across PubMed/MEDLINE, Web of Science, Scopus, ScienceDirect, and Google Scholar covering peer-reviewed studies published up to 2026. **Study selection:** Articles encompassing molecular mechanisms of antimicrobial resistance, CRISPR-Cas antimicrobials, non-viral nucleic acid delivery systems, and bilosomal formulations were critically scrutinized. **Summary of contents of the article:** Multidrug-resistant bacteria make regular antibiotics less effective and complicate clinical therapy. CRISPR-Cas technology can identify specific genomic loci and target resistance genes, virulence determinants, and plasmid scaffolds. However, therapeutic utilization is severely impeded by nuclease vulnerability, poor bacterial penetration, and transient stability. Bilosomes—lipid vesicles incorporated with deoxycholate, glycocholate, or taurocholate—exhibit superior membrane flexibility, resistance to gastrointestinal and enzymatic degradation, and enhanced cellular permeation compared to conventional liposomes. Functionalizing bilosomes with targeting ligands enables localized delivery and sustained release of Cas9 ribonucleoprotein complexes, driving sequence-specific chromosomal double-strand breaks, plasmid curing, and resensitization to conventional antibiotics. **Conclusion:** CRISPR-loaded bilosomal nanocarriers offer an effective non-antibiotic delivery platform capable of degrading bacterial chromosomes, curing high-risk resistance plasmids (blaKPC, blaNDM, mcr-1), and selectively reducing pathogen load without triggering broad microbiome dysbiosis.

Keywords: Multidrug-resistant bacteria (MDR), CRISPR-Cas, Bilosomes, Nanocarrier delivery, Antimicrobial resistance (AMR), Plasmid curing, Precision antimicrobial therapy.

Graphical Abstract



Graphical Abstract Synopsis: Schematic representation illustrating the precision antimicrobial strategy of CRISPR-loaded bilosomal nanocarriers against multidrug-resistant (MDR) bacterial superbugs across three coordinated stages: (1) Carrier Formulation & Pathogen Recognition: Vesicular bilosomes assembled with phospholipids, cholesterol, and intercalated bile salts encapsulate Cas9–sgRNA RNP complexes in a protective core, functionalized with surface ligands to recognize priority ESKAPE pathogens; (2) Delivery & Barrier Penetration: Deformable bilosomes penetrate dense extracellular polymeric substance (EPS) biofilm matrices and fluidize bacterial outer membrane lipopolysaccharides (LPS) and peptidoglycan for direct cytosolic cargo translocation; (3) Molecular Action & Outcomes: PAM-guided double-strand break (DSB) cleavage executes lethal chromosomal fragmentation or cures plasmid-borne resistance determinants (blaKPC, blaNDM, mcr-1), neutralizing virulence and restoring resusceptibility to first-line antibiotics.

INTRODUCTION

Antimicrobial resistance (AMR) has become one of the primary public health challenges of the contemporary era. Bacterial infections that are resistant to treatments increase illness, cause deaths, raise healthcare costs, and complicate clinical therapies.[1] In 2019, bacterial AMR was directly associated with 4.95 million deaths globally, with 1.27 million deaths attributable directly to resistant bacterial pathogens.[1] The global issue of AMR has been connected to severe illness and mortality worldwide.[1-4] Several bacterial pathogens are of critical clinical significance due to their exceptional capacity to acquire resistance against conventional antibacterial agents. These critical priority pathogens—frequently classified within the 'ESKAPE' complex—encompass *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Enterococcus faecium*.[2] Using antibiotics in a manner that entails prolonged exposure and improper eradication leads to environmental pollution with antibiotic residues and promotes the selection and transmission of resistance factors.[3,4]

Bacterial resistance is frequently mediated by mobile genetic elements, such as plasmids, transposons, integrons, and bacteriophages, which facilitate the horizontal dissemination of resistance genes across divergent bacterial taxa.[5] Plasmids are especially critical vectors because they carry multidrug resistance determinants and propagate rapidly across interspecies boundaries.[6] Additionally, bacterial biofilms establish protected multicellular communities enclosed within extracellular polymeric substances, which impede antibiotic penetration and foster phenotypic tolerance.[7] The growing challenge of AMR has catalyzed the development of precision therapeutic modalities that are far more targeted than standard broad-spectrum antibiotics. CRISPR-Cas systems, which evolved naturally as adaptive immune mechanisms in prokaryotes, have been re-engineered into versatile tools capable of sequence-specific DNA and RNA cleavage.[8,9] Unlike conventional antibiotics, CRISPR-based antimicrobials can specifically attack antibiotic resistance genes, virulence factors, essential metabolic genes, or transmissible plasmids. Experimental investigations have confirmed that CRISPR-Cas platforms act as sequence-directed bactericidal agents.[10,11] Advances in structural and mechanistic characterization have further enabled the precise programming of these genetic scissors for diverse antimicrobial applications.[12,13] Research has demonstrated that CRISPR can selectively kill

pathogens, eliminate plasmid-borne resistance determinants, and resensitize resistant bacteria to conventional antibiotics.[14-18]

To introduce CRISPR components into bacterial cells, researchers have investigated multiple delivery modalities, including bacteriophages, conjugative plasmids, and synthetic nanocarriers.[19-24] However, achieving high delivery efficiency across complex bacterial cell envelopes remains a major obstacle before clinical implementation. Nanocarriers offer an attractive approach to encapsulate fragile CRISPR cargo, enhance stability, facilitate intracellular translocation, and provide spatiotemporally controlled release.[25,26] Among diverse nanocarriers, bilosomes have attracted widespread interest as deformable lipid-surfactant vesicles incorporating bile salts, which impart superior membrane elasticity, enhanced stability against physiological biofluids, and improved membrane permeability.[27-30] Integrating Cas9 ribonucleoprotein or mRNA cargo into deformable bilosomal vesicles presents a targeted, sequence-directed alternative to overcome outer membrane exclusion and thick biofilm barriers. Nevertheless, bilosome-facilitated CRISPR delivery remains at an early preclinical stage, requiring systematic evaluation of lipid–bile salt membrane fusion kinetics, encapsulation stability, and in vivo biodistribution.

REVIEW METHODOLOGY

This narrative review was synthesized by analyzing literature spanning antimicrobial resistance, multidrug-resistant bacterial pathogens, CRISPR-Cas precision antimicrobial approaches, non-viral delivery platforms, and bilosomal pharmaceutical formulations. Peer-reviewed literature was retrieved from primary bibliographic databases including PubMed/MEDLINE, Web of Science, Scopus, ScienceDirect, and Google Scholar from January 2000 through October 2026. Search algorithms combined key terms: 'multidrug-resistant bacteria', 'CRISPR-Cas', 'bilosomes', 'nanocarrier delivery', 'plasmid curing', and 'precision antimicrobial therapy'. Emphasis was placed on studies investigating sequence-specific gene knockout, plasmid clearance, biofilm penetration, and vesicular delivery of nucleic acids and ribonucleoproteins. The synthesized evidence was systematically structured into: (i) the global AMR crisis and resistance mechanisms; (ii) CRISPR-Cas endonuclease architecture and precision antibacterial targeting; (iii) pharmaceutical attributes and comparative advantages of bilosomal vesicles; and (iv) the rational formulation, mechanism of action, delivery barriers, and experimental roadmap of CRISPR-loaded bilosomes.

ANTIMICROBIAL RESISTANCE AND MULTIDRUG-RESISTANT BACTERIA

Mechanisms of Antimicrobial Resistance

Bacteria develop antimicrobial resistance through several multifaceted mechanisms, including enzymatic degradation or modification of antibiotics, alteration of drug targets, reduction of outer membrane permeability, active multidrug efflux, metabolic reprogramming, biofilm formation, and horizontal gene transfer.[3-7] Resistance genes can be located on the bacterial chromosome or mobilized onto plasmids, transposons, and integrons. Plasmid-mediated resistance is of paramount clinical concern because conjugative plasmids facilitate rapid intra- and inter-species dissemination of multidrug resistance determinants.[5,6]

Important MDR Bacterial Pathogens

The clinical burden of MDR infections is predominantly driven by priority bacterial pathogens:

Escherichia coli: MDR E. coli isolates frequently cause severe urinary tract infections, bloodstream bacteremia, and intra-abdominal infections. The proliferation of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated colistin resistance (mcr-1) severely restricts effective antimicrobial regimens.[2,46]

Klebsiella pneumoniae: A critical pathogen capable of acquiring extensive resistance determinants, including blaKPC and metallo-β-lactamases (blaNDM, blaOXA-48). Plasmid-mediated transfer drives rapid nosocomial outbreak dissemination.[2,19]

Acinetobacter baumannii: Exhibits widespread resistance to carbapenems, fluoroquinolones, and aminoglycosides via class D carbapenemases (OXA-23), porin loss, and AdeABC efflux systems, leaving colistin as a toxic last resort.[2]

Pseudomonas aeruginosa: Possesses formidable intrinsic resistance due to low outer membrane permeability (OprF) and Mex-family efflux pumps, alongside acquired metallo-β-lactamases and thick alginate biofilms.[2,7]

Staphylococcus aureus: Methicillin-resistant S. aureus (MRSA) expresses the mecA gene encoding PBP2a, which possesses drastically reduced affinity for β-lactam antibiotics, requiring glycopeptides or oxazolidinones.[2,14]

Enterococcus faecium: Vancomycin-resistant E. faecium (VRE) isolates harbor plasmid- or transposon-borne vanA/vanB gene clusters that remodel peptidoglycan precursors, conferring high-level glycopeptide tolerance.[2,15]

Limitations of Conventional Antibiotic Therapy

Standard antibiotics target fundamental bacterial processes, such as cell wall synthesis, translation, replication, or folate metabolism. However, this broad selection pressure creates major drawbacks: (1) selective enrichment of resistant mutants; (2) broad-spectrum disruption of host commensal microflora causing dysbiosis; (3) failure against extensively drug-resistant phenotypes; (4) physical exclusion by biofilm matrices; (5) induction of horizontal resistance gene dissemination; and (6) exhaustion of the antibiotic discovery pipeline.[1-7] These deficiencies underscore the need for programmable sequence-specific precision antimicrobials.

CRISPR-CAS SYSTEMS AS PRECISION ANTIMICROBIAL TOOLS

Core Principle of CRISPR-Cas Endonucleases

CRISPR-Cas systems function as adaptive immune networks in bacteria and archaea.[8,9] In engineered antimicrobial applications, guide RNA molecules direct Cas endonucleases to sequence-specific DNA or RNA targets. Cas9-based Type II systems introduce double-strand breaks at complementary 20-nucleotide target sites flanked by a protospacer adjacent motif (PAM).[12,13] Because bacteria frequently lack efficient non-homologous end-joining (NHEJ) repair pathways, chromosomal cleavage triggers lethal genomic degradation.[14-16] Additionally, Cas12 systems (DNA-targeting) and Cas13 systems (RNA-targeting) expand programmable antimicrobial options.[18,20]

Mechanisms of CRISPR-Mediated Antibacterial Activity

CRISPR antimicrobials operate through distinct genetic targeting strategies: (A) **Targeting essential bacterial genes:** Cleavage of indispensable genomic loci (e.g., dnaA, ftsZ, gyrA) causes direct bactericidal lysis.[14,15] (B) **Targeting antibiotic-resistance genes:** Cleavage of carbapenemase (blaKPC, blaNDM) or colistin resistance (mcr-1) genes eliminates resistance phenotypes.[19,46] (C) **Targeting resistance plasmids:** Cleaving conserved plasmid replication or partitioning sequences cures plasmids from bacterial populations.[14-17] (D) **Targeting virulence determinants:** Disrupting toxin or adhesin genes disarms pathogens without cell lysis.[15,17] (E) **CRISPR interference (CRISPRi):** Catalytically dead Cas9 (dCas9) sterically represses transcription elongation without inducing DNA breaks.[22]

Evidence Supporting CRISPR-Based Antibacterial Strategies

Seminal proof-of-concept investigations have confirmed that CRISPR nucleases function as sequence-specific antimicrobials. Citorik et al. demonstrated sequence-specific killing and resistance gene elimination in E. coli.[14] Bikard et al. demonstrated targeted strain-specific depletion of S. aureus and plasmid clearance in vivo.[15] Gomaa et al. showed programmable removal of specific bacterial strains from mixed cultures.[16] Yosef et al. developed temperate and lytic phage-based delivery systems to resensitize resistant bacteria.[17] Subsequent investigations demonstrated CRISPR-Cas13a sequence-specific RNA targeting and plasmid curing across clinical isolates.[18-24] However, non-viral vesicular delivery platforms remain relatively unexplored.

NANOCARRIER-MEDIATED DELIVERY OF CRISPR COMPONENTS

The therapeutic success of CRISPR antimicrobials depends upon safe and efficient cellular delivery. Therapeutic cargo can be formulated as Cas proteins, Cas mRNA, plasmid DNA, or Cas-sgRNA ribonucleoprotein (RNP) complexes.[25,27] Naked nucleic acids and proteins undergo rapid enzymatic degradation and cannot spontaneously traverse the negatively charged lipopolysaccharide or peptidoglycan barriers.[26,44] Nanocarriers enhance delivery by shielding cargo from nucleases, improving colloidal stability, facilitating cellular translocation, enabling surface ligand conjugation, and providing sustained cargo release.[25-33]

BILOSOMES: STRUCTURE, COMPOSITION, AND PHARMACEUTICAL PROPERTIES

Bilosomes are vesicular nanostructures composed of non-ionic surfactants or phospholipids stabilized by the incorporation of bile salts.[34-37] A typical bilosome formulation contains neutral lipids/surfactants, cholesterol, and bile salts (sodium deoxycholate, sodium glycocholate, or sodium taurocholate).[34-36] The intercalation of steroid bile salts within the bilayer imparts exceptional membrane flexibility and fluidity, protects encapsulated drugs from enzymatic and gastric degradation, enhances permeation across biological membranes, and accommodates both hydrophilic cargo in the aqueous core and lipophilic agents within the bilayer leaflets.[34-40] Bilosomes have been widely explored for antimicrobial and macromolecular drug delivery.[38-40]

Table 1: Comparative assessment of naked CRISPR cargo versus nanocarrier-associated and bilosomal delivery systems.

Delivery Metric / Parameter	Naked CRISPR Cargo	Conventional Nanocarriers	Bilosomal Vesicles
Nuclease protection & serum stability	Extremely poor; rapid degradation by nucleases within minutes[25,27]	Moderate to high; physical shielding inside core/shell[28,29]	Superior; bile salts prevent enzymatic degradation and vesicle lysis[34-37]
Bacterial envelope penetration	Negligible; severe electrostatic repulsion by LPS/peptidoglycan[26,44]	Variable; depends heavily on surface charge and functionalization[26,32]	High; bile salts act as natural penetration enhancers fluidizing membranes[35,38]
Gastrointestinal & biofluid resilience	Completely degraded by gastric acid and proteases[27,30]	Poor; liposomes aggregate or dissolve in physiological bile salts[26,34]	Outstanding; bile salt intercalation imparts exceptional stability against bile/acid[34,37]

Biofilm matrix diffusion	Severely restricted; trapped in extracellular polymeric substance[7,26]	Limited penetration; hindered by dense negative charge of eDNA[7,32]	Enhanced; flexible deformable lamellae facilitate deep biofilm matrix diffusion[38,40]
Cargo packaging versatility	Limited; susceptible to immediate environmental denaturation[13,27]	Encapsulates plasmid DNA, Cas9 mRNA, or RNP complexes[25,28]	High; hydrophilic core encapsulates RNP/mRNA; bilayer accommodates hydrophobic adjuvants[34,39]
Carrier immunogenicity & toxicity	Minimal carrier toxicity, but rapid clearance by host immune cells[25,28]	Variable; cationic polymers can provoke cytotoxicity and inflammation[28,32]	Low; utilizes biocompatible, physiological bile salts with excellent safety[34,37]
Surface targeting customizability	Incapable of stable ligand functionalization[17,25]	Capable of antibody, aptamer, or peptide ligand conjugation[26,32]	Excellent; easily functionalized with pathogen-specific targeting ligands[35,38]

Footnote: LPS = lipopolysaccharide; eDNA = extracellular DNA; RNP = ribonucleoprotein. Data compiled and synthesized from foundational literature.[7,13,17,25-40,44]

CRISPR-LOADED BILOSOMES: CONCEPTUAL DESIGN AND FORMULATION

The conceptual formulation of CRISPR-loaded bilosomes bridges the gap between precision genetic scissors and membrane-permeating vesicular nanobiotechnology (Figure 1).[34-37,42-45] To engineer a therapeutic CRISPR-bilosome system against multidrug-resistant pathogens, the formulation architecture comprises several coordinated components: (1) vesicular bilayer scaffolding formed by neutral or zwitterionic phospholipids (e.g., DOPC, SPC) and cholesterol (10–30 mol%) to maintain bilayer stability; (2) intercalated bile salt membrane modulators (sodium deoxycholate, glycocholate, or taurocholate, 5–15 mol%) that expand membrane deformability and enhance bacterial cell envelope permeation; (3) CRISPR therapeutic cargo comprising Cas9–sgRNA ribonucleoprotein (RNP) complexes or mRNA/gRNA encapsulated within the aqueous core for rapid cleavage without transcription delays; and (4) surface targeting ligands (monoclonal antibodies, antimicrobial peptide fragments, or aptamers) functionalized onto the bilayer surface for pathogen-selective homing.[10,11,17,25,34-36]

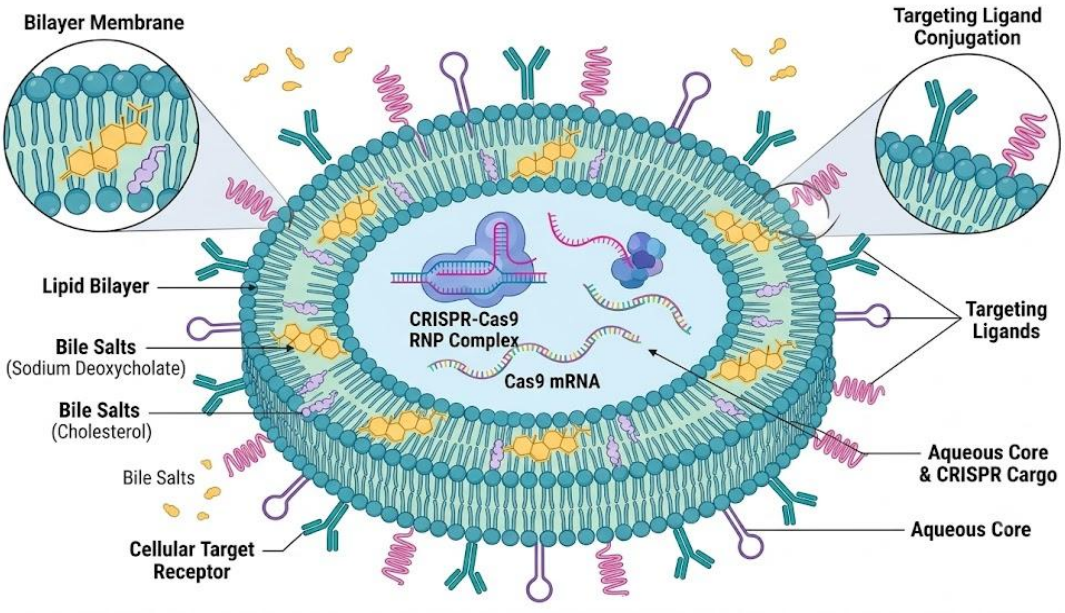


Figure 1: Structural organization and functional components of CRISPR-loaded bilosomal nanocarriers.

Schematic representation depicting the vesicular architecture of a bilosome composed of a phospholipid bilayer with interspersed cholesterol molecules, functionally stabilized by the intercalation of physiological bile salts (e.g., sodium deoxycholate) into the lipid bilayer leaflets. The central hydrophilic aqueous core encapsulates Cas9–sgRNA ribonucleoprotein (RNP) complexes and mRNA, providing physical shielding against nucleolytic and environmental degradation. The outer surface is functionalized with pathogen-specific targeting ligands (such as monoclonal antibodies or affinity peptides) to mediate selective recognition and docking onto multidrug-resistant bacterial envelopes.

PROPOSED MECHANISM OF ACTION OF CRISPR-LOADED BILOSOMES

The targeted eradication of multidrug-resistant bacteria by CRISPR-loaded bilosomes proceeds through a multi-stage sequential cascade (Figure 2):[14-17,25,34-40]

Stage 1: Nanocarrier Encapsulation and Protection — CRISPR payloads (Cas9 RNPs or mRNA/gRNA) are efficiently entrapped within the aqueous core or electrostatically condensed at the interior lamellae, shielding them from degradation by host nucleases, gastric acid, and extracellular bacterial enzymes.[27,34-37]

Stage 2: Pathogen-Directed Homing — Upon systemic or localized administration, the functionalized bilosomes migrate to infection sites. Surface ligands selectively anchor to bacterial outer membrane proteins (in Gram-negative species) or lipoteichoic acids (in Gram-positive species), establishing close interfacial contact.[10,11,17]

Stage 3: Bile Salt-Facilitated Membrane Fusion and Uptake — Intercalated bile salts lower interfacial tension and destabilize bacterial outer membranes by transiently displacing divalent cations (Mg2+, Ca2+) from lipopolysaccharide bridges. The flexible deformable vesicles undergo local fusion or endocytic-like uptake, delivering the intact CRISPR cargo directly into the microbial periplasm or cytoplasm.[35-40,43]

Stage 4: Intracellular Release and Sequence Interrogation — Intracellular biochemical triggers release the active Cas9-sgRNA RNP complex. The RNP interrogates the bacterial genome via rapid three-dimensional diffusion, seeking the appropriate PAM sequence.[12,13]

Stage 5: High-Fidelity Sequence-Specific Cleavage — Upon PAM recognition, the sgRNA pairs with the target resistance gene (e.g., blaNDM, mcr-1), activating Cas9 nuclease domains to generate irreversible double-strand breaks.[12,14,46]

Stage 6: Phenotypic Outcomes (Bactericidal Death vs. Resensitization) — If an essential chromosomal gene is cleaved, catastrophic DNA degradation kills the pathogen. If an antibiotic-resistance plasmid is cleaved, the plasmid is cured, completely stripping the bacterium of its resistance phenotype and restoring clinical efficacy to inexpensive first-line antibiotics.[14-16,19,46]

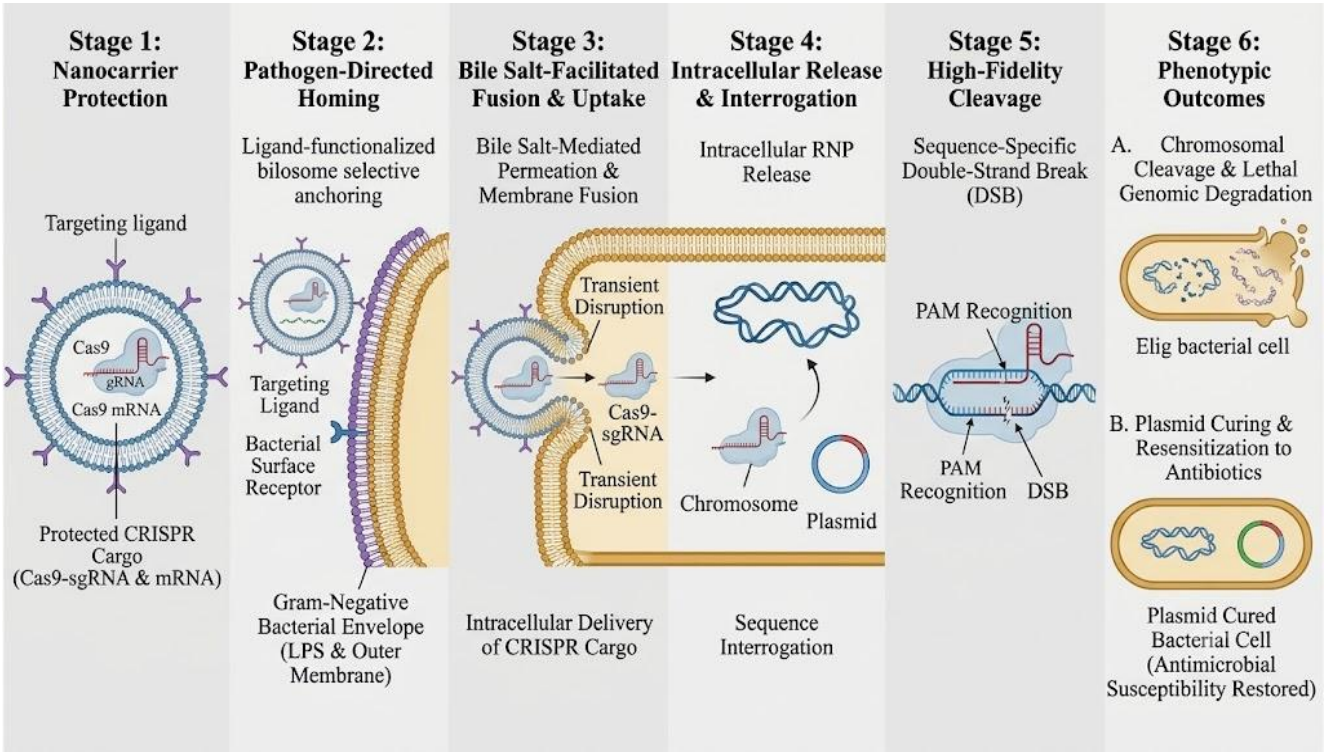


Figure 2: Stepwise cascade of bilosome-mediated intracellular CRISPR delivery and bacterial eradication.

Schematic representation of the six-stage antibacterial mechanism: Stage 1 (Nanocarrier Protection): Encapsulation shields Cas9–sgRNA RNP payloads from nucleases and harsh biofluids; Stage 2 (Pathogen-Directed Homing): Surface ligands anchor selectively to outer membrane epitopes of target superbugs; Stage 3 (Bile Salt-Facilitated Fusion & Uptake): Intercalated bile salts transiently fluidize and perturb the bacterial envelope (lipopolysaccharide/peptidoglycan), enabling direct cytosolic translocation; Stage 4 (Intracellular Release & Interrogation): Liberation of active RNPs and rapid scanning of genomic/plasmid DNA; Stage 5 (High-Fidelity Cleavage): PAM-guided formation of lethal double-strand breaks (DSBs); Stage 6 (Phenotypic Outcomes): Divergent therapeutic results, encompassing lethal genomic degradation from chromosomal targeting and plasmid curing with restoration of antimicrobial susceptibility following resistance gene targeting.

Table 2: Representative CRISPR genetic targets, target resistance mechanisms, and therapeutic outcomes in MDR bacteria.

Target Pathogen	Genetic Target	Encoded Resistance / Virulence Function	Targeted Cleavage Outcome & Significance
Escherichia coli	mcr-1 plasmid gene	Phosphoethanolamine transferase mediating colistin resistance	Disruption of mcr-1 gene; cures resistance plasmid; completely restores susceptibility to colistin.[19,46]
Klebsiella pneumoniae	blaKPC-2 / blaNDM-1	Serine carbapenemase / Metallo-β-lactamase hydrolyzing carbapenems	Cures carbapenem resistance plasmids; restores bactericidal efficacy of meropenem and imipenem.[14,19]
Pseudomonas aeruginosa	oprD porin / gyrA	Downregulated porin channel / altered DNA gyrase subunit A	Sequence-specific lethal cleavage; disables fluoroquinolone resistance and halts DNA replication.[2,10,14]
Acinetobacter baumannii	blaOXA-23 / adeB	Class D carbapenemase / AdeABC multidrug efflux pump component	Eliminates carbapenem resistance determinants; suppresses active efflux of tigecycline and aminoglycosides.[2,19]
Staphylococcus aureus (MRSA)	mecA chromosomal gene	PBP2a transpeptidase with reduced β-lactam antibiotic affinity	Induces lethal chromosomal double-strand break or restores susceptibility to oxacillin and methicillin.[14,15]
Enterococcus faecium (VRE)	vanA operon (vanA/vanB)	Altered peptidoglycan cell wall precursor (D-Ala-D-Lac synthesis)	Eliminates glycopeptide resistance; re-establishes vancomycin and teicoplanin bactericidal potency.[2,15]
Pan-pathogen biofilms	icaA / pel/psl operons	Biosynthesis of intercellular adhesins and extracellular biofilm matrix	Impedes biofilm formation; disperses mature biofilms; enhances penetration of companion therapeutics.[7,26]

Footnote: MRSA = methicillin-resistant Staphylococcus aureus; VRE = vancomycin-resistant Enterococcus; PBP2a = penicillin-binding protein 2a. Data compiled from references.[2,7,10,14,15,19,46]

Table 3: Comprehensive comparison between conventional antibiotics and the proposed CRISPR-bilosome precision system.

Feature/Criterion	Conventional Antibiotic Chemotherapy	Proposed CRISPR-Bilosome Platform
Primary molecular target	Conserved bacterial physiological pathways (cell wall, ribosome, gyrase)[3,4]	Specific, unique DNA or RNA nucleotide sequences (resistance or essential genes) [12-15]
Spectrum of activity	Broad or class-dependent; indiscriminate killing of microflora[3,4]	Sequence-restricted and programmable; selectively spares non-target commensal microflora.[14-16]
Resistance-gene targeting	Indirect; exerts Darwinian selection pressure driving resistance amplification[5-7]	Direct; cleaves, disrupts, or permanently eliminates resistance determinants[14,19,46]
Plasmid curing capability	Extremely limited; cannot selectively eliminate transmissible resistance plasmids[5,6]	High; targeted cleavage of plasmid replication or resistance loci cures plasmids[16,19]
Cargo protection mechanism	Not applicable; small molecules face metabolic and enzymatic inactivation[3,4]	Bilosomal encapsulation shields nucleic acids from nucleases and acidic environments[34-37]
Adaptability to new mutations	Slow; requires multi-year chemical scaffold discovery and synthesis[1,3]	Rapid; requires redesigning only the 20-nucleotide guide RNA sequence[12,13]
Combination therapy potential	Commonly used (e.g., β-lactam + β-lactamase inhibitor), but resistance develops[3,5]	Ideal; co-delivers CRISPR resensitizer + antibiotic in a single bilosomal vesicle[34,39]
Biofilm penetration efficacy	Poor; diffusion impeded by extracellular matrix, leaving persister cells[7]	Enhanced; deformable bile salt vesicles penetrate dense biofilm layers[38,40]
Clinical development maturity	Fully established clinical standard of care[1-3]	Emerging preclinical proof-of-concept requiring formal translational validation[24,26,43]

Footnote: Tabulated comparative parameters compiled and synthesized from literature.[1-7,12-16,19,24,26,34-40,43,46]

MAJOR CHALLENGES, KNOWLEDGE GAPS, AND TRANSLATIONAL ROADMAP

Critical Translational Bottlenecks

Despite the compelling theoretical rationale, transitioning CRISPR-loaded bilosomes to clinical reality entails addressing key technical challenges:[21,24,26,35,41-45]

1. **Bacterial Cell Envelope and Biofilm Matrix Barriers:** The outer membrane of Gram-negative bacteria presents a hydrophobic barrier reinforced by divalent cation-stabilized LPS leaflets, while Gram-positive walls contain thick peptidoglycan layers. Furthermore, dense extracellular polymeric substance (EPS) matrices exclude conventional therapeutics and trap naked nucleic acids, a physiological barrier circumvented by deformable, membrane-fluidizing bilosomes (Figure 3).[7,26,38,40,44]

2. **Bacterial Anti-CRISPR Defenses:** Bacteriophages and plasmids have evolved anti-CRISPR proteins (Acr families) that directly inhibit Cas9 binding or nuclease activation, potentially neutralizing therapeutic gene editing.[41]

3. **Development of CRISPR Resistance:** Bacterial pathogens can acquire mutations in the target protospacer or PAM sequence, rendering single sgRNAs ineffective; multiplexed gRNAs directed against independent loci are essential to suppress mutational escape.[14,18,21]

4. **Nanomanufacturing Quality Control:** Achieving high batch reproducibility under GMP standards requires precise control over vesicle diameter (<150 nm), low polydispersity index (PDI < 0.2), high encapsulation efficiency (>75%), and long-term colloidal stability.[34,36,38]

5. **Regulatory and Safety Standards:** Demonstrating absence of host genomic off-target cleavage, lack of immunotoxicity, and safety across non-target commensal microbiomes.[24,26,43]

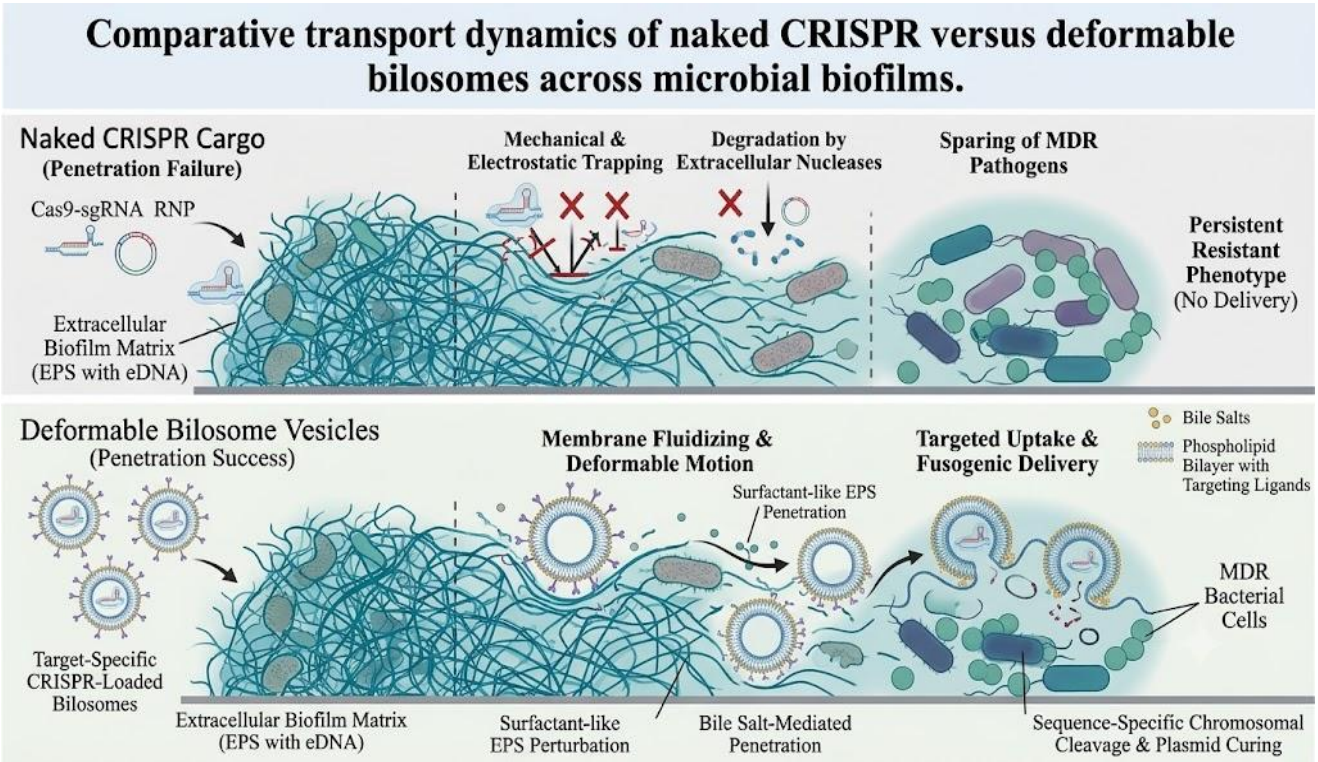


Figure 3: Comparative transport dynamics of naked CRISPR versus deformable bilosomes across microbial biofilms.

Diagrammatic comparison illustrating the transport failure of naked CRISPR cargo (top panel), which undergoes mechanical and electrostatic entrapment and extracellular nuclease degradation within the negative extracellular polymeric substance (EPS with eDNA) matrix, leaving deep-seated MDR pathogens untouched and phenotypically tolerant. In contrast, deformable bilosome vesicles (bottom panel) exploit surfactant-like EPS perturbation, enhanced membrane deformability, and bile salt-mediated fluidization to traverse dense biofilm barriers, fuse with recalcitrant bacteria, and execute sequence-specific chromosomal cleavage and plasmid curing.

Identification of Research Gaps

Current literature confirms progress in CRISPR antimicrobial mechanisms, non-viral gene delivery, and bilosomal formulation for small molecules. However, there is a distinct lack of published experimental research evaluating bilosomes specifically engineered to deliver CRISPR-Cas machinery into MDR bacteria. The membrane fusion kinetics, intracellular release efficiency, and in vivo pharmacokinetics of CRISPR-bilosomes remain entirely unexplored, representing an important research gap.[14-26,34-40]

Table 4: Stepwise experimental research roadmap and prioritization framework for CRISPR-loaded bilosome development.

Development Phase	Experimental Objective & Specific Investigation	Primary Evaluation Metrics	Translational Priority
Phase I: Nanocarrier Optimization	Formulate bilosomes with varying lipid-to-bile-salt ratios (DOPC:SDC:Chol)[34-36]	Particle size (<150 nm), PDI (<0.2), zeta potential, cryo-TEM lamellar morphology[34-37]	High
Phase II: CRISPR Cargo Loading	Evaluate loading of Cas9 RNP, Cas9 mRNA, and pDNA; assess nuclease protection[25,27,43]	Encapsulation efficiency (>75%), gel electrophoresis integrity, in vitro release profile[27,36]	High
Phase III: Pathogen Targeting & Uptake	Functionalize bilosomes with targeting ligands; evaluate bacterial membrane fusion[10,11,17]	Flow cytometry internalization, confocal fluorescence microscopy, membrane permeability assays[38,40]	High
Phase IV: Genetic Cleavage & Plasmid Curing	Target blaKPC, blaNDM, and mcr-1 resistance determinants in MDR isolates[14,19,46]	PCR amplicon sequencing, plasmid loss frequency, Western blot for target proteins[14,19]	High
Phase V: In Vitro Antibacterial Efficacy	Measure bactericidal kill curves, MIC/MBC shifts, and antibiotic resensitization[14-16]	Colony-forming unit (CFU) reduction, time-kill kinetics, fractional inhibitory concentration index[14,16]	High
Phase VI: Biofilm Eradication	Evaluate penetration and eradication of mature biofilms in microtiter plates[7,26]	Crystal violet biomass assay, confocal live/dead imaging, eDNA reduction assays[7,38]	Medium–High
Phase VII: Safety & Toxicological Profile	Screen biocompatibility against mammalian red blood cells and macrophage cell lines[34,37]	Hemolysis rate (<2%), MTT cytotoxicity assay, pro-inflammatory cytokine profiling[34,37]	High
Phase VIII: In Vivo Preclinical Validation	Assess therapeutic efficacy in murine bacteremia, pneumonia, and wound models[17,28,40]	Animal survival rates, organ bacterial clearance, tissue histopathology, pharmacokinetic profile[28,40]	High

Footnote: DOPC = 1,2-dioleoyl-sn-glycero-3-phosphocholine; SDC = sodium deoxycholate; Chol = cholesterol; PDI = polydispersity index; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration. Synthesized from references.[7,10,11,14,16,17,19,25-28,34-40,43,46]

CONCLUSION

Multidrug-resistant bacterial superbugs pose a catastrophic threat to modern healthcare, rendering empirical antibiotic therapies increasingly obsolete.[1-4] CRISPR-Cas systems provide a transformative paradigm shift from indiscriminate chemical poisoning to sequence-specific genetic eradication, capable of executing targeted chromosomal double-strand breaks, curing transmissible multi-resistance plasmids, and reversing antimicrobial resistance phenotypes.[14-19,46] Nevertheless, the realization of CRISPR antimicrobials has been bottlenecked by physiological delivery barriers.[23-26]

Bilosomes—flexible vesicular nanostructures stabilized by physiological deoxycholate or taurocholate—exhibit high bilayer deformability (deformability index > 15), protection against gastric and mucosal degradation, and natural membrane-permeating activity.[34-40] Formulating CRISPR payloads into these bile salt–modified vesicles provides a feasible route to cross dense lipopolysaccharide leaflets and penetrate the protective extracellular polymeric matrix of mature biofilms.[35-40,42-45] While this delivery platform remains in preclinical stages, standardizing vesicle size distribution (80–130 nm), optimizing guide multiplexing to curb protospacer escape mutations, and verifying safety in systemic animal models will be necessary steps to establish bilosomal CRISPR nanomedicines as viable countermeasures against multi-resistant pathogens.[1-4,14-19,34-40]

DECLARATIONS

Author's Contribution

Dipu Pan (Principal & Corresponding Author): Conceptualized the study, formulated the central research hypothesis of CRISPR-bilosome integration, developed the overarching review design, conducted extensive literature retrieval across primary scientific databases, synthesized the molecular mechanisms of CRISPR-Cas and bilosomal nanocarriers, drafted the original manuscript, designed and curated all four scientific tables, performed critical revision of the manuscript for rigorous intellectual content, coordinated feedback among co-authors, and approved the final version for submission.

Shubhajit Sarkar (Co-Author): Contributed significantly to literature collection and screening regarding multidrug-resistant ESKAPE pathogens, assisted in detailing bacterial resistance mechanisms (enzymatic degradation, efflux systems, and mobile genetic elements), critically reviewed and revised the mechanistic sections of the manuscript, and participated in structuring the comparative tables.

Bipasha De (Co-Author): Assisted in gathering literature on non-viral nanocarriers and compiling data on vesicular drug delivery systems.

Amit Mia (Co-Author): Contributed to literature retrieval concerning Class 2 CRISPR-Cas9 and Cas12a endonuclease mechanisms.

Ratnaprava Das (Co-Author): Aided in literature screening related to bacterial biofilm formation and extracellular polymeric substance penetration.

Soumita Guha (Co-Author): Assisted in collating data on bile salt chemistry and physicochemical attributes of bilosomal bilayers.

Sachin Biswas (Co-Author): Contributed to reviewing literature on plasmid-mediated horizontal gene transfer and plasmid curing strategies.

Risha Saha (Co-Author): Aided in searching publications regarding preclinical animal models of bacterial sepsis and pneumonia.

Sudipta Mukherjee (Co-Author): Pharmacological review, contributed to compiling literature on regulatory considerations and quality-by-design standards for nanomedicines.

Sagarika Ghosh (Co-Author): Pharmacological review, Assisted with bibliographic cross-referencing and formatting citations in accordance with journal guidelines.

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