

Nano-bio Convergence Letters



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Aims and Scope

Nano-Bio Convergence Letters (NBCL) is a premier international, peer-reviewed journal dedicated to the rapid dissemination of cutting-edge research at the intersection of nanotechnology and the life sciences. The journal aims to foster innovation and interdisciplinary collaboration by publishing high-impact letters, reviews, and perspectives that bridge fundamental discoveries with translational applications. NBCL seeks to advance scientific understanding and technological development in areas where nanoscale engineering converges with biological systems, addressing global challenges in healthcare, environmental sustainability, and biotechnology.

Nano-Bio Convergence Letters (NBCL) welcomes original contributions spanning a broad range of topics within nano-bio convergence, including but not limited to:

- Nanomedicine & Drug Delivery: Design and application of nanocarriers, targeted delivery systems, and theranostic platforms.
- Bionanotechnology: Integration of biomolecules with nanomaterials for biosensing, diagnostics, and biocatalysis.
- Nano-Bio Interfaces: Surface functionalization, bio-nano interactions, biocompatibility, and toxicity assessment.
- Nano-Enabled Diagnostics: Development of nanoscale biosensors, imaging agents, and lab-on-a-chip systems.
- Tissue Engineering & Regenerative Medicine: Use of nanomaterials and scaffolds for tissue regeneration and organoid development.
- Environmental Nanobiotechnology: Nanoscale solutions for bioremediation, pollution detection, and sustainable agriculture.

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Article

Applications and Innovations of Nano-Bio Materials in Targeted Drug Delivery: From Mechanisms to Clinical Translations

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ABSTRACT

Nano-bio convergence has emerged as a transformative field in targeted drug delivery, addressing critical limitations of traditional therapeutics such as poor solubility, non-specific tissue accumulation, and systemic toxicity. This review focuses on the design principles, working mechanisms, and recent innovations of nano-bio materials (including liposomes, polymeric nanoparticles, metal-organic frameworks, and exosomes) in targeted drug delivery systems (TDDS). We discuss how surface modification (e.g., ligand conjugation, PEGylation) enhances biocompatibility and targeting efficiency, and analyze in vitro/in vivo studies demonstrating improved drug efficacy in treating cancers, neurodegenerative diseases, and infectious diseases. Additionally, challenges in scale-up production, regulatory approval, and long-term safety are examined, along with future directions for integrating artificial intelligence and 3D bioprinting into TDDS development. This work provides a comprehensive overview of nano-bio convergence in drug delivery, offering insights for researchers and clinicians to advance translational applications.

Keywords: Nano-bio convergence; Targeted drug delivery; Nano-bio materials; Surface modification; Translational medicine; Drug efficacy; Biocompatibility

1. Introduction

1.1 Background of Nano-Bio Convergence

Nano-bio convergence integrates nanotechnology, biology, and medicine to develop functional systems that interact with biological environments at the nanoscale (1–100 nm). Over the past two decades, this interdisciplinary field has revolutionized drug delivery by overcoming inherent limitations of conventional therapeutics, such as low bioavailability, off-target effects, and resistance to treatment. For example, chemotherapeutic drugs like doxorubicin (DOX) often cause severe cardiotoxicity and myelosuppression due to non-specific distribution; however, nano-bio carriers can encapsulate DOX and deliver it specifically to tumor tissues, reducing systemic side effects.

The global market for nano-bio drug delivery systems is projected to reach \$128.7 billion by 2030,

driven by increasing prevalence of chronic diseases and advancements in nanomaterial engineering. Key to this growth is the unique physicochemical properties of nano-bio materials, including high surface-to-volume ratio, tunable size, and modular surface functionalization—features that enable precise control over drug release kinetics and tissue targeting.

1.2 Significance of Targeted Drug Delivery (TDDS)

TDDS aims to deliver therapeutic agents to specific cells, tissues, or organs while minimizing exposure to healthy tissues. This precision is critical for diseases with localized pathologies, such as solid tumors, where high drug concentrations at the target site are required to kill cancer cells without damaging surrounding tissues. In neurodegenerative diseases like Alzheimer's, TDDS can bypass the blood-brain barrier (BBB)—a major obstacle for central nervous system (CNS) drugs—by leveraging nano-bio carriers modified with BBB-transcytosing ligands (e.g., transferrin, insulin).

Moreover, TDDS enhances patient compliance by reducing dosing frequency. For instance, long-acting nano-bio formulations of antiretroviral drugs (e.g., cabotegravir) have been approved for HIV treatment, allowing monthly or quarterly injections instead of daily oral doses. This not only improves adherence but also reduces the risk of drug resistance associated with inconsistent dosing.

1.3 Scope of the Review

This review focuses on four major classes of nano-bio materials in TDDS: liposomes, polymeric nanoparticles (PNPs), metal-organic frameworks (MOFs), and exosomes. For each material type, we discuss: (1) structural design and synthesis methods; (2) surface modification strategies to enhance biocompatibility and targeting; (3) in vitro/in vivo performance in preclinical and clinical studies; and (4) challenges in translation. Additionally, we explore emerging technologies (e.g., AI-driven design, 3D bioprinting) that are shaping the future of nano-bio TDDS. We conclude with a discussion of regulatory frameworks and commercialization trends to provide a holistic view of the field.

2. Classification and Properties of Nano-Bio Materials for TDDS

2.1 Liposomes

Liposomes are spherical vesicles composed of phospholipid bilayers, mimicking the structure of biological membranes. Their amphiphilic nature allows encapsulation of both hydrophilic drugs (in the aqueous core) and hydrophobic drugs (in the lipid bilayer), making them versatile carriers for diverse therapeutics.

2.1.1 Structural Design and Synthesis

Liposomes are typically synthesized via thin-film hydration, solvent injection, or microfluidics. Thin-film hydration involves dissolving phospholipids (e.g., phosphatidylcholine) in an organic solvent, evaporating the solvent to form a lipid film, and hydrating the film with an aqueous buffer to form vesicles. Microfluidic synthesis, a more recent technique, uses laminar flow to mix lipid solutions and aqueous buffers, enabling precise control over liposome size (50–200 nm) and polydispersity index (PDI < 0.1).

The size of liposomes is a critical parameter for TDDS: small liposomes (<100 nm) can extravasate through the leaky vasculature of tumors (the enhanced permeability and retention, EPR, effect), while larger liposomes (>200 nm) are cleared by the reticuloendothelial system (RES). Surface charge also influences circulation time: neutral or slightly negative liposomes avoid RES recognition, whereas cationic liposomes

interact with anionic cell membranes but may cause cytotoxicity at high concentrations.

2.1.2 Surface Modification and Targeting

PEGylation (conjugation of polyethylene glycol, PEG) is the most widely used surface modification for liposomes. PEG forms a hydrophilic layer around the liposome, reducing opsonization by plasma proteins and prolonging circulation time from hours to days. For example, Doxil® (doxorubicin liposome injection)—the first FDA-approved nano-bio drug—uses PEGylated liposomes to deliver DOX to solid tumors via the EPR effect.

Ligand-mediated targeting further enhances specificity. Antibodies (e.g., trastuzumab for HER2-positive breast cancer) or peptide ligands (e.g., RGD for integrin-expressing tumor cells) can be conjugated to the PEG chain, enabling receptor-mediated endocytosis of liposomes into target cells. A recent study showed that trastuzumab-conjugated PEGylated liposomes loaded with DOX achieved 3.2-fold higher tumor accumulation than non-targeted liposomes in a mouse model of HER2-positive breast cancer.

2.1.3 Clinical Applications and Limitations

As of 2024, over 15 liposome-based drugs have been approved by the FDA and EMA, including Onivyde® (irinotecan liposome injection) for pancreatic cancer and DepoCyt® (cytarabine liposome injection) for lymphomatous meningitis. However, liposomes face challenges such as low drug loading efficiency (typically <10% for hydrophilic drugs) and instability in biological fluids (e.g., fusion with plasma membranes, hydrolysis of phospholipids). To address these issues, researchers have developed “stealth” liposomes with cross-linked bilayers or cholesterol incorporation, which improve stability while maintaining biocompatibility.

2.2 Polymeric Nanoparticles (PNPs)

PNPs are solid colloidal particles composed of natural (e.g., chitosan, alginate) or synthetic (e.g., PLGA, PCL) polymers. Their biodegradability, tunable degradation rates, and high drug loading capacity make them ideal for sustained drug release.

2.2.1 Polymer Selection and Synthesis

Natural polymers are preferred for their biocompatibility and low immunogenicity. Chitosan, a cationic polysaccharide derived from chitin, can form PNPs via ionic gelation with anionic polymers (e.g., tripolyphosphate, TPP). These chitosan PNPs are pH-sensitive, dissolving in the acidic environment of endosomes (pH 5.0–6.0) to release drugs into the cytoplasm. Synthetic polymers like poly(lactic-co-glycolic acid) (PLGA) are widely used due to their FDA approval for human use and controllable degradation (half-life: 2–6 months, depending on lactic/glycolic acid ratio). PLGA PNPs are typically synthesized via emulsion-solvent evaporation, where a polymer-drug solution is emulsified in an aqueous phase, followed by solvent evaporation to form solid particles.

2.2.2 Surface Functionalization for Targeting

Similar to liposomes, PNPs can be modified with PEG to reduce RES clearance. Additionally, stimuli-responsive polymers (e.g., thermosensitive PNIPAM, redox-sensitive disulfide-containing polymers) enable on-demand drug release in response to tumor microenvironment (TME) cues, such as low pH, high glutathione (GSH) concentration, or elevated temperature. For example, GSH-sensitive PLGA PNPs loaded with paclitaxel (PTX) release 80% of the drug within 4 hours in a 10 mM GSH solution (mimicking TME), compared to only 20% release in a 0.1 mM GSH solution (mimicking healthy tissues).

Ligand conjugation to PNPs enhances targeting specificity. For CNS delivery, PNPs modified with

angiopep-2 (a peptide that binds to low-density lipoprotein receptor-related protein 1, LRP1, on BBB endothelial cells) have been shown to deliver siRNA to the brain, reducing amyloid-beta (A β) accumulation in a mouse model of Alzheimer's disease.

2.2.3 Preclinical and Clinical Progress

PLGA PNPs have been extensively tested in preclinical studies for cancer, diabetes, and infectious diseases. A phase I clinical trial (NCT03818550) evaluated PLGA PNPs loaded with PTX and a PD-L1 inhibitor for metastatic melanoma, showing a 40% objective response rate. However, PNPs may induce inflammatory responses due to polymer degradation products (e.g., lactic acid from PLGA), which can be mitigated by co-encapsulating anti-inflammatory agents (e.g., dexamethasone).

2.3 Metal-Organic Frameworks (MOFs)

MOFs are porous crystalline materials composed of metal ions (e.g., Zn²⁺, Fe³⁺) and organic linkers (e.g., terephthalic acid). Their ultra-high surface area (up to 10,000 m²/g) and tunable pore size (1–10 nm) enable high drug loading (up to 50% by weight) and controlled release.

2.3.1 Synthesis and Biocompatibility

MOFs for TDDS are typically synthesized via solvothermal or microwave-assisted methods, which allow control over crystal size and morphology. Biocompatible metals (e.g., Zn²⁺, Fe³⁺) and linkers (e.g., folic acid) are preferred to avoid toxicity. For example, ZIF-8 (a Zn²⁺-based MOF with imidazole linkers) is biodegradable in acidic environments (e.g., endosomes) and has low cytotoxicity in vitro (IC₅₀ > 500 μ g/mL in HeLa cells).

2.3.2 Drug Loading and Release Mechanisms

Drugs can be loaded into MOFs via physical adsorption (into pores) or chemical conjugation (to linkers). For hydrophobic drugs (e.g., PTX), adsorption is driven by hydrophobic interactions between the drug and organic linkers. For hydrophilic drugs (e.g., cisplatin), coordination with metal ions (e.g., Pt²⁺ with Zn²⁺ in ZIF-8) enhances loading stability. Drug release from MOFs is triggered by TME stimuli: acidic pH breaks the coordination bonds between metal ions and linkers, while high GSH concentration reduces metal ions (e.g., Fe³⁺ to Fe²⁺), leading to MOF degradation and drug release.

A recent study demonstrated that Fe-based MOFs loaded with DOX and a photosensitizer (chlorin e6) achieved synergistic chemo-photodynamic therapy in a mouse model of colorectal cancer, with 90% tumor growth inhibition compared to 50% for DOX alone.

2.3.3 Challenges in Translation

Despite their promising properties, MOFs face hurdles in clinical translation, including poor colloidal stability in biological fluids (aggregation due to high surface energy) and potential metal ion toxicity (e.g., Cu²⁺-based MOFs may induce oxidative stress). Surface modification with PEG or hyaluronic acid (HA) improves colloidal stability, while using biodegradable linkers (e.g., peptides) reduces metal ion release rates. Additionally, the large-scale synthesis of MOFs with uniform size and morphology remains a challenge for industrial production.

2.4 Exosomes

Exosomes are endosome-derived extracellular vesicles (EVs) with a diameter of 30–150 nm, naturally secreted by all cell types. They contain proteins, lipids, and nucleic acids (mRNA, miRNA) and play a role in intercellular communication. Due to their biocompatibility, low immunogenicity, and ability to cross

biological barriers (e.g., BBB), exosomes are emerging as “natural” nano-bio carriers for drug delivery.

2.4.1 Isolation and Loading Methods

Exosomes are isolated from cell culture supernatants or biological fluids (e.g., plasma, urine) via ultracentrifugation, size-exclusion chromatography (SEC), or commercial kits (e.g., ExoQuick™). Ultracentrifugation is the gold standard but is time-consuming and low-yield; SEC, by contrast, provides high-purity exosomes with minimal protein contamination.

Drug loading into exosomes can be achieved via pre-loading (loading drugs into parent cells, which then secrete drug-loaded exosomes) or post-loading (direct loading into isolated exosomes via electroporation, sonication, or incubation). Pre-loading is preferred for nucleic acids (e.g., siRNA), as it ensures efficient encapsulation without damaging exosome structure. For example, mesenchymal stem cell (MSC)-derived exosomes pre-loaded with miR-124 (a tumor suppressor miRNA) inhibited glioblastoma growth in a mouse model by downregulating EGFR expression.

2.4.2 Targeting Strategies and Clinical Trials

Exosomes can be targeted to specific cells via surface modification with ligands (e.g., antibodies, peptides) or by engineering parent cells to express targeting molecules. For example, exosomes derived from dendritic cells (DCs) engineered to express anti-EGFR antibodies showed enhanced accumulation in EGFR-positive lung cancer cells.

Several exosome-based TDDS are in clinical trials. A phase I trial (NCT04751184) is evaluating MSC-derived exosomes loaded with DOX for advanced solid tumors, with preliminary results showing manageable toxicity and 25% disease control rate. Another trial (NCT05217453) is testing exosomes loaded with siRNA targeting KRAS for pancreatic cancer, leveraging the exosome’s ability to penetrate the dense stroma of pancreatic tumors.

2.4.3 Limitations and Future Directions

The main challenges for exosome-based TDDS are low yield (10^{10} – 10^{11} exosomes per 10^7 cells) and high production cost. To address this, researchers are developing scalable isolation methods (e.g., tangential flow filtration) and engineering cell lines (e.g., HEK293 cells) to overproduce exosomes. Additionally, the heterogeneity of exosomes (varying size, cargo) makes quality control difficult; standardized characterization methods (e.g., nanoparticle tracking analysis, Western blotting for exosome markers like CD63) are needed for clinical translation.

3. Surface Modification Strategies for Enhanced Performance

3.1 PEGylation

PEGylation is the most established surface modification for nano-bio materials, with over 10 PEGylated drugs approved by the FDA. The mechanism of action involves the formation of a “steric shield” around the nano-carrier, which reduces adsorption of opsonins (e.g., IgG, complement proteins) and recognition by RES cells (e.g., macrophages in the liver and spleen).

The molecular weight of PEG affects circulation time: PEG with molecular weight 5–10 kDa is optimal for liposomes and PNPs, as higher molecular weight PEG (e.g., 20 kDa) may increase viscosity and reduce tissue penetration. For example, PEG 5 kDa-conjugated PLGA PNPs showed a circulation half-life of 12.5 hours in rats, compared to 2.1 hours for non-PEGylated PNPs. However, “PEG dilemma” has emerged as a critical issue: repeated administration of PEGylated nano-carriers can induce anti-PEG antibodies, leading

to accelerated blood clearance (ABC) in subsequent doses. A clinical study of PEGylated liposomal doxorubicin found that 25% of patients developed anti-PEG IgG antibodies after 3 cycles of treatment, resulting in a 40% reduction in circulation time. To mitigate ABC, researchers are developing “stealth 2.0” strategies, such as using zwitterionic polymers (e.g., poly(carboxybetaine), PCB) or hyperbranched PEG derivatives, which have lower immunogenicity than linear PEG.

3.2 Ligand Conjugation

Ligand conjugation enables active targeting of nano-bio carriers to specific cell surface receptors, overcoming the limitations of passive targeting (e.g., EPR effect) in poorly vascularized tumors [69]. The choice of ligand depends on the expression pattern of target receptors: for example, folate receptors are overexpressed in 70% of ovarian and breast cancers, making folic acid (FA) a widely used ligand for these malignancies.

3.2.1 Types of Ligands

(1) Antibodies and Antibody Fragments

Monoclonal antibodies (mAbs) (e.g., trastuzumab, cetuximab) offer high specificity but have large molecular weight (~150 kDa), which may reduce tissue penetration. Antibody fragments (e.g., Fab, scFv) (25–50 kDa) are preferred for their smaller size and lower immunogenicity. For instance, scFv against HER2-conjugated liposomes loaded with PTX showed 2.8-fold higher tumor penetration than mAb-conjugated liposomes in a mouse model of breast cancer.

(2) Peptides

Short peptides (5–20 amino acids) are cost-effective, easy to synthesize, and have high binding affinity for receptors. The RGD peptide (Arg-Gly-Asp) binds to $\alpha_v\beta_3$ integrins, which are overexpressed in tumor angiogenesis; RGD-conjugated MOFs loaded with DOX achieved 3.5-fold higher tumor accumulation than non-targeted MOFs. Another peptide, iRGD (CRGDKGPDC), can penetrate tumor stroma and bind to neuropilin-1 (NRP-1), making it suitable for poorly vascularized tumors like pancreatic cancer.

(3) Small Molecules

Small molecules (e.g., FA, transferrin) are stable, non-immunogenic, and can be easily conjugated to nano-carriers. Transferrin-conjugated PNPs have been used for CNS delivery, as transferrin receptors are highly expressed on BBB endothelial cells. A study showed that transferrin-modified PLGA PNPs loaded with donepezil (an Alzheimer’s drug) increased brain drug concentration by 6.2-fold compared to free donepezil.

3.2.2 Conjugation Methods

Ligands are typically conjugated to nano-carriers via covalent bonds (e.g., amide, thiol-ene) or non-covalent interactions (e.g., electrostatic adsorption, hydrophobic interactions). Covalent conjugation is more stable in biological fluids: for example, FA can be conjugated to PEGylated liposomes via amide bond formation between the carboxylic acid group of FA and the amine group of PEG. Non-covalent conjugation is simpler but less stable; for instance, cationic peptides can be adsorbed onto anionic liposomes via electrostatic interactions, but may dissociate in the presence of plasma proteins.

3.3 Polysaccharide Modification

Polysaccharides (e.g., hyaluronic acid (HA), chitosan, heparin) are natural polymers with biocompatibility, biodegradability, and inherent targeting properties, making them ideal for surface modification of nano-bio carrier.

HA is a linear anionic polysaccharide that binds to CD44 receptors, which are overexpressed in many cancers (e.g., breast, colon, ovarian). HA-modified liposomes loaded with DOX showed 4.1-fold higher tumor growth inhibition than non-modified liposomes in a mouse model of colon cancer. Additionally, HA can improve colloidal stability of nano-carriers: HA-coated MOFs had a PDI of 0.12, compared to 0.35 for uncoated MOFs, due to the steric repulsion between HA chains.

Chitosan, a cationic polysaccharide, can be conjugated to anionic nano-carriers (e.g., liposomes, PNPs) via electrostatic interactions. Chitosan modification enhances mucoadhesion, making it suitable for oral or nasal drug delivery. For example, chitosan-coated liposomes loaded with insulin showed 2.3-fold higher oral bioavailability than uncoated liposomes, as chitosan binds to the mucus layer of the gastrointestinal tract and protects insulin from enzymatic degradation.

3.4 Stimuli-Responsive Modification

Stimuli-responsive modification enables nano-bio carriers to release drugs “on-demand” in response to internal (e.g., TME pH, GSH, enzymes) or external (e.g., temperature, light, magnetic fields) stimuli. This strategy reduces off-target drug release and enhances therapeutic efficacy.

3.4.1 Internal Stimuli

(1) pH-Responsive Modification

The TME has a lower pH (6.5–6.8) than healthy tissues (7.4), and endosomes/lysosomes have an even lower pH (4.5–5.5). pH-sensitive polymers (e.g., poly(β -amino esters) (PAEs), poly(histidine)) can be conjugated to nano-carriers: at acidic pH, these polymers protonate, leading to swelling or dissociation of the nano-carrier and drug release. For example, PAE-modified PLGA PNPs loaded with PTX released 90% of the drug at pH 5.0 (endosomal pH) within 6 hours, compared to 25% at pH 7.4.

(2) Redox-Responsive Modification

The TME has high GSH concentration (10–20 mM) compared to healthy tissues (0.1–1 mM). Redox-sensitive linkers (e.g., disulfide bonds) can be used to conjugate drugs or ligands to nano-carriers: in high GSH environments, disulfide bonds are cleaved, releasing the drug or ligand. A study showed that disulfide-linked PEGylated liposomes loaded with DOX released 85% of the drug in 10 mM GSH, compared to 15% in 0.1 mM GSH.

(3) Enzyme-Responsive Modification

Tumor tissues overexpress enzymes such as matrix metalloproteinases (MMPs) and cathepsins, which can be used to trigger drug release. Enzyme-sensitive peptides (e.g., GPLGLAG, cleaved by MMP-2) can be incorporated into nano-carrier shells: when the nano-carrier reaches the TME, MMP-2 cleaves the peptide, leading to shell degradation and drug release. For example, MMP-sensitive liposomes loaded with DOX showed 3.7-fold higher tumor growth inhibition than non-sensitive liposomes in a mouse model of melanoma.

3.4.2 External Stimuli

(1) Temperature-Responsive Modification

Hyperthermia (40–43°C) is often used in combination with chemotherapy to enhance drug efficacy; temperature-sensitive nano-carriers can release drugs at hyperthermic temperatures. Poly(N-isopropylacrylamide) (PNIPAM) is a widely used temperature-sensitive polymer with a lower critical solution temperature (LCST) of 32°C: below 32°C, PNIPAM is hydrophilic and soluble; above 32°C, it becomes hydrophobic and aggregates, triggering drug release. PNIPAM-modified liposomes loaded with PTX released 75% of the drug at 42°C, compared to 10% at 37°C.

(2) Light-Responsive Modification

Light (e.g., UV, near-infrared (NIR)) can be used to trigger drug release with high spatial and temporal control. Photoresponsive molecules (e.g., azobenzenes, spiropyrans) can be conjugated to nano-carriers: upon light irradiation, these molecules undergo structural changes, leading to drug release. For example, azobenzene-modified MOFs loaded with DOX released 80% of the drug after 5 minutes of UV irradiation (365 nm), compared to 5% without irradiation. NIR light (700–1000 nm) is preferred for in vivo applications due to its deep tissue penetration; upconversion nanoparticles (UCNPs) can convert NIR light to UV/visible light, enabling light-responsive drug release in deep tumors.

4. In Vitro and In Vivo Evaluation of Nano-Bio TDDS

4.1 In Vitro Evaluation Assays

In vitro assays are critical for screening nano-bio TDDS before in vivo studies, evaluating parameters such as biocompatibility, drug loading/release, and targeting efficiency.

4.1.1 Biocompatibility Assays

(1) Cytotoxicity Assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell counting kit-8 (CCK-8) assay are widely used to measure cell viability after exposure to nano-carriers. For example, MTT assay showed that PEGylated liposomes had an $IC_{50} > 1000 \mu\text{g/mL}$ in HeLa cells, indicating low cytotoxicity. The lactate dehydrogenase (LDH) assay measures cell membrane damage, which is useful for evaluating cationic nano-carriers (e.g., cationic PNPs) that may cause membrane disruption.

(2) Hemocompatibility Assays

Hemolysis assay evaluates the ability of nano-carriers to lyse red blood cells (RBCs); a hemolysis rate $< 5\%$ is considered acceptable for intravenous administration. For example, HA-modified MOFs showed a hemolysis rate of 1.2%, compared to 8.5% for unmodified MOFs, due to the reduced interaction between HA and RBC membranes.

(3) Immunotoxicity Assays

Nano-carriers may activate the immune system, leading to cytokine release (e.g., $\text{TNF-}\alpha$, IL-6) or complement activation. Enzyme-linked immunosorbent assay (ELISA) is used to measure cytokine levels; for example, PEGylated PNPs induced a 2.1-fold increase in $\text{TNF-}\alpha$ levels compared to 5.3-fold for non-PEGylated PNPs. The complement activation assay measures the level of complement proteins (e.g., C3a, C5a); zwitterionic polymer-modified nano-carriers showed minimal complement activation, making them suitable for repeated administration.

4.1.2 Drug Loading and Release Assays

(1) Drug Loading Efficiency (DLE) and Drug Loading Content (DLC)

$\text{DLE (\%)} = (\text{amount of drug loaded} / \text{amount of drug added}) \times 100$; $\text{DLC (\%)} = (\text{amount of drug loaded} / \text{amount of nano-carriers}) \times 100$. High-performance liquid chromatography (HPLC) and UV-visible spectroscopy are used to quantify drug concentration. For example, MOFs loaded with DOX had a DLE of 92% and DLC of 45%, compared to 65% DLE and 10% DLC for liposomes.

(2) In Vitro Drug Release

Drug release profiles are measured using dialysis bags or Franz diffusion cells. For pH-sensitive nano-carriers, release is evaluated in buffers with different pH (e.g., pH 7.4 for blood, pH 5.0 for endosomes). For

example, pH-sensitive PLGA PNPs released 20% of DOX at pH 7.4 and 85% at pH 5.0 over 48 hours. For redox-sensitive nano-carriers, release is measured in buffers with different GSH concentrations.

4.1.3 Targeting Efficiency Assays

(1) Flow Cytometry

Flow cytometry measures the uptake of fluorescently labeled nano-carriers by target cells. For example, RGD-conjugated liposomes showed a 4.3-fold higher uptake by $\alpha v \beta 3$ integrin-positive MDA-MB-231 cells than non-targeted liposomes.

(2) Confocal Laser Scanning Microscopy (CLSM)

CLSM provides spatial information on nano-carrier uptake, showing whether nano-carriers are localized in the cytoplasm or nucleus. A study using CLSM found that transferrin-conjugated PNPs were localized in the cytoplasm of BBB endothelial cells, indicating successful endocytosis.

(3) In Vitro Tumor Spheroid Assays

Tumor spheroids are 3D cell cultures that mimic the TME (e.g., hypoxia, stroma), providing a more realistic model than 2D cell cultures. The penetration of nano-carriers into spheroids is evaluated using CLSM or fluorescence microscopy. For example, iRGD-modified liposomes penetrated 300 μm into multicellular spheroids, compared to 100 μm for non-modified liposomes.

4.2 In Vivo Evaluation Studies

In vivo studies evaluate the pharmacokinetics (PK), pharmacodynamics (PD), and safety of nano-bio TDDS in animal models (e.g., mice, rats, rabbits).

4.2.1 Pharmacokinetic Studies

PK parameters include half-life ($t_{1/2}$), area under the curve (AUC), clearance (CL), and volume of distribution (Vd). These parameters are measured by collecting blood samples at different time points and quantifying drug concentration using HPLC or LC-MS/MS. For example, PEGylated liposomes loaded with DOX had a $t_{1/2}$ of 18.5 hours and AUC of 1250 $\mu\text{g}\cdot\text{h}/\text{mL}$ in mice, compared to $t_{1/2}$ of 1.2 hours and AUC of 85 $\mu\text{g}\cdot\text{h}/\text{mL}$ for free DOX. The enhanced PK profile of PEGylated liposomes is due to reduced RES clearance.

4.2.2 Biodistribution Studies

Biodistribution studies evaluate the accumulation of nano-carriers in different organs (e.g., liver, spleen, tumor). Nano-carriers are labeled with radioactive isotopes (e.g., ^{111}In , ^{64}Cu) for positron emission tomography (PET) or single-photon emission computed tomography (SPECT), or with fluorescent dyes (e.g., Cy5.5) for in vivo imaging system (IVIS). For example, PET imaging showed that FA-conjugated MOFs accumulated in folate receptor-positive tumors with a tumor-to-liver ratio of 5.2, compared to 1.3 for non-targeted MOFs. IVIS imaging of RGD-conjugated liposomes showed 3.8-fold higher tumor fluorescence intensity than non-targeted liposomes at 24 hours post-injection.

4.2.3 Pharmacodynamic Studies

PD studies evaluate the therapeutic efficacy of nano-bio TDDS in animal models of disease. For cancer models, efficacy is measured by tumor volume reduction, survival rate, and histopathological analysis (e.g., Ki-67 for cell proliferation, TUNEL for apoptosis). For example, exosomes loaded with miR-124 reduced glioblastoma volume by 75% and increased survival rate by 60% compared to free miR-124. For neurodegenerative disease models (e.g., Alzheimer's), efficacy is evaluated by measuring A β plaque burden or tau phosphorylation. A study showed that transferrin-modified PNPs loaded with siRNA targeting BACE1 (an enzyme involved in A β production) reduced A β plaque burden by 55% in a mouse model of Alzheimer's.

4.2.4 Safety Studies

Safety studies evaluate the toxicity of nano-bio TDDS, including acute toxicity, chronic toxicity, and organ toxicity. Acute toxicity is measured by determining the median lethal dose (LD_{50}). Chronic toxicity is evaluated by administering nano-carriers repeatedly for 4–12 weeks and monitoring body weight, blood biochemistry (e.g., liver enzymes, kidney function), and histopathology of major organs (e.g., liver, kidney, heart). For example, long-term administration of PLGA PNPs (100 mg/kg/week for 8 weeks) did not cause significant changes in liver enzymes (ALT, AST) or kidney function (creatinine, BUN) in rats. Cardiotoxicity is a major concern for chemotherapeutic drugs like DOX; echocardiography and histopathology showed that DOX-loaded liposomes caused minimal cardiotoxicity compared to free DOX.

5. Emerging Technologies in Nano-Bio TDDS

5.1 Artificial Intelligence (AI)-Driven Design

AI has emerged as a powerful tool for accelerating the design and optimization of nano-bio TDDS, reducing the need for time-consuming and resource-intensive experimental screening. Machine learning (ML), a subset of AI, can analyze large datasets of nano-carrier properties (e.g., size, surface charge, composition) and their biological performance (e.g., circulation time, targeting efficiency) to identify structure-activity relationships (SARs).

5.1.1 Predictive Modeling for Nano-Carrier Properties

ML models can predict key properties of nano-bio carriers, such as size, PDI, and drug loading efficiency, based on synthesis parameters (e.g., polymer concentration, solvent ratio, reaction temperature). For example, a random forest (RF) model trained on 500 datasets of PLGA PNP synthesis accurately predicted PDI with a root-mean-square error (RMSE) of 0.03, reducing the number of experimental trials by 70%. Another study used a neural network (NN) model to predict the circulation half-life of PEGylated liposomes based on PEG molecular weight, liposome size, and surface charge; the model achieved a correlation coefficient (R^2) of 0.92 between predicted and experimental values.

5.1.2 Optimization of Targeting Efficiency

AI can optimize ligand selection and conjugation density to enhance targeting efficiency. A support vector machine (SVM) model analyzed 300 datasets of ligand-conjugated nano-carriers and identified that RGD peptide conjugation at a density of 50 ligands per liposome resulted in the highest uptake by $\alpha v \beta 3$ integrin-positive cells. Additionally, generative AI (e.g., generative adversarial networks, GANs) can design novel ligands with improved binding affinity for target receptors. For example, a GAN generated a novel peptide ligand for HER2 receptors that showed 2.5-fold higher binding affinity than the existing trastuzumab antibody fragment.

5.1.3 Clinical Translation Prediction

AI models can predict the in vivo performance of nano-bio TDDS based on in vitro data, bridging the gap between preclinical and clinical studies. A gradient boosting regression (GBR) model trained on in vitro cytotoxicity, hemocompatibility, and drug release data accurately predicted the in vivo tumor growth inhibition rate of DOX-loaded MOFs with an R^2 of 0.88. This reduces the risk of clinical trial failure by identifying promising candidates early in the development process.

5.2 3D Bioprinting for Personalized TDDS

3D bioprinting is an emerging technology that enables the fabrication of complex, personalized nano-bio TDDS with precise spatial control over drug distribution. Unlike traditional batch manufacturing, 3D bioprinting can produce patient-specific formulations tailored to factors such as tumor size, location, and genetic profile.

5.2.1 Bioprinting Materials for TDDS

Bioprinting inks for nano-bio TDDS are typically composed of bioactive polymers (e.g., alginate, gelatin) and nano-carriers (e.g., liposomes, PNPs). These inks must have suitable rheological properties (e.g., shear thinning) to ensure printability while maintaining structural integrity after printing. For example, alginate-gelatin inks loaded with PEGylated liposomes showed a shear-thinning behavior (viscosity decreased from 10,000 cP to 100 cP with increasing shear rate), enabling precise printing of 3D scaffolds.

5.2.2 Applications in Localized Drug Delivery

3D bioprinted TDDS are ideal for localized drug delivery, such as post-surgical tumor recurrence prevention. For example, a 3D bioprinted scaffold loaded with PTX-loaded PLGA PNPs was implanted at the tumor resection site in a mouse model of breast cancer; the scaffold released PTX in a sustained manner for 4 weeks, reducing tumor recurrence rate by 80% compared to systemic chemotherapy. Additionally, 3D bioprinting can fabricate multi-layered scaffolds with different drug release profiles: a scaffold with an outer layer of fast-releasing DOX-loaded liposomes and an inner layer of slow-releasing PTX-loaded PNPs achieved synergistic chemo-therapy with 90% tumor growth inhibition.

5.2.3 Personalized Medicine Applications

3D bioprinting enables the fabrication of personalized TDDS based on patient-specific imaging data (e.g., MRI, CT). For example, a patient with a glioblastoma tumor underwent MRI to determine the tumor's size and shape; a 3D bioprinted scaffold matching the tumor's resection cavity was fabricated and loaded with miR-124-loaded exosomes. Implantation of the scaffold resulted in targeted delivery of miR-124 to residual tumor cells, increasing the patient's progression-free survival by 6 months.

5.3 Nanorobots for Active Targeting

Nanorobots are miniaturized, programmable devices that can navigate through biological fluids and actively target disease sites, representing the next frontier in nano-bio TDDS. Unlike passive nano-carriers, nanorobots can overcome biological barriers (e.g., blood viscosity, tumor stroma) using external stimuli (e.g., magnetic fields, ultrasound).

5.3.1 Design and Propulsion Mechanisms

Nanorobots are typically composed of a metallic core (e.g., Fe_3O_4 , Au) for propulsion and a biocompatible coating (e.g., PEG, HA) for stealth properties. Propulsion mechanisms include:

Magnetic Propulsion: Magnetic nanorobots are driven by external magnetic fields; for example, Fe_3O_4 -based nanorobots with a rod-like shape showed a speed of 5 $\mu\text{m/s}$ under a 0.5 T magnetic field.

Ultrasound Propulsion: Ultrasound waves can induce cavitation in the fluid surrounding nanorobots, generating thrust; Au nanorods showed a speed of 3 $\mu\text{m/s}$ under 1 MHz ultrasound.

Chemical Propulsion: Nanorobots can use chemical reactions (e.g., decomposition of H_2O_2) to generate thrust; ZnO-based nanorobots showed a speed of 2 $\mu\text{m/s}$ in a 1% H_2O_2 solution (mimicking the TME's high reactive oxygen species (ROS) concentration).

5.3.2 Targeting and Drug Delivery Applications

Nanorobots can be equipped with targeting ligands (e.g., antibodies, peptides) and drug-loading compartments for active targeting. For example, magnetic nanorobots conjugated with anti-EGFR antibodies and loaded with DOX were navigated to EGFR-positive lung cancer tumors in a mouse model using a magnetic field; the nanorobots achieved 4.3-fold higher tumor accumulation than non-propelled liposomes. Additionally, nanorobots can penetrate dense biological barriers: ultrasound-propelled Au nanorods penetrated 500 μm into the stroma of pancreatic tumors, compared to 100 μm for passive PNPs.

5.3.3 Challenges and Future Directions

The main challenges for nanorobots include biocompatibility (metallic cores may induce toxicity), control precision (navigating through complex biological fluids), and scale-up production. To address biocompatibility, researchers are developing biodegradable nanorobots using materials like magnesium (Mg) or silk fibroin; Mg-based nanorobots degraded completely in mouse blood within 7 days, with no significant organ toxicity. For control precision, AI-based navigation algorithms are being developed to adjust the nanorobot's path based on real-time imaging data (e.g., ultrasound).

6. Challenges and Future Directions in Nano-Bio TDDS

6.1 Current Challenges

6.1.1 Scale-Up Production

Despite significant preclinical progress, scaling up the production of nano-bio TDDS to meet clinical demand remains a major challenge. Traditional synthesis methods (e.g., thin-film hydration for liposomes, emulsion-solvent evaporation for PNPs) are labor-intensive, low-yield, and prone to batch-to-batch variation. For example, the batch production of MOFs typically yields 10–50 mg of material, which is insufficient for clinical trials requiring grams of material. Additionally, quality control during scale-up is difficult: variations in size, PDI, and drug loading efficiency between batches can affect therapeutic efficacy and safety.

To address this, continuous manufacturing technologies (e.g., continuous flow microfluidics, spray drying) are being developed. Continuous flow microfluidics can produce liposomes with uniform size (PDI < 0.1) at a rate of 100 mL/h, compared to 10 mL/h for batch methods. Spray drying enables large-scale production of PNPs with high yield (up to 90%) and minimal batch variation. However, these technologies require high initial investment and specialized equipment, limiting their adoption by small and medium-sized enterprises.

6.1.2 Regulatory Approval

The regulatory approval process for nano-bio TDDS is complex due to their unique physicochemical properties and potential long-term toxicity. Regulatory agencies such as the FDA and EMA require extensive characterization of nano-carriers (e.g., size, surface charge, stability) and long-term safety data (e.g., chronic toxicity, immunogenicity). For example, the FDA required 5 years of long-term safety data for the approval of Onivyde® (irinotecan liposome injection), delaying its market launch by 2 years.

Additionally, there is a lack of standardized testing guidelines for nano-bio TDDS. For instance, in vitro drug release assays use different buffer compositions and agitation rates, making it difficult to compare data between studies. The International Organization for Standardization (ISO) is developing guidelines for nano-bio material characterization, but their implementation is still in progress.

6.1.3 Long-Term Safety

Long-term safety of nano-bio TDDS is a major concern, as nano-carriers may accumulate in organs (e.g., liver, spleen) over time, leading to chronic toxicity. For example, unmodified Au nanoparticles accumulated in the liver of rats after repeated administration, causing liver fibrosis after 6 months. Additionally, nano-carriers may induce immunological responses, such as the production of anti-nano-carrier antibodies or activation of the complement system. A study showed that repeated administration of chitosan PNPs induced a 3.2-fold increase in IL-6 levels in mice, indicating chronic inflammation.

To mitigate long-term toxicity, researchers are developing biodegradable nano-carriers that degrade into non-toxic byproducts. For example, PLGA PNPs degrade into lactic acid and glycolic acid, which are metabolized by the body via the Krebs cycle. However, the degradation rate of PLGA PNPs is slow (half-life of 2–6 months), leading to potential accumulation in organs.

6.2 Future Directions

6.2.1 Multifunctional Nano-Bio TDDS

The future of nano-bio TDDS lies in the development of multifunctional systems that combine multiple therapeutic modalities (e.g., chemotherapy, immunotherapy, phototherapy) for synergistic efficacy. For example, a multifunctional MOF loaded with DOX (chemotherapy), anti-PD-L1 antibody (immunotherapy), and chlorin e6 (photodynamic therapy) achieved 95% tumor growth inhibition in a mouse model of melanoma, compared to 50–70% for single-modal therapy. Additionally, multifunctional TDDS can integrate diagnostic capabilities (e.g., imaging agents) for theranostics—simultaneous diagnosis and treatment. A liposome loaded with DOX (therapy) and Cy5.5 (fluorescent imaging agent) enabled real-time monitoring of tumor accumulation and treatment response in mice.

6.2.2 Personalized Nano-Bio Medicine

Advancements in AI and 3D bioprinting will enable the development of personalized nano-bio TDDS tailored to individual patients. AI models can analyze patient data (e.g., genetic profile, tumor biomarkers, imaging data) to design optimal nano-carrier formulations, while 3D bioprinting can fabricate patient-specific delivery devices. For example, a patient with colorectal cancer underwent genetic testing to identify overexpression of EGFR and MMP-2; an AI model designed an MMP-sensitive liposome conjugated with anti-EGFR antibodies, and 3D bioprinting produced a personalized scaffold for localized delivery of the liposomes. This personalized approach resulted in a 60% increase in progression-free survival compared to standard chemotherapy.

6.2.3 Integration of Omics Technologies

Omics technologies (e.g., genomics, proteomics, metabolomics) will play a key role in understanding the interaction between nano-bio carriers and biological systems. Genomics can identify genetic variations that affect the efficacy of nano-bio TDDS; for example, patients with a specific polymorphism in the ABCB1 gene (which encodes P-glycoprotein, a drug efflux pump) showed 2.3-fold higher tumor accumulation of DOX-loaded liposomes than patients without the polymorphism. Proteomics can analyze the protein corona formed around nano-carriers in biological fluids, which affects their circulation time and targeting efficiency. Metabolomics can evaluate the metabolic changes induced by nano-bio TDDS, providing insights into their mechanism of action and potential side effects.

7. Conclusion

Nano-bio convergence has revolutionized targeted drug delivery by leveraging the unique properties of nano-bio materials (liposomes, PNPs, MOFs, exosomes) and advanced surface modification strategies (PEGylation, ligand conjugation, stimuli-responsive modification). In vitro and in vivo studies have demonstrated that nano-bio TDDS enhance drug efficacy, reduce systemic toxicity, and improve patient compliance in the treatment of cancers, neurodegenerative diseases, and infectious diseases.

Emerging technologies such as AI-driven design, 3D bioprinting, and nanorobots are pushing the boundaries of nano-bio TDDS, enabling personalized medicine and multifunctional therapeutic systems. However, significant challenges remain, including scale-up production, regulatory approval, and long-term safety. Addressing these challenges will require collaboration between researchers, engineers, clinicians, and regulatory agencies to develop standardized manufacturing processes, testing guidelines, and safety assessment protocols.

As the field continues to advance, nano-bio TDDS have the potential to transform healthcare by providing precise, effective, and personalized treatments for a wide range of diseases. This review provides a comprehensive overview of the current state and future directions of nano-bio convergence in targeted drug delivery, serving as a valuable resource for researchers, clinicians, and industry professionals working in this rapidly evolving field.

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Article

pH-Responsive Mesoporous Silica Nanoparticles Functionalized with Aptamers for Targeted siRNA Delivery in Triple-Negative Breast Cancer Therapy

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ABSTRACT

Triple-negative breast cancer (TNBC) remains a recalcitrant malignancy due to its lack of hormone receptors and HER2 overexpression, limiting targeted therapy options. Small interfering RNA (siRNA)-based therapy holds promise for TNBC by silencing oncogenes, but its clinical translation is hindered by poor stability, low cellular uptake, and off-target effects. Herein, we developed a pH-responsive mesoporous silica nanoparticle (MSN) system functionalized with AS1411 aptamers for targeted siRNA delivery to TNBC cells. The MSNs were engineered with a pH-sensitive poly(β -amino ester) (PBAE) coating to facilitate endosomal escape and surface-conjugated with AS1411, which binds to nucleolin overexpressed on TNBC cell membranes. The siRNA targeting MCL-1 (a prosurvival oncogene) was loaded into the MSN pores via electrostatic interaction. In vitro studies showed that the aptamer-functionalized MSNs (Apt-MSNs) exhibited 3.2-fold higher cellular uptake in MDA-MB-231 TNBC cells than non-targeted MSNs, leading to 82.3% MCL-1 silencing and 67.5% cell apoptosis. In vivo, Apt-MSN/siMCL-1 significantly inhibited tumor growth in TNBC xenograft mice (tumor volume reduction of 71.2% vs. saline control) and reduced systemic toxicity, as evidenced by normal liver/kidney function and minimal organ damage. This work demonstrates the potential of aptamer-functionalized, pH-responsive MSNs as a targeted nanoplatform for siRNA delivery in TNBC therapy, highlighting the convergence of nanomaterial engineering and biomedicine for precision cancer treatment.

Keywords: Mesoporous Silica Nanoparticles; Aptamer Targeting; pH-Responsive Delivery; siRNA; Triple-Negative Breast Cancer; Nanomedicine; Oncogene Silencing; Drug Delivery Systems

1. Introduction

1.1 Background

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related death among women worldwide, with triple-negative breast cancer (TNBC) accounting for 15–20% of all cases. TNBC is characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human

epidermal growth factor receptor 2 (HER2) expression, rendering it unresponsive to hormone therapy and anti-HER2 agents. Current standard treatments for TNBC include chemotherapy and radiation therapy, but these approaches are associated with severe systemic toxicity and high rates of recurrence, with a 5-year overall survival rate of only 40–50% for advanced-stage disease.

Small interfering RNA (siRNA) technology has emerged as a promising strategy for TNBC treatment by enabling sequence-specific silencing of oncogenes critical for tumor proliferation, survival, and metastasis. Myeloid cell leukemia 1 (MCL-1), a member of the B-cell lymphoma 2 (Bcl-2) family of prosurvival proteins, is frequently overexpressed in TNBC and contributes to chemotherapy resistance and tumor progression. Silencing MCL-1 with siRNA (siMCL-1) can sensitize TNBC cells to apoptosis and inhibit tumor growth. However, the clinical application of siRNA is limited by several challenges: (1) poor stability in biological fluids due to degradation by nucleases; (2) low cellular uptake caused by the negatively charged siRNA backbone and large molecular weight; (3) inefficient endosomal escape after internalization; and (4) off-target effects leading to systemic toxicity.

Nanoparticle-based delivery systems have been widely explored to overcome these barriers. Mesoporous silica nanoparticles (MSNs) are particularly attractive for siRNA delivery due to their large surface area, tunable pore size, high loading capacity, and excellent biocompatibility. MSNs can be functionalized with targeting ligands to enhance tumor accumulation and cellular uptake, and stimuli-responsive coatings to trigger siRNA release at the tumor microenvironment (TME), which is characterized by acidic pH (pH 6.5–6.8) compared to normal tissues (pH 7.4).

1.2 Research Gaps

Despite significant progress in MSN-based siRNA delivery, existing systems still face limitations. Non-targeted MSNs often exhibit low tumor selectivity, leading to off-target siRNA accumulation in normal organs (e.g., liver, kidneys). While some MSNs are functionalized with antibodies for targeting, antibodies are large molecules that can induce immune responses and reduce nanoparticle stability. Aptamers, short single-stranded nucleic acids that bind to target molecules with high affinity and specificity, offer a promising alternative to antibodies. AS1411, a 26-mer G-rich aptamer, binds to nucleolin, a protein overexpressed on the surface of TNBC cells and tumor endothelial cells, making it an ideal targeting ligand for TNBC.

Another gap is the inefficient endosomal escape of MSN/siRNA complexes. After cellular internalization, nanoparticles are trafficked to endosomes, where the acidic environment (pH 5.0–6.0) can degrade siRNA if not released promptly. pH-responsive polymers, such as poly(β -amino ester) (PBAE), can protonate in acidic endosomes, leading to osmotic swelling and endosomal rupture, facilitating siRNA release into the cytoplasm. However, few studies have integrated aptamer targeting, pH-responsive PBAE coating, and MSN-based siRNA delivery for TNBC therapy.

1.3 Research Objectives and Contributions

The primary objective of this study is to develop a targeted, pH-responsive MSN system for efficient siMCL-1 delivery to TNBC cells. Specific objectives include:

Synthesize and characterize pH-responsive MSNs functionalized with PBAE and AS1411 aptamers.

Evaluate the siRNA loading capacity, pH-triggered release behavior, and stability of the Apt-MSN/siRNA system in biological fluids.

Assess the in vitro cellular uptake, endosomal escape, MCL-1 silencing efficiency, and cytotoxicity of

Apt-MSN/siMCL-1 in TNBC cell lines.

Investigate the in vivo tumor targeting, anti-tumor efficacy, and systemic toxicity of Apt-MSN/siMCL-1 in TNBC xenograft mouse models.

The key contributions of this research are:

- **Nanomaterial Engineering Contribution:** The integration of AS1411 aptamers and pH-responsive PBAE into MSNs creates a dual-functional nanoplatform that enhances targeted delivery and endosomal escape, addressing critical limitations of existing siRNA delivery systems.

- **Biomedical Translation Contribution:** The Apt-MSN/siMCL-1 system demonstrates significant anti-tumor efficacy and low toxicity in vivo, providing a clinically relevant strategy for TNBC treatment.

- **Methodological Contribution:** The study establishes a comprehensive characterization and evaluation pipeline for aptamer-functionalized, stimuli-responsive MSNs, which can be adapted for other oncogene-targeting siRNAs and cancer types.

2. Literature Review

2.1 MSNs in siRNA Delivery for Cancer Therapy

Mesoporous silica nanoparticles (MSNs) have gained considerable attention as siRNA delivery carriers due to their unique structural properties. MSNs typically have a pore size of 2–50 nm, a surface area of 500–1,500 m²/g, and a pore volume of 0.5–2.0 cm³/g, allowing for high siRNA loading via electrostatic interaction, hydrophobic interaction, or covalent conjugation. The surface of MSNs can be modified with various functional groups (e.g., amino, carboxyl, thiol) to improve biocompatibility and enable ligand conjugation.

Several studies have demonstrated the potential of MSNs for siRNA delivery in cancer therapy. For example, amino-functionalized MSNs loaded with siRNA targeting vascular endothelial growth factor (VEGF) showed enhanced tumor accumulation and inhibited angiogenesis in colorectal cancer xenografts. However, these non-targeted MSNs exhibited high accumulation in the liver and spleen, leading to potential toxicity. To improve targeting, MSNs have been functionalized with ligands such as folic acid, transferrin, and antibodies. Folic acid-functionalized MSNs loaded with siBcl-2 showed increased uptake in folate receptor-overexpressing breast cancer cells and inhibited tumor growth. However, folic acid is expressed in some normal tissues (e.g., kidney proximal tubules), leading to off-target effects.

2.2 Aptamers as Targeting Ligands in Nanomedicine

Aptamers are single-stranded DNA or RNA molecules that fold into unique three-dimensional structures, enabling specific binding to target proteins, peptides, or small molecules. Aptamers offer several advantages over antibodies: (1) small size (5–25 kDa vs. 150 kDa for antibodies), which enhances tissue penetration and reduces immunogenicity; (2) high stability and easy chemical modification; (3) low production cost and batch-to-batch consistency.

AS1411 is one of the most widely studied aptamers for cancer targeting. It binds to nucleolin, a protein that is overexpressed on the surface of various cancer cells, including TNBC, lung cancer, and pancreatic cancer, and is involved in cell proliferation, angiogenesis, and metastasis. AS1411-functionalized nanoparticles have been shown to enhance targeted delivery to cancer cells. For example, AS1411-conjugated liposomes loaded with doxorubicin showed increased uptake in TNBC cells and improved anti-tumor efficacy compared to non-targeted liposomes. However, liposomes have low siRNA loading capacity

and poor stability in biological fluids, limiting their application for siRNA delivery.

2.3 pH-Responsive Polymers for Endosomal Escape

The endosomal escape is a critical barrier for nanoparticle-mediated siRNA delivery. After internalization via endocytosis, nanoparticles are enclosed in endosomes, which mature into late endosomes (pH 5.0–5.5) and lysosomes (pH 4.5–5.0). If nanoparticles do not escape the endosome, siRNA will be degraded by lysosomal nucleases. pH-responsive polymers can overcome this barrier by exploiting the acidic endosomal environment.

Poly(β -amino ester) (PBAE) is a class of pH-responsive polymers that have shown great promise for endosomal escape. PBAE contains tertiary amines that protonate in acidic endosomes, leading to an increase in positive charge and osmotic pressure, which causes endosomal swelling and rupture. PBAE has been used to coat nanoparticles such as liposomes and MSNs to improve endosomal escape. For example, PBAE-coated MSNs loaded with siRNA showed increased cytoplasmic delivery and gene silencing efficiency in HeLa cells. However, the combination of PBAE coating and aptamer targeting in MSNs for siRNA delivery in TNBC has not been fully explored.

3. Methodology

3.1 Synthesis and Characterization of Apt-MSN/siRNA

3.1.1 Synthesis of MSNs

MSNs were synthesized using a modified sol-gel method. Briefly, 1.0 g of cetyltrimethylammonium bromide (CTAB) was dissolved in 480 mL of deionized water (DI) and 3.5 mL of 2 M NaOH. The solution was heated to 80°C with stirring, and 5.0 mL of tetraethyl orthosilicate (TEOS) was added dropwise. The mixture was stirred for 2 h at 80°C, and the resulting white precipitate was collected by centrifugation (8,000 rpm, 15 min). The CTAB template was removed by refluxing the nanoparticles in a solution of 1.0 g of ammonium nitrate in 100 mL of ethanol for 6 h. The MSNs were washed with ethanol and DI water three times and dried at 60°C overnight.

3.1.2 Functionalization of MSNs with PBAE

PBAE was synthesized via Michael addition polymerization of 1,4-butanediol diacrylate and 4,4'-trimethylenedipiperidine. The MSNs were amino-functionalized by reacting with 3-aminopropyltriethoxysilane (APTES) in ethanol. Briefly, 100 mg of MSNs was dispersed in 50 mL of ethanol, and 1.0 mL of APTES was added. The mixture was refluxed for 4 h, and the amino-functionalized MSNs (NH₂-MSNs) were collected by centrifugation, washed with ethanol, and dried. The NH₂-MSNs were then coated with PBAE by mixing 50 mg of NH₂-MSNs with 100 mg of PBAE in 10 mL of DI water. The mixture was stirred for 2 h at room temperature, and the PBAE-coated MSNs (PBAE-MSNs) were collected by centrifugation and washed with DI water.

3.1.3 Conjugation of AS1411 Aptamer to PBAE-MSNs

AS1411 aptamer (5'-GGT GGT GGT GGT TGT GGT GGT GGT GG-3') with a 5'-thiol modification was purchased from Integrated DNA Technologies (Coralville, IA, USA). The PBAE-MSNs were activated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) to introduce carboxyl groups. Briefly, 50 mg of PBAE-MSNs was dispersed in 10 mL of 0.1 M MES buffer (pH 6.0), and 10 mg of EDC and 10 mg of sulfo-NHS were added. The mixture was stirred for 1

h at room temperature, and the activated MSNs were collected by centrifugation. The activated MSNs were then reacted with 100 μ M AS1411 aptamer in 10 mL of 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 4 h at room temperature. The aptamer-functionalized MSNs (Apt-MSNs) were collected by centrifugation, washed with PBS, and stored in PBS at 4°C.

3.1.4 Loading of siMCL-1 into Apt-MSNs

siRNA targeting MCL-1 (siMCL-1: 5'-GGA UUC UUG UCA ACA UGA ATT-3', sense; 5'-UUC AUG UUG ACA AGA AUC CTT-3', antisense) and scrambled siRNA (siScr: 5'-UUC UCC GAA CGU GUC ACG UTT-3', sense; 5'-ACG UGA CAC GUU CGG AGA ATT-3', antisense) were purchased from Ambion (Austin, TX, USA). siMCL-1 was loaded into Apt-MSNs via electrostatic interaction. Briefly, 10 mg of Apt-MSNs was dispersed in 1 mL of PBS, and different concentrations of siMCL-1 (0.1–1.0 μ M) were added. The mixture was incubated for 30 min at room temperature, and the Apt-MSN/siMCL-1 complexes were collected by centrifugation. The siRNA loading efficiency was determined by measuring the absorbance of free siRNA in the supernatant at 260 nm using a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan).

3.1.5 Characterization of Nanoparticles

The morphology of MSNs, PBAE-MSNs, and Apt-MSNs was observed using transmission electron microscopy (TEM, JEOL JEM-2100, Tokyo, Japan) and scanning electron microscopy (SEM, FEI Quanta 250, Hillsboro, OR, USA). The particle size and zeta potential were measured using dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS, Worcestershire, UK). The specific surface area and pore size distribution of MSNs were determined by Brunauer-Emmett-Teller (BET) analysis (Micromeritics ASAP 2020, Norcross, GA, USA). The conjugation of AS1411 aptamer was confirmed by Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet iS50, Waltham, MA, USA) and X-ray photoelectron spectroscopy (XPS, Thermo Scientific K-Alpha, Waltham, MA, USA).

3.2 In Vitro Evaluation of Apt-MSN/siMCL-1

3.2.1 Cell Culture

Human TNBC cell lines MDA-MB-231 and BT-549, and normal breast epithelial cell line MCF-10A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 and BT-549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 U/mL penicillin, 100 μ g/mL streptomycin) at 37°C in a humidified atmosphere with 5% CO₂. MCF-10A cells were cultured in Mammary Epithelial Growth Medium (MEGM, Lonza, Basel, Switzerland) supplemented with MEGM SingleQuots (Lonza) and 1% penicillin-streptomycin under the same conditions. Cells were passaged every 2–3 days when they reached 80–90% confluence.

3.2.2 Cellular Uptake Assay

Cellular uptake of Apt-MSNs was evaluated using flow cytometry and confocal laser scanning microscopy (CLSM). For flow cytometry, MDA-MB-231, BT-549, and MCF-10A cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight. The cells were treated with fluorescein isothiocyanate (FITC)-labeled Apt-MSNs or non-targeted MSNs (NT-MSNs) at a concentration of 100 μ g/mL for 1, 2, 4, and 6 h. For competition assays, MDA-MB-231 cells were pre-incubated with 1 μ M free AS1411 aptamer for 1 h before treatment with FITC-labeled Apt-MSNs. After incubation, the cells were washed three times with cold PBS, trypsinized, and resuspended in PBS. The fluorescence intensity of each sample was measured using a flow cytometer (BD FACSCanto II, BD Biosciences, San Jose, CA, USA), and the mean

fluorescence intensity (MFI) was calculated to quantify cellular uptake.

For CLSM analysis, MDA-MB-231 cells were seeded on glass coverslips in 24-well plates at a density of 1×10^5 cells/well and incubated overnight. The cells were treated with FITC-labeled Apt-MSNs or NT-MSNs (100 $\mu\text{g/mL}$) for 4 h. After incubation, the cells were washed three times with cold PBS, fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 10 min. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) for 5 min. The coverslips were mounted on glass slides using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA), and images were acquired using a CLSM (Zeiss LSM 880, Carl Zeiss, Oberkochen, Germany) with a 63 $\times$ oil immersion objective.

3.2.3 Endosomal Escape Assay

Endosomal escape of Apt-MSN/siRNA complexes was evaluated using a pH-sensitive fluorescent probe, LysoTracker Red DND-99 (Thermo Fisher Scientific, Waltham, MA, USA). MDA-MB-231 cells were seeded on glass coverslips in 24-well plates at a density of 1×10^5 cells/well and incubated overnight. The cells were treated with FITC-labeled Apt-MSN/siRNA complexes (100 $\mu\text{g/mL}$ MSNs, 100 nM siRNA) for 1, 2, and 4 h. After incubation, the cells were incubated with 50 nM LysoTracker Red for 30 min to label endosomes/lysosomes. The cells were washed three times with cold PBS, fixed with 4% paraformaldehyde, and stained with DAPI. CLSM images were acquired to observe the colocalization of FITC-labeled Apt-MSNs (green) and LysoTracker Red (red). The colocalization coefficient was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) to quantify endosomal escape.

3.2.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The MCL-1 mRNA expression level was measured using qRT-PCR. MDA-MB-231 and BT-549 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight. The cells were treated with different formulations: (1) saline; (2) free siMCL-1; (3) NT-MSN/siScr; (4) NT-MSN/siMCL-1; (5) Apt-MSN/siScr; (6) Apt-MSN/siMCL-1. The siRNA concentration was 100 nM for all treatments. After 48 h of incubation, total RNA was extracted from the cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qRT-PCR was performed using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The primer sequences for MCL-1 were: forward 5'-GGA GGA GTT GGA CGG ACA AC-3', reverse 5'-TCC TCC GTT TCA GTT TCC CA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control, with primer sequences: forward 5'-GAA GGT GAA GGT CGG AGT C-3', reverse 5'-GAA GAT GGT GAT GGG ATT TC-3'. The relative MCL-1 mRNA expression level was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

3.2.5 Western Blot Analysis

The MCL-1 protein expression level was measured using Western blot analysis. MDA-MB-231 and BT-549 cells were treated with the same formulations as in the qRT-PCR assay for 72 h. The cells were lysed in RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). The protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein (30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Burlington, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies

against MCL-1 (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) and GAPDH (1:5000 dilution, Cell Signaling Technology). After washing three times with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution, Cell Signaling Technology) for 1 h at room temperature. The protein bands were visualized using the ECL Western Blotting Substrate (Thermo Fisher Scientific) and quantified using ImageJ software.

3.2.6 Cell Apoptosis Assay

Cell apoptosis was evaluated using the Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences). MDA-MB-231 and BT-549 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and treated with the same formulations as above for 72 h. The cells were washed three times with cold PBS, trypsinized, and resuspended in $1 \times$ binding buffer. The cells were stained with 5 μ L of Annexin V-FITC and 5 μ L of PI for 15 min in the dark at room temperature. The apoptotic rate was measured using a flow cytometer, and the cells were divided into four quadrants: viable cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), late apoptotic cells (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁻/PI⁺). The total apoptotic rate was calculated as the sum of early and late apoptotic cells.

3.2.7 Cytotoxicity Assay

The cytotoxicity of Apt-MSN/siMCL-1 was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDA-MB-231, BT-549, and MCF-10A cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were treated with different concentrations of Apt-MSNs (0, 25, 50, 100, 200, 400 μ g/mL) or different formulations (saline, free siMCL-1, NT-MSN/siScr, NT-MSN/siMCL-1, Apt-MSN/siScr, Apt-MSN/siMCL-1) for 72 h. For the formulation treatments, the siRNA concentration was 100 nM. After incubation, 20 μ L of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for 4 h at 37°C. The supernatant was removed, and 150 μ L of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability was calculated as (Absorbance of treated group / Absorbance of control group) $\times$ 100%.

3.3 In Vivo Evaluation of Apt-MSN/siMCL-1

3.3.1 Animal Model Establishment

Female BALB/c nude mice (4–6 weeks old, 18–22 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a specific pathogen-free (SPF) environment with a 12 h light/dark cycle, controlled temperature ($22 \pm 2^\circ\text{C}$), and humidity ($50 \pm 5\%$). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Duke University (Protocol Number: A225-23-07). To establish the TNBC xenograft model, 1×10^7 MDA-MB-231 cells suspended in 100 μ L of PBS/matrigel (1:1, v/v) were injected subcutaneously into the right flank of each mouse. Tumor volume was measured every 3 days using a digital caliper and calculated using the formula: Tumor volume = (length $\times$ width²) / 2. When the tumor volume reached 100–150 mm³, the mice were randomly divided into six groups (n=6 per group): (1) saline; (2) free siMCL-1; (3) NT-MSN/siScr; (4) NT-MSN/siMCL-1; (5) Apt-MSN/siScr; (6) Apt-MSN/siMCL-1.

3.3.2 In Vivo Tumor Targeting and Biodistribution

The in vivo tumor targeting and biodistribution of Apt-MSNs were evaluated using near-infrared (NIR) fluorescence imaging. FITC-labeled Apt-MSNs or NT-MSNs (100 μ g/mL, 200 μ L) were intravenously injected into MDA-MB-231 xenograft mice via the tail vein. At 1, 4, 8, 12, and 24 h post-injection, the mice

were anesthetized with isoflurane, and NIR fluorescence images were acquired using an IVIS Spectrum Imaging System (PerkinElmer, Waltham, MA, USA) with excitation at 488 nm and emission at 520 nm. The fluorescence intensity in the tumor and major organs (heart, liver, spleen, lungs, kidneys) was quantified using Living Image software (PerkinElmer). At 24 h post-injection, the mice were euthanized, and the tumor and major organs were harvested, weighed, and imaged *ex vivo*. The fluorescence intensity of each organ was measured to calculate the organ/tumor fluorescence ratio.

3.3.3 Anti-Tumor Efficacy Study

The anti-tumor efficacy of Apt-MSN/siMCL-1 was evaluated in MDA-MB-231 xenograft mice. The mice were treated with the six formulations via tail vein injection every 3 days for a total of five treatments. The siRNA dose was 2 mg/kg for all siRNA-containing formulations. Tumor volume and body weight were measured every 3 days. At the end of the treatment (15 days), the mice were euthanized, and the tumors were harvested, weighed, and photographed. The tumor growth inhibition rate (TGIR) was calculated using the formula: $TGIR = [(Average\ tumor\ weight\ of\ control\ group - Average\ tumor\ weight\ of\ treated\ group) / Average\ tumor\ weight\ of\ control\ group] \times 100\%$.

3.3.4 Histopathological Analysis

Histopathological analysis was performed to evaluate tumor cell apoptosis and organ toxicity. The harvested tumors and major organs (heart, liver, spleen, lungs, kidneys) were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 μ m slices. The tumor sections were stained with hematoxylin and eosin (H&E) to observe tumor morphology and with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) to detect apoptotic cells. The organ sections were stained with H&E to evaluate tissue damage. The stained sections were observed under a light microscope (Olympus BX53, Tokyo, Japan), and the number of TUNEL-positive cells in tumor sections was quantified using ImageJ software.

3.3.5 Biochemical Analysis

Biochemical analysis was performed to evaluate liver and kidney function. At the end of the treatment, blood samples were collected from the mice via cardiac puncture and centrifuged at 3,000 rpm for 15 min to separate serum. The serum levels of alanine transaminase (ALT), aspartate transaminase (AST) (liver function markers), blood urea nitrogen (BUN), and creatinine (Cr) (kidney function markers) were measured using commercial kits (Sigma-Aldrich) according to the manufacturer's instructions.

3.4 Statistical Analysis

All experiments were performed in triplicate, and the data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 Synthesis and Characterization of Apt-MSN/siRNA

4.1.1 Morphology and Size Distribution

TEM and SEM images showed that MSNs, PBAE-MSNs, and Apt-MSNs had a spherical morphology with uniform particle size (Figure 1A–C). The average particle size of MSNs measured by DLS was 125.6 ± 8.4

nm, which increased to 142.3 ± 9.1 nm after PBAE coating (PBAE-MSNs) and 151.7 ± 10.2 nm after AS1411 aptamer conjugation (Apt-MSNs) (Figure 1D). The increase in particle size was attributed to the PBAE coating and aptamer conjugation on the MSN surface.

4.1.2 Zeta Potential

The zeta potential of MSNs was -28.5 ± 3.2 mV due to the presence of silanol groups on the surface. After amino-functionalization with APTES, the zeta potential changed to $+35.7 \pm 4.1$ mV. Coating with PBAE further increased the zeta potential to $+42.3 \pm 4.5$ mV, as PBAE contains positively charged tertiary amines. Conjugation of AS1411 aptamer (negatively charged) reduced the zeta potential to $+28.9 \pm 3.8$ mV, confirming successful aptamer conjugation (Figure 1E).

4.1.3 BET Surface Area and Pore Size

BET analysis showed that MSNs had a specific surface area of $1,256 \pm 58$ m²/g, a pore volume of 1.85 ± 0.12 cm³/g, and an average pore size of 5.2 ± 0.3 nm. After PBAE coating, the specific surface area and pore volume decreased to 876 ± 45 m²/g and 1.23 ± 0.09 cm³/g, respectively, due to the partial filling of MSN pores by PBAE. Conjugation of AS1411 aptamer further reduced the specific surface area and pore volume to 789 ± 41 m²/g and 1.05 ± 0.08 cm³/g, respectively (Table 1).

Table 1: BET Surface Area, Pore Volume, and Pore Size of MSNs, PBAE-MSNs, and Apt-MSNs

Nanoparticle Type	Specific Surface Area (m ² /g)	Pore Volume (cm ³ /g)	Pore Size (nm)
MSNs	$1,256 \pm 58$	1.85 ± 0.12	5.2 ± 0.3
PBAE-MSNs	876 ± 45	1.23 ± 0.09	4.8 ± 0.2
Apt-MSNs	789 ± 41	1.05 ± 0.08	4.5 ± 0.2

4.1.4 siRNA Loading and Release Behavior

The siRNA loading efficiency of Apt-MSNs was evaluated by measuring the absorbance of free siRNA in the supernatant. At a siRNA concentration of 0.5 μ M, the loading efficiency reached $92.3 \pm 4.1\%$, with a loading capacity of 46.2 ± 2.0 μ g siRNA/mg MSNs. The loading efficiency increased with increasing siRNA concentration up to 0.5 μ M, after which it plateaued due to saturation of the MSN pores (Figure 2A).

The pH-responsive siRNA release behavior of Apt-MSN/siMCL-1 was investigated in vitro using PBS buffers at pH 7.4 (simulating normal tissue) and pH 5.0 (simulating endosomal/lysosomal environment). At pH 7.4, only $18.5 \pm 3.2\%$ of siRNA was released after 48 h, indicating good stability of the complex in normal tissue. In contrast, at pH 5.0, the cumulative siRNA release reached $89.2 \pm 5.4\%$ after 48 h, with a burst release of $45.3 \pm 4.6\%$ in the first 6 h (Figure 2B). This pH-responsive release profile was attributed to the protonation of PBAE in acidic conditions, which weakened the electrostatic interaction between PBAE and siRNA, facilitating siRNA release.

4.1.5 Stability in Biological Fluids

The stability of Apt-MSN/siMCL-1 in serum was evaluated by measuring the particle size and siRNA integrity over time. In 10% fetal bovine serum (FBS), the particle size of Apt-MSN/siMCL-1 increased slightly from 151.7 ± 10.2 nm to 168.3 ± 12.5 nm after 24 h, indicating minimal aggregation. In contrast, free siRNA was completely degraded within 4 h in 10% FBS, while siRNA in Apt-MSN/siMCL-1 remained intact for 24 h, as confirmed by agarose gel electrophoresis (Figure 2C). These results demonstrated that Apt-MSNs protect siRNA from nuclease degradation in biological fluids.

4.2 In Vitro Cellular Evaluation

4.2.1 Cellular Uptake

Flow cytometry results showed that the cellular uptake of Apt-MSNs in MDA-MB-231 cells was time-dependent, with the mean fluorescence intensity (MFI) increasing from 234 ± 28 at 1 h to 896 ± 64 at 6 h (Figure 3A). At 4 h, the MFI of Apt-MSNs was 3.2-fold higher than that of NT-MSNs (821 ± 58 vs. 256 ± 32 , $p < 0.001$), indicating enhanced targeted uptake. In BT-549 cells (another TNBC cell line with high nucleolin expression), Apt-MSNs also showed 2.9-fold higher uptake than NT-MSNs (765 ± 49 vs. 264 ± 29 , $p < 0.001$). In contrast, in MCF-10A cells (normal breast epithelial cells with low nucleolin expression), the uptake of Apt-MSNs was similar to that of NT-MSNs (218 ± 25 vs. 196 ± 23 , $p > 0.05$) (Figure 3B).

Competition assays showed that pre-incubation of MDA-MB-231 cells with free AS1411 aptamer reduced the uptake of Apt-MSNs by 68.3% (MFI: 261 ± 31 vs. 821 ± 58 , $p < 0.001$), confirming that the enhanced uptake of Apt-MSNs was mediated by specific binding to nucleolin (Figure 3C). CLSM images further confirmed that Apt-MSNs (green fluorescence) were more abundant in the cytoplasm of MDA-MB-231 cells than NT-MSNs, with minimal fluorescence observed in MCF-10A cells (Figure 3D).

4.2.2 Endosomal Escape

CLSM images showed that at 1 h post-treatment, most Apt-MSN/siRNA complexes (green) colocalized with LysoTracker Red (red) in endosomes/lysosomes, with a colocalization coefficient of 0.82 ± 0.06 . At 2 h, the colocalization coefficient decreased to 0.45 ± 0.05 , and at 4 h, it further decreased to 0.18 ± 0.03 , indicating efficient endosomal escape (Figure 4A,B). In contrast, the colocalization coefficient of NT-MSN/siRNA complexes remained high at 4 h (0.63 ± 0.07), suggesting that PBAE coating was critical for endosomal escape. The pH-responsive protonation of PBAE in endosomes led to osmotic swelling and endosomal rupture, facilitating the release of siRNA into the cytoplasm.

4.2.3 MCL-1 Silencing Efficiency

qRT-PCR results showed that Apt-MSN/siMCL-1 significantly reduced MCL-1 mRNA expression in MDA-MB-231 cells by $82.3 \pm 5.7\%$, compared to $45.6 \pm 4.2\%$ for NT-MSN/siMCL-1 and $12.5 \pm 3.1\%$ for free siMCL-1 ($p < 0.001$ vs. all other groups) (Figure 5A). Similar results were observed in BT-549 cells, where Apt-MSN/siMCL-1 reduced MCL-1 mRNA expression by $78.6 \pm 4.9\%$ (Figure 5B). Western blot analysis confirmed that MCL-1 protein expression was reduced by $76.4 \pm 5.3\%$ in MDA-MB-231 cells and $72.8 \pm 4.8\%$ in BT-549 cells after treatment with Apt-MSN/siMCL-1 (Figure 5C,D). The scrambled siRNA groups (NT-MSN/siScr and Apt-MSN/siScr) had no significant effect on MCL-1 expression, confirming the sequence-specific silencing effect of siMCL-1.

4.2.4 Cell Apoptosis

Flow cytometry results showed that Apt-MSN/siMCL-1 induced a total apoptotic rate of $67.5 \pm 4.8\%$ in MDA-MB-231 cells, which was significantly higher than that of NT-MSN/siMCL-1 ($38.2 \pm 3.9\%$), free siMCL-1 ($15.3 \pm 2.7\%$), and control groups (saline: $5.2 \pm 1.3\%$; NT-MSN/siScr: $6.8 \pm 1.5\%$; Apt-MSN/siScr: $7.2 \pm 1.6\%$) ($p < 0.001$) (Figure 6A). In BT-549 cells, Apt-MSN/siMCL-1 induced a total apoptotic rate of $62.3 \pm 4.5\%$ (Figure 6B). TUNEL staining of MDA-MB-231 cells further confirmed that Apt-MSN/siMCL-1 increased the number of apoptotic cells (brown staining) compared to other groups (Figure 6C).

4.2.5 Cytotoxicity

MTT assay results showed that Apt-MSNs had low cytotoxicity in MDA-MB-231, BT-549, and MCF-10A cells, with cell viability $> 85\%$ even at a concentration of $400 \mu\text{g/mL}$ (Figure 7A). For the formulation

treatments, Apt-MSN/siMCL-1 reduced the viability of MDA-MB-231 cells to $32.5 \pm 3.6\%$, compared to $58.7 \pm 4.3\%$ for NT-MSN/siMCL-1 and $89.2 \pm 5.1\%$ for free siMCL-1 ($p < 0.001$) (Figure 7B). In BT-549 cells, Apt-MSN/siMCL-1 reduced cell viability to $36.8 \pm 4.1\%$ (Figure 7C). In contrast, Apt-MSN/siMCL-1 had minimal cytotoxicity in MCF-10A cells (cell viability: $82.3 \pm 5.2\%$), indicating good biocompatibility with normal cells.

4.3 In Vivo Evaluation

4.3.1 Tumor Targeting and Biodistribution

NIR fluorescence imaging showed that Apt-MSNs accumulated in the tumor tissue of MDA-MB-231 xenograft mice, with the highest fluorescence intensity observed at 8 h post-injection (Figure 8A). At 24 h post-injection, the fluorescence intensity in the tumor was 3.8-fold higher for Apt-MSNs than for NT-MSNs ($p < 0.001$) (Figure 8B). Ex vivo imaging of major organs showed that Apt-MSNs had higher accumulation in the tumor and lower accumulation in the liver and spleen compared to NT-MSNs (Figure 8C). The tumor-to-liver fluorescence ratio was 2.7 for Apt-MSNs, compared to 0.9 for NT-MSNs ($p < 0.001$) (Figure 8D). These results demonstrated the efficient tumor targeting of Apt-MSNs mediated by AS1411 aptamer.

4.3.2 Anti-Tumor Efficacy

Tumor growth curves showed that Apt-MSN/siMCL-1 significantly inhibited tumor growth compared to other groups (Figure 9A). At the end of the treatment (15 days), the average tumor volume in the Apt-MSN/siMCL-1 group was $185.6 \pm 23.8 \text{ mm}^3$, which was 71.2% smaller than that in the saline group ($644.3 \pm 58.7 \text{ mm}^3$, $p < 0.001$). The tumor growth inhibition rate (TGIR) of Apt-MSN/siMCL-1 was $73.5 \pm 4.8\%$, compared to $42.3 \pm 3.9\%$ for NT-MSN/siMCL-1 and $12.5 \pm 2.7\%$ for free siMCL-1 ($p < 0.001$) (Figure 9B,C). The body weight of mice in all groups remained stable throughout the treatment, indicating no significant systemic toxicity (Figure 9D).

4.3.3 Histopathological Analysis

H&E staining of tumor sections showed that Apt-MSN/siMCL-1 treatment led to significant tumor necrosis and reduced tumor cell density compared to other groups (Figure 10A). TUNEL staining showed that Apt-MSN/siMCL-1 increased the number of apoptotic cells in the tumor to $58.7 \pm 4.9\%$, compared to $26.3 \pm 3.8\%$ for NT-MSN/siMCL-1 and $8.2 \pm 2.1\%$ for saline ($p < 0.001$) (Figure 10B,C). H&E staining of major organs (heart, liver, spleen, lungs, kidneys) showed no significant tissue damage in the Apt-MSN/siMCL-1 group, while minor liver inflammation was observed in the NT-MSN/siMCL-1 group (Figure 10D).

4.3.4 Biochemical Analysis

Serum biochemical analysis showed that the levels of ALT, AST, BUN, and Cr in the Apt-MSN/siMCL-1 group were within the normal range and not significantly different from the saline group ($p > 0.05$) (Figure 11A–D). In contrast, the NT-MSN/siMCL-1 group showed a slight increase in ALT and AST levels ($p < 0.05$ vs. saline), indicating mild liver injury. These results confirmed that Apt-MSN/siMCL-1 had no significant systemic toxicity.

5. Discussion

5.1 Key Findings and Mechanisms

This study successfully developed a pH-responsive, aptamer-functionalized MSN system for targeted siMCL-1 delivery in TNBC therapy. The key findings are:

5.1.1 Efficient siRNA Loading and pH-Responsive Release

The Apt-MSNs exhibited high siRNA loading efficiency ($92.3 \pm 4.1\%$) and pH-responsive release, with minimal release in normal tissue (pH 7.4) and rapid release in endosomes (pH 5.0). This was attributed to the large pore volume of MSNs and the pH-sensitive protonation of PBAE, which weakened the electrostatic interaction between PBAE and siRNA in acidic conditions.

5.1.2 Targeted Cellular Uptake and Endosomal Escape

The AS1411 aptamer mediated specific binding to nucleolin overexpressed on TNBC cells, leading to 3.2-fold higher cellular uptake compared to non-targeted MSNs. The PBAE coating facilitated efficient endosomal escape (colocalization coefficient: 0.18 ± 0.03 at 4 h), overcoming the major barrier of siRNA delivery to the cytoplasm.

5.1.3 Potent MCL-1 Silencing and Anti-Tumor Efficacy

Apt-MSN/siMCL-1 achieved $82.3 \pm 5.7\%$ MCL-1 mRNA silencing and $67.5 \pm 4.8\%$ cell apoptosis in vitro, and reduced tumor volume by 71.2% in vivo. MCL-1 silencing induced apoptosis by disrupting the balance between prosurvival and proapoptotic Bcl-2 family proteins, sensitizing TNBC cells to cell death.

5.1.4 Low Systemic Toxicity

The Apt-MSNs showed good biocompatibility in vitro (cell viability $> 85\%$ at $400 \mu\text{g/mL}$) and in vivo (normal liver/kidney function, no organ damage), due to their targeted accumulation in tumors and minimal uptake by normal cells.

5.2 Comparison with Existing Nanoplatforms

Compared to existing siRNA delivery systems for TNBC, the Apt-MSN/siMCL-1 system offers several advantages:

5.2.1 Targeting Specificity

Unlike folate or transferrin-targeted nanoparticles, which bind to receptors expressed on some normal cells, AS1411 aptamer specifically targets nucleolin, which is overexpressed on TNBC cells and tumor endothelial cells but not on most normal cells. This reduces off-target effects and systemic toxicity.

5.2.2 pH-Responsive Release

Many MSN-based systems rely on passive siRNA release, leading to premature release in normal tissue. The PBAE coating of Apt-MSNs ensures that siRNA is only released in endosomes, improving the efficiency of cytoplasmic delivery.

5.2.3 Combined Efficacy and Safety

Previous studies have reported MSN-based siRNA delivery systems with high anti-tumor efficacy but significant liver toxicity due to non-targeted accumulation. The Apt-MSNs minimize liver accumulation (tumor-to-liver ratio: 2.7 vs. 0.9 for NT-MSNs) and show no significant liver/kidney damage, making them more clinically relevant.

5.3 Limitations and Future Directions

Despite its promising results, this study has several limitations:

5.3.1 In Vivo Model Limitations

The study used a subcutaneous TNBC xenograft model, which does not fully recapitulate the complex tumor microenvironment (e.g., immune cells, extracellular matrix) and metastasis of human TNBC. Future

studies should use orthotopic or metastatic TNBC models to evaluate the anti-metastatic efficacy of Apt-MSN/siMCL-1.

5.3.2 Long-Term Toxicity and Biodistribution

The *in vivo* study lasted 15 days, and long-term toxicity (e.g., 3–6 months) and biodistribution of Apt-MSNs remain unknown. Future studies should investigate the long-term fate of Apt-MSNs in the body, including their clearance by the reticuloendothelial system (RES).

5.3.3 Combination Therapy Potential

MCL-1 silencing can sensitize TNBC cells to chemotherapy or immunotherapy. Future work should explore combining Apt-MSN/siMCL-1 with paclitaxel (a standard chemotherapy drug for TNBC) or immune checkpoint inhibitors (e.g., anti-PD-1 antibodies) to enhance anti-tumor efficacy.

5.3.4 Clinical Translation Challenges

Scaling up the synthesis of Apt-MSNs with consistent properties (e.g., particle size, siRNA loading) and obtaining regulatory approval (e.g., FDA) are major challenges for clinical translation. Future studies should optimize the manufacturing process to ensure batch-to-batch consistency and conduct preclinical safety studies in larger animals (e.g., rabbits, dogs).

6. Conclusion

In this study, we developed a pH-responsive, AS1411 aptamer-functionalized mesoporous silica nanoparticle system for targeted siMCL-1 delivery to triple-negative breast cancer (TNBC) cells. The systematic characterization confirmed that the Apt-MSNs had uniform spherical morphology, appropriate particle size (~ 150 nm), high siRNA loading efficiency ($92.3 \pm 4.1\%$), and pH-responsive release behavior—critical properties for effective siRNA delivery.

In vitro studies demonstrated that the AS1411 aptamer mediated specific binding to nucleolin overexpressed on TNBC cells, resulting in 3.2-fold higher cellular uptake compared to non-targeted MSNs. The pH-responsive PBAE coating enabled efficient endosomal escape (colocalization coefficient: 0.18 ± 0.03 at 4 h), leading to potent MCL-1 silencing ($82.3 \pm 5.7\%$ mRNA reduction) and high cell apoptosis ($67.5 \pm 4.8\%$) in MDA-MB-231 cells. Importantly, the Apt-MSNs showed minimal cytotoxicity in normal breast epithelial cells (MCF-10A), highlighting their excellent biocompatibility.

In vivo experiments in MDA-MB-231 xenograft mice further validated the system's efficacy: Apt-MSN/siMCL-1 reduced tumor volume by 71.2% and achieved a tumor growth inhibition rate of $73.5 \pm 4.8\%$, significantly outperforming free siMCL-1 and non-targeted MSN/siMCL-1. NIR fluorescence imaging confirmed efficient tumor targeting (tumor-to-liver ratio: 2.7), while histopathological and biochemical analyses showed no significant systemic toxicity—normal liver/kidney function and no organ damage were observed.

This work bridges nanomaterial engineering and biomedicine by developing a targeted, stimuli-responsive nanoplatform that addresses key challenges in siRNA-based TNBC therapy, including poor stability, low cellular uptake, and off-target effects. The Apt-MSN/siMCL-1 system not only provides a promising strategy for TNBC treatment but also serves as a versatile platform for delivering other therapeutic siRNAs to various cancer types, advancing the field of precision nanomedicine.

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Article

Dual-Enzyme-Mimicking MOF-Derived Carbon Nanoparticles Loaded with Antimicrobial Peptides for Smart Photothermal-Assisted Bacterial Wound Infection Therapy

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ABSTRACT

Bacterial wound infections, particularly those caused by drug-resistant strains (e.g., methicillin-resistant *Staphylococcus aureus*, MRSA), pose a severe threat to public health due to limited treatment options and high mortality rates. Conventional antibiotics often fail to eliminate biofilms and induce drug resistance, while single-modal antimicrobial strategies lack efficiency in complex infection microenvironments. Herein, we report a smart nanotherapeutic platform based on metal-organic framework (MOF)-derived carbon nanoparticles (MDC NPs) with dual enzyme-mimetic activities (peroxidase-like and catalase-like) and photothermal properties, loaded with antimicrobial peptides (AMPs) for synergistic bacterial wound infection therapy. The MDC NPs were synthesized by pyrolyzing zeolitic imidazolate framework-8 (ZIF-8) at 800°C, followed by surface modification with polyethylene glycol (PEG) to enhance biocompatibility. The dual enzyme-mimetic activities of MDC NPs enable them to catalytically generate reactive oxygen species (ROS) via peroxidase-like activity in the presence of H₂O₂ (abundant in infected wounds) and decompose excess H₂O₂ into O₂ via catalase-like activity to relieve oxidative stress in normal tissues. Under near-infrared (NIR) laser irradiation (808 nm), MDC NPs exhibit excellent photothermal conversion efficiency (42.3%), which not only directly ablates bacteria but also promotes AMP release from the nanoparticle surface. In vitro studies show that the MDC-AMP nanosystem achieves 99.8% and 99.2% antibacterial efficiency against *E. coli* (Gram-negative) and MRSA (Gram-positive), respectively, and effectively disrupts MRSA biofilms (biofilm degradation rate: 87.6%). In a MRSA-infected mouse full-thickness skin wound model, MDC-AMP + NIR treatment accelerates wound closure (wound healing rate: 92.3% at day 7 vs. 45.6% for saline control), reduces inflammatory cell infiltration, and promotes collagen deposition. This work demonstrates a versatile nanotherapeutic strategy that integrates enzyme catalysis, photothermal therapy, and AMP-based antimicrobial action, providing a promising solution for drug-resistant bacterial wound infections and advancing the convergence of nanomaterial science and clinical microbiology.

Keywords: MOF-Derived Carbon Nanoparticles; Nanozymes; Antimicrobial Peptides; Photothermal Therapy; Bacterial Wound Infection; MRSA; Reactive Oxygen Species; Wound Healing

1. Introduction

1.1 Background

Bacterial wound infections affect over 100 million people worldwide annually, with drug-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β -lactamase (ESBL)-producing *E. coli* accounting for 30–50% of cases. These infections delay wound healing, increase the risk of sepsis, and lead to approximately 700,000 deaths each year. Conventional antibiotic therapy faces two major challenges: (1) the formation of bacterial biofilms—extracellular polymeric substance (EPS) matrices that shield bacteria from antibiotics and the host immune system; (2) the rapid evolution of drug resistance, with over 2.8 million antibiotic-resistant infections reported annually in the United States alone.

Alternative antimicrobial strategies, such as antimicrobial peptides (AMPs), photothermal therapy (PTT), and reactive oxygen species (ROS)-based therapy, have gained attention in recent years. AMPs are short cationic peptides that disrupt bacterial membranes without inducing resistance, but their clinical application is limited by poor stability, high cytotoxicity, and rapid degradation in vivo. PTT uses nanomaterials with NIR light absorption to generate local heat (45–55°C) for bacterial ablation, but it may damage surrounding normal tissues and fail to eliminate bacteria in deep wound layers. ROS-based therapy relies on the production of highly toxic ROS (e.g., $\cdot\text{OH}$, O_2^-) to kill bacteria, but excessive ROS can cause oxidative stress and delay wound healing.

1.2 Research Gaps

Existing nanotherapeutic platforms for bacterial wound infections often rely on single-modal action, leading to suboptimal efficacy in complex infection microenvironments. For example, AMP-loaded nanoparticles improve peptide stability but lack the ability to disrupt mature biofilms. Photothermal nanomaterials can ablate bacteria but require high laser power, increasing tissue damage risk. Nanozymes—nanomaterials with enzyme-mimetic activities—have been used for ROS generation, but most single-enzyme-mimetic nanozymes cannot balance ROS production and oxidative stress relief. Additionally, few platforms integrate multiple therapeutic modalities (e.g., enzyme catalysis, PTT, AMPs) into a single nanosystem for synergistic antimicrobial action, and even fewer are validated in clinically relevant wound infection models.

1.3 Research Objectives and Contributions

The primary objective of this study is to develop a multi-modal nanotherapeutic platform based on MOF-derived carbon nanoparticles (MDC NPs) for smart, synergistic treatment of drug-resistant bacterial wound infections. Specific objectives include:

- (1) Synthesize and characterize MDC NPs with dual enzyme-mimetic activities (peroxidase-like and catalase-like) and photothermal properties.
- (2) Load AMPs onto MDC NPs and evaluate AMP loading efficiency, NIR-triggered release behavior, and biocompatibility.
- (3) Assess the in vitro antibacterial efficacy of MDC-AMP against planktonic bacteria (Gram-positive MRSA and Gram-negative *E. coli*) and MRSA biofilms.
- (4) Validate the in vivo therapeutic efficacy of MDC-AMP + NIR in a MRSA-infected mouse full-thickness skin wound model, focusing on wound healing rate, inflammation reduction, and tissue regeneration.

The key contributions of this research are:

•**Nanomaterial Engineering Contribution:** The MDC NPs exhibit dual enzyme-mimetic activities and high photothermal conversion efficiency, enabling simultaneous ROS generation, oxidative stress relief, and photothermal ablation—addressing the limitations of single-modal nanozymes or photothermal agents.

•**Therapeutic Synergy Contribution:** The integration of enzyme catalysis, PTT, and AMPs creates a synergistic antimicrobial effect that enhances antibacterial efficacy against planktonic bacteria and biofilms while reducing AMP dosage and minimizing tissue damage.

•**Clinical Translation Contribution:** The MDC-AMP nanosystem accelerates wound healing in a clinically relevant mouse model, providing a translatable strategy for treating drug-resistant bacterial wound infections.

2. Literature Review

2.1 MOF-Derived Carbon Nanoparticles in Biomedical Applications

Metal-organic frameworks (MOFs) are porous crystalline materials composed of metal ions/clusters and organic ligands, with high surface area, tunable pore size, and excellent biocompatibility. MOF-derived carbon nanoparticles (MDC NPs), synthesized by pyrolyzing MOFs at high temperatures, inherit the porous structure of MOFs and exhibit unique properties such as high electrical conductivity, photothermal activity, and enzyme-mimetic behavior. ZIF-8, a widely used MOF composed of Zn^{2+} ions and 2-methylimidazole ligands, is an ideal precursor for MDC NPs due to its high thermal stability and low toxicity (Zn^{2+} is biodegradable and essential for human metabolism).

Recent studies have demonstrated the potential of MDC NPs in biomedical applications. For example, ZIF-8-derived carbon NPs have been used for drug delivery, with a loading capacity of up to 400 μg drug/mg NPs due to their large surface area. MDC NPs also exhibit photothermal properties, with photothermal conversion efficiencies ranging from 35–50% under NIR irradiation, making them suitable for PTT. Additionally, MDC NPs can mimic the activity of natural enzymes (e.g., peroxidase, catalase) by virtue of their surface defects and metal dopants, enabling ROS-based therapy. However, few studies have explored MDC NPs with dual enzyme-mimetic activities for bacterial infection therapy, and even fewer have integrated them with AMPs for synergistic action.

2.2 Nanozymes for Bacterial Infection Therapy

Nanozymes offer several advantages over natural enzymes for antimicrobial therapy: (1) high stability under harsh conditions (e.g., acidic wound microenvironment, high temperature); (2) low cost and easy large-scale synthesis; (3) tunable enzyme-mimetic activity via surface modification. Peroxidase-like nanozymes catalyze the decomposition of H_2O_2 into highly toxic $\cdot\text{OH}$, which disrupts bacterial membranes and DNA. Catalase-like nanozymes decompose H_2O_2 into O_2 and H_2O , relieving oxidative stress in normal tissues.

Single-enzyme-mimetic nanozymes have limitations: peroxidase-like nanozymes require exogenous H_2O_2 (often not present in sufficient quantities in wounds), while catalase-like nanozymes cannot generate ROS for antibacterial action. Dual-enzyme-mimetic nanozymes that combine peroxidase and catalase activities can overcome these limitations: in the presence of low H_2O_2 concentrations (e.g., infected wounds, where H_2O_2 is produced by immune cells), they act as peroxidases to generate ROS; in high H_2O_2 concentrations, they switch to catalase activity to prevent excessive ROS-induced tissue damage. However,

current dual-enzyme-mimetic nanozymes often have low catalytic efficiency and poor targeting ability, limiting their antimicrobial efficacy.

2.3 Antimicrobial Peptides (AMPs) in Wound Therapy

Antimicrobial peptides are short (10–50 amino acids) cationic, amphipathic peptides that kill bacteria by disrupting their membranes or interfering with intracellular processes. Unlike antibiotics, AMPs do not induce resistance because they target conserved membrane structures, making them ideal for treating drug-resistant infections. However, AMPs have poor stability in vivo (degraded by proteases), high cytotoxicity at high concentrations, and low bioavailability—challenges that can be addressed by loading them onto nanocarriers.

Nanoparticle-based AMP delivery systems, such as liposomes, polymersomes, and mesoporous silica NPs, improve AMP stability and reduce cytotoxicity by controlling release. For example, AMP-loaded liposomes show a 5–10-fold increase in half-life compared to free AMPs. However, most AMP delivery systems rely on passive release, leading to premature release in normal tissues and reduced efficacy at infection sites. Stimuli-responsive release (e.g., NIR light, acidic pH, bacterial enzymes) can address this issue by triggering AMP release only at the infection site. Few studies have combined stimuli-responsive AMP delivery with nanozyme and photothermal activities for synergistic antimicrobial therapy.

3. Methodology

3.1 Synthesis and Characterization of MDC-AMP Nanoparticles

3.1.1 Synthesis of ZIF-8 MOFs

ZIF-8 MOFs were synthesized using a solvothermal method. Briefly, 0.61 g of zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) was dissolved in 20 mL of methanol, and 1.64 g of 2-methylimidazole was dissolved in 20 mL of methanol. The two solutions were mixed under vigorous stirring at room temperature, and the mixture was incubated for 24 h. The resulting white ZIF-8 precipitate was collected by centrifugation (8,000 rpm, 15 min), washed with methanol three times, and dried at 60°C overnight.

3.1.2 Synthesis of MDC NPs

MDC NPs were prepared by pyrolyzing ZIF-8 at high temperature. ZIF-8 powder (1.0 g) was placed in a quartz tube and heated to 800°C at a rate of 5°C/min under a nitrogen atmosphere. The pyrolysis was maintained for 2 h, then cooled to room temperature. The resulting black MDC NPs were ground into a fine powder and dispersed in deionized water (DI) for further modification.

3.1.3 Surface Modification with PEG

To enhance biocompatibility and reduce aggregation, MDC NPs were modified with polyethylene glycol (PEG). MDC NPs (100 mg) were dispersed in 10 mL of DI water, and 50 mg of NH_2 -PEG-COOH (molecular weight: 5 kDa) was added. The mixture was stirred at room temperature for 4 h, and the PEG-modified MDC NPs (MDC-PEG) were collected by centrifugation (10,000 rpm, 20 min), washed with DI water three times, and resuspended in DI water.

3.1.4 Loading of Antimicrobial Peptides (AMPs)

The antimicrobial peptide LL-37 (sequence: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES) was purchased from ChinaPeptides Co., Ltd. (Shanghai, China). AMPs were loaded onto MDC-PEG via electrostatic interaction. MDC-PEG (50 mg) was dispersed in 5 mL of 0.1 M PBS (pH 7.4), and different

concentrations of LL-37 (0.1–1.0 mg/mL) were added. The mixture was incubated at 4°C for 12 h, and the MDC-AMP nanoparticles were collected by centrifugation (10,000 rpm, 20 min). The loading efficiency and capacity were determined by measuring the absorbance of free LL-37 in the supernatant at 280 nm using a UV-Vis spectrophotometer (Shimadzu UV-2600, Kyoto, Japan).

3.1.5 Characterization of Nanoparticles

The morphology of ZIF-8, MDC NPs, MDC-PEG, and MDC-AMP was observed using transmission electron microscopy (TEM, JEOL JEM-2100, Tokyo, Japan) and scanning electron microscopy (SEM, FEI Quanta 250, Hillsboro, OR, USA). The crystal structure was analyzed by X-ray diffraction (XRD, Bruker D8 Advance, Karlsruhe, Germany) with Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$). The specific surface area and pore size distribution were measured by Brunauer-Emmett-Teller (BET) analysis (Micromeritics ASAP 2020, Norcross, GA, USA). The surface functional groups were characterized by Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet iS50, Waltham, MA, USA) and X-ray photoelectron spectroscopy (XPS, Thermo Scientific K-Alpha, Waltham, MA, USA). The zeta potential and particle size were measured using dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS, Worcestershire, UK).

3.2 Enzyme-Mimetic Activity Assays

3.2.1 Peroxidase-Like Activity

Peroxidase-like activity was evaluated using 3,3',5,5'-tetramethylbenzidine (TMB) as a chromogenic substrate. Different concentrations of MDC-PEG (0–200 $\mu\text{g/mL}$) were mixed with 0.1 mM TMB and 100 μM H_2O_2 in 0.1 M acetate buffer (pH 4.0). The mixture was incubated at 37°C for 30 min, and the absorbance was measured at 652 nm. The catalytic rate was calculated based on the change in absorbance over time.

3.2.2 Catalase-Like Activity

Catalase-like activity was evaluated by measuring the volume of O_2 generated from H_2O_2 decomposition. MDC-PEG (100 $\mu\text{g/mL}$) was added to 10 mL of 10 mM H_2O_2 in 0.1 M PBS (pH 7.4) at 37°C. The volume of O_2 released was measured using a gas burette, and the catalytic efficiency was calculated as moles of O_2 generated per minute per gram of MDC-PEG.

3.3 Photothermal Properties and NIR-Triggered AMP Release

3.3.1 Photothermal Conversion Efficiency

The photothermal properties of MDC-PEG were evaluated under NIR laser irradiation (808 nm, 1.0 W/cm^2). MDC-PEG solutions (0–200 $\mu\text{g/mL}$) were irradiated for 10 min, and the temperature change was recorded every 30 seconds using a thermal imaging camera (FLIR E60, Wilsonville, OR, USA). The photothermal conversion efficiency (η) was calculated using the following formula:

$$\eta = (hS\Delta T_{\text{max}} - Q_0) / I(1 - 10^{-A_{808}})$$

where hS is the heat transfer coefficient, ΔT_{max} is the maximum temperature change, Q_0 is the heat generated by the solvent, I is the laser power density, and A_{808} is the absorbance of MDC-PEG at 808 nm.

3.3.2 NIR-Triggered AMP Release

To evaluate the NIR-triggered AMP release behavior, MDC-AMP (100 $\mu\text{g/mL}$) was dispersed in 0.1 M PBS (pH 7.4) and irradiated with an 808 nm NIR laser (1.0 W/cm^2) for 0, 5, 10, 15, and 20 min. At each time point, the solution was centrifuged (10,000 rpm, 20 min), and the concentration of released LL-37 in the supernatant was measured at 280 nm. The cumulative release percentage was calculated as (Released AMP amount / Total loaded AMP amount) $\times$ 100%. For comparison, the release behavior of MDC-AMP without

NIR irradiation was also measured over 24 h.

3.4 In Vitro Antibacterial Experiments

3.4.1 Bacterial Strains and Culture Conditions

Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300) and Gram-negative *Escherichia coli* (E. coli, ATCC 25922) were used in this study. Bacteria were cultured in Luria-Bertani (LB) broth at 37°C with shaking (180 rpm) for 12 h to reach the logarithmic growth phase. The bacterial concentration was adjusted to 1×10^6 colony-forming units (CFU)/mL using LB broth for subsequent experiments.

3.4.2 Antibacterial Efficacy Against Planktonic Bacteria

The antibacterial efficacy of different formulations was evaluated using the colony counting method. Bacterial suspensions (1×10^6 CFU/mL) were treated with: (1) saline; (2) free LL-37 (20 µg/mL); (3) MDC-PEG (100 µg/mL); (4) MDC-PEG + NIR (100 µg/mL, 808 nm, 1.0 W/cm², 10 min); (5) MDC-AMP (100 µg/mL MDC-PEG, 20 µg/mL LL-37); (6) MDC-AMP + NIR (100 µg/mL MDC-PEG, 20 µg/mL LL-37, 808 nm, 1.0 W/cm², 10 min). After incubation at 37°C for 4 h, the treated bacterial suspensions were serially diluted (10^0 – 10^6) with sterile PBS, and 100 µL of each dilution was spread on LB agar plates. The plates were incubated at 37°C for 24 h, and the number of colonies was counted. The antibacterial efficiency was calculated as:

Antibacterial efficiency = [(CFU of control group - CFU of treated group) / CFU of control group] × 100%

Additionally, the bacterial viability was visualized using LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bacteria were stained with SYTO 9 (green, live bacteria) and propidium iodide (PI, red, dead bacteria) for 15 min in the dark, then observed under a confocal laser scanning microscope (CLSM, Zeiss LSM 880, Oberkochen, Germany).

3.4.3 MRSA Biofilm Disruption Assay

MRSA biofilms were formed in 24-well plates. Briefly, 1 mL of MRSA suspension (1×10^6 CFU/mL) was added to each well and incubated at 37°C for 48 h. The medium was refreshed every 12 h to promote biofilm formation. After 48 h, the supernatant was removed, and the biofilms were gently washed with PBS to remove planktonic bacteria. The biofilms were then treated with the same formulations as in the planktonic antibacterial assay for 6 h (MDC-AMP + NIR group was irradiated for 10 min at the start of incubation).

After treatment, the biofilm biomass was measured using the crystal violet (CV) staining method. The biofilms were fixed with 4% paraformaldehyde for 15 min, stained with 0.1% CV for 30 min, and washed with PBS to remove excess dye. The bound CV was dissolved in 33% acetic acid, and the absorbance was measured at 570 nm. The biofilm degradation rate was calculated as:

Biofilm degradation rate = [(Absorbance of control group - Absorbance of treated group) / Absorbance of control group] × 100%

The viability of bacteria within the biofilm was evaluated using the LIVE/DEAD staining kit, followed by CLSM observation. The biofilm structure was also observed using scanning electron microscopy (SEM). After treatment, the biofilms were fixed with 2.5% glutaraldehyde for 24 h, dehydrated with a gradient ethanol series (30%, 50%, 70%, 90%, 100%), and sputter-coated with gold. SEM images were acquired using a FEI Quanta 250 SEM.

3.5 Biocompatibility Evaluation

3.5.1 Hemolysis Assay

The hemocompatibility of MDC-PEG and MDC-AMP was evaluated using a hemolysis assay. Fresh mouse blood was collected from BALB/c mice (4–6 weeks old) and centrifuged at 3,000 rpm for 10 min to separate red blood cells (RBCs). The RBCs were washed three times with PBS and resuspended in PBS to a concentration of 2% (v/v). Different concentrations of MDC-PEG or MDC-AMP (0–400 µg/mL) were mixed with 0.5 mL of 2% RBC suspension and incubated at 37°C for 1 h. Distilled water was used as a positive control (100% hemolysis), and PBS was used as a negative control (0% hemolysis). After incubation, the mixtures were centrifuged at 3,000 rpm for 10 min, and the absorbance of the supernatant was measured at 540 nm. The hemolysis rate was calculated as:

$$\text{Hemolysis rate} = \frac{[\text{Absorbance of sample} - \text{Absorbance of negative control}]}{[\text{Absorbance of positive control} - \text{Absorbance of negative control}]} \times 100\%$$

3.5.2 Cytotoxicity Assay

The cytotoxicity of MDC-PEG and MDC-AMP against mouse embryonic fibroblasts (NIH/3T3 cells) was evaluated using the MTT assay. NIH/3T3 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight at 37°C with 5% CO₂. The cells were then treated with different concentrations of MDC-PEG or MDC-AMP (0–400 µg/mL) for 24 h. For the NIR groups, the cells were irradiated with an 808 nm laser (1.0 W/cm²) for 10 min after adding the nanoparticles. After incubation, 20 µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 h. The supernatant was removed, and 150 µL of DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability was calculated as:

$$\text{Cell viability} = \frac{\text{Absorbance of treated group}}{\text{Absorbance of control group}} \times 100\%$$

3.5.3 In Vivo Biocompatibility

To evaluate in vivo biocompatibility, MDC-PEG or MDC-AMP (200 µL, 100 µg/mL) was subcutaneously injected into BALB/c mice (n=3 per group). Saline was injected as a control. At 1, 3, and 7 days post-injection, the mice were euthanized, and the injection site tissues were harvested. The tissues were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The sections were observed under a light microscope (Olympus BX53, Tokyo, Japan) to evaluate inflammatory cell infiltration and tissue damage.

3.6 In Vivo MRSA-Infected Wound Healing Model

3.6.1 Wound Model Establishment

Female BALB/c nude mice (4–6 weeks old, 18–22 g) were used to establish the MRSA-infected full-thickness skin wound model. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan (Protocol Number: PRO00010256). The mice were anesthetized with isoflurane, and the dorsal hair was removed using a depilatory cream. A 10 mm diameter full-thickness skin wound was created on the dorsal side using a sterile biopsy punch. The wound was inoculated with 100 µL of MRSA suspension (1×10^8 CFU/mL) to induce infection.

3.6.2 Treatment Protocol

Twenty-four hours after infection, the mice were randomly divided into six groups (n=6 per group): (1) Saline; (2) Free LL-37; (3) MDC-PEG; (4) MDC-PEG + NIR; (5) MDC-AMP; (6) MDC-AMP + NIR. For each

treatment:

Saline group: 100 μ L of saline was applied topically to the wound.

Free LL-37 group: 100 μ L of free LL-37 (20 μ g/mL) was applied topically.

MDC-PEG group: 100 μ L of MDC-PEG (100 μ g/mL) was applied topically.

MDC-PEG + NIR group: 100 μ L of MDC-PEG (100 μ g/mL) was applied, followed by 808 nm NIR irradiation (1.0 W/cm²) for 10 min.

MDC-AMP group: 100 μ L of MDC-AMP (100 μ g/mL MDC-PEG, 20 μ g/mL LL-37) was applied topically.

MDC-AMP + NIR group: 100 μ L of MDC-AMP was applied, followed by 808 nm NIR irradiation (1.0 W/cm²) for 10 min.

Treatments were administered once every 2 days for a total of 7 days.

3.6.3 Wound Healing Evaluation

The wound images were captured every 2 days using a digital camera. The wound area was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the wound healing rate was calculated as:

Wound healing rate = [(Initial wound area - Wound area at day n) / Initial wound area] $\times$ 100%

At 7 days post-treatment, the mice were euthanized, and the wound tissues were harvested for further analysis.

3.6.4 Bacterial Load Determination

The bacterial load in the wound tissues was measured using the colony counting method. The wound tissues were homogenized in 1 mL of sterile PBS, and the homogenates were serially diluted. 100 μ L of each dilution was spread on LB agar plates containing methicillin (10 μ g/mL) to select MRSA. The plates were incubated at 37°C for 24 h, and the number of colonies was counted to determine the CFU per gram of tissue.

3.6.5 Histopathological and Immunohistochemical Analysis

The wound tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 μ m slices. The sections were stained with H&E to evaluate tissue regeneration (e.g., re-epithelialization, granulation tissue formation) and inflammatory cell infiltration. Masson's trichrome staining was used to assess collagen deposition.

Immunohistochemical staining was performed to detect the expression of inflammatory markers (tumor necrosis factor- α , TNF- α ; interleukin-6, IL-6) and angiogenesis markers (cluster of differentiation 31, CD31). The sections were incubated with primary antibodies against TNF- α (1:200, Abcam, Cambridge, UK), IL-6 (1:200, Abcam), and CD31 (1:200, Abcam) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibodies (1:500, Abcam) for 1 h at room temperature. The staining was visualized using 3,3'-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin. The positive staining area was quantified using ImageJ software.

3.7 Statistical Analysis

All experiments were performed in triplicate, and the data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 Synthesis and Characterization of MDC-AMP Nanoparticles

4.1.1 Morphology and Structure

TEM images showed that ZIF-8 had a typical rhombic dodecahedral morphology with a uniform size of ~ 100 nm (Figure 1A). After pyrolysis at 800°C , ZIF-8 was converted into MDC NPs with a porous carbon structure, retaining partial rhombic dodecahedral shape but with a reduced size of ~ 80 nm (Figure 1B). Surface modification with PEG and loading of AMPs did not significantly change the morphology of MDC NPs, as observed in TEM images of MDC-PEG (Figure 1C) and MDC-AMP (Figure 1D). SEM images further confirmed the porous structure of MDC NPs and the uniform distribution of MDC-AMP (Figure 1E,F).

XRD patterns (Figure 2A) showed that ZIF-8 exhibited characteristic diffraction peaks at 7.3° , 10.3° , 12.7° , 14.7° , 16.4° , 18.0° , 22.1° , and 24.5° , which matched the standard pattern of ZIF-8 (JCPDS No. 00-062-1030). After pyrolysis, the diffraction peaks of ZIF-8 disappeared, and a broad peak at $\sim 23^{\circ}$ was observed for MDC NPs, corresponding to the (002) plane of amorphous carbon, indicating successful conversion of ZIF-8 to carbon-based NPs. No significant changes in XRD patterns were observed for MDC-PEG and MDC-AMP, confirming that PEG modification and AMP loading did not affect the crystal structure of MDC NPs.

4.1.2 BET Surface Area and Pore Size

BET analysis (Figure 2B,C) showed that MDC NPs had a high specific surface area of 867 ± 32 m^2/g and a pore volume of 1.25 ± 0.08 cm^3/g , with an average pore size of 5.8 ± 0.4 nm. This porous structure is beneficial for AMP loading and enzyme-mimetic activity. After PEG modification, the specific surface area and pore volume of MDC-PEG decreased to 654 ± 28 m^2/g and 0.92 ± 0.06 cm^3/g , respectively, due to the partial filling of pores by PEG. Loading of AMPs further reduced the specific surface area and pore volume to 589 ± 25 m^2/g and 0.78 ± 0.05 cm^3/g , confirming successful AMP loading into the pores of MDC-PEG.

4.1.3 Surface Chemistry and Zeta Potential

FTIR spectra (Figure 2D) showed that ZIF-8 had characteristic peaks at 420 cm^{-1} (Zn-N stretching), 750 cm^{-1} (C-H bending of imidazole), 1140 cm^{-1} (C-N stretching), and 1580 cm^{-1} (C=C stretching of imidazole). After pyrolysis, these peaks disappeared, and new peaks at 1620 cm^{-1} (C=C stretching of carbon) and 3400 cm^{-1} (O-H stretching) were observed for MDC NPs, indicating the formation of carbon structures with surface hydroxyl groups. MDC-PEG exhibited additional peaks at 2880 cm^{-1} (C-H stretching of PEG) and 1080 cm^{-1} (C-O-C stretching of PEG), confirming successful PEG modification. MDC-AMP showed a peak at 1650 cm^{-1} (amide I band of AMPs), verifying AMP loading.

XPS analysis (Figure 2E,F) revealed that MDC NPs contained C (82.3%), O (12.5%), and N (5.2%) elements. The high-resolution C 1s spectrum of MDC NPs showed peaks at 284.8 eV (C=C), 285.6 eV (C-N/C-O), and 288.2 eV (C=O), confirming the presence of various carbon-containing functional groups. After AMP loading, the N content increased to 7.8%, and a new peak at 400.2 eV (N-H of amide groups in AMPs) was observed in the high-resolution N 1s spectrum of MDC-AMP, further confirming successful AMP conjugation.

The zeta potential of ZIF-8 was $+25.6 \pm 2.3$ mV due to the protonation of imidazole ligands. After pyrolysis, MDC NPs exhibited a negative zeta potential of -32.4 ± 3.1 mV, attributed to surface hydroxyl and carboxyl groups. PEG modification (with negatively charged carboxyl groups) slightly decreased the zeta potential to -38.7 ± 3.5 mV. Loading of positively charged AMPs (LL-37, isoelectric point ~ 9.5) reversed the zeta potential to $+18.9 \pm 2.7$ mV, confirming electrostatic interaction between MDC-PEG and AMPs (Figure

2G).

4.1.4 AMP Loading and NIR-Triggered Release

The AMP loading efficiency and capacity of MDC-PEG were evaluated at different LL-37 concentrations. At an LL-37 concentration of 0.5 mg/mL, the loading efficiency reached $91.2 \pm 4.3\%$, with a loading capacity of $45.6 \pm 2.1 \mu\text{g AMP/mg MDC-PEG}$. Higher LL-37 concentrations ($\square 0.5 \text{ mg/mL}$) did not significantly increase the loading efficiency, indicating saturation of the MDC-PEG pores (Figure 3A).

The NIR-triggered AMP release behavior of MDC-AMP is shown in Figure 3B. Without NIR irradiation, only $12.5 \pm 2.3\%$ of AMPs were released after 24 h, indicating good stability of the complex under physiological conditions. Under 808 nm NIR irradiation (1.0 W/cm^2), the cumulative AMP release increased with irradiation time: $35.2 \pm 3.1\%$ after 5 min, $68.7 \pm 4.5\%$ after 10 min, $82.3 \pm 5.2\%$ after 15 min, and $90.1 \pm 5.7\%$ after 20 min. This rapid NIR-triggered release was attributed to heat-induced weakening of electrostatic interactions between MDC-PEG and AMPs, as well as pore expansion of MDC NPs under thermal stimulation.

4.2 Enzyme-Mimetic Activities

4.2.1 Peroxidase-Like Activity

The peroxidase-like activity of MDC-PEG was evaluated using TMB as a substrate. MDC-PEG catalyzed the oxidation of TMB by H_2O_2 , resulting in a blue color change (absorbance at 652 nm) in a concentration-dependent manner (Figure 4A). The catalytic rate of MDC-PEG ($100 \mu\text{g/mL}$) was $0.87 \pm 0.06 \mu\text{M/min}$, which was 3.2-fold higher than that of bare carbon NPs ($0.27 \pm 0.03 \mu\text{M/min}$) and comparable to that of natural horseradish peroxidase (HRP, $1.02 \pm 0.08 \mu\text{M/min}$). The peroxidase-like activity was pH-dependent, with maximum activity at pH 4.0 (simulating the acidic wound microenvironment) and minimal activity at pH 7.4 (normal tissue pH) (Figure 4B). This pH responsiveness ensures selective ROS generation at infection sites, reducing off-target oxidative damage.

4.2.2 Catalase-Like Activity

MDC-PEG exhibited efficient catalase-like activity, decomposing H_2O_2 into O_2 and H_2O . The volume of O_2 generated increased linearly with MDC-PEG concentration, with a catalytic efficiency of $12.5 \pm 0.8 \text{ mmol O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ for MDC-PEG ($100 \mu\text{g/mL}$) (Figure 4C). In the presence of high H_2O_2 concentrations ($\square 500 \mu\text{M}$), the catalase-like activity of MDC-PEG was enhanced, while the peroxidase-like activity was inhibited (Figure 4D). This dual-enzyme switching behavior allows MDC-PEG to scavenge excess H_2O_2 in normal tissues (preventing oxidative stress) and generate ROS in infected wounds (for antibacterial action), addressing the limitations of single-enzyme nanozymes.

4.3 Photothermal Properties

MDC-PEG exhibited excellent photothermal performance under 808 nm NIR irradiation. The temperature of MDC-PEG solutions increased in a concentration- and power-dependent manner. At a concentration of $200 \mu\text{g/mL}$ and power density of 1.0 W/cm^2 , the temperature increased from 25.6°C to 58.7°C within 10 min (Figure 5A,B). The photothermal conversion efficiency (η) of MDC-PEG was calculated as $42.3 \pm 2.1\%$, which is higher than that of other carbon-based nanomaterials (e.g., graphene oxide, $\eta \approx 30\%$; carbon nanotubes, $\eta \approx 35\%$) (Figure 5C). MDC-PEG also showed good photothermal stability, with no significant decrease in temperature after 5 cycles of NIR irradiation (Figure 5D), ensuring repeated use for PTT.

4.4 In Vitro Antibacterial Efficacy

4.4.1 Antibacterial Activity Against Planktonic Bacteria

Colony counting results showed that MDC-AMP + NIR exhibited the highest antibacterial efficiency against both *E. coli* and MRSA (Figure 6A,B). For *E. coli*, the antibacterial efficiency of MDC-AMP + NIR was $99.8 \pm 0.2\%$, compared to $65.3 \pm 4.1\%$ for free LL-37, $32.5 \pm 3.6\%$ for MDC-PEG, and $58.7 \pm 4.8\%$ for MDC-AMP ($p < 0.001$). For MRSA, MDC-AMP + NIR achieved an antibacterial efficiency of $99.2 \pm 0.5\%$, significantly higher than other groups (free LL-37: $61.2 \pm 3.8\%$; MDC-PEG: $28.9 \pm 3.2\%$; MDC-AMP: $54.3 \pm 4.5\%$, $p < 0.001$).

LIVE/DEAD staining further confirmed these results. For *E. coli* (Figure 6C) and MRSA (Figure 6D), the MDC-AMP + NIR group showed intense red fluorescence (dead bacteria) and minimal green fluorescence (live bacteria), while the saline group showed mostly green fluorescence. The synergistic effect of enzyme-catalyzed ROS generation, photothermal ablation, and NIR-triggered AMP release contributed to the superior antibacterial efficacy.

4.4.2 MRSA Biofilm Disruption

MRSA biofilms are highly resistant to conventional antibiotics, but MDC-AMP + NIR effectively disrupted biofilms. CV staining showed that MDC-AMP + NIR reduced biofilm biomass by $87.6 \pm 5.3\%$, compared to $32.1 \pm 3.7\%$ for free LL-37, $21.5 \pm 2.9\%$ for MDC-PEG, and $48.9 \pm 4.2\%$ for MDC-AMP ($p < 0.001$) (Figure 7A).

CLSM images of LIVE/DEAD-stained biofilms showed that MDC-AMP + NIR significantly reduced the number of live bacteria (green) within the biofilm, with extensive red fluorescence (dead bacteria) throughout the biofilm structure (Figure 7B). SEM images revealed that the compact biofilm structure in the saline group was disrupted by MDC-AMP + NIR, with visible bacterial lysis and EPS degradation (Figure 7C). The photothermal effect of MDC-PEG likely melted the EPS matrix, while ROS and AMPs killed the exposed bacteria, achieving efficient biofilm eradication.

4.5 Biocompatibility Evaluation

4.5.1 Hemolysis Assay

MDC-PEG and MDC-AMP showed excellent hemocompatibility. At concentrations up to $400 \mu\text{g/mL}$, the hemolysis rate of MDC-PEG was $2.3 \pm 0.5\%$, and that of MDC-AMP was $3.1 \pm 0.7\%$, both well below the 5% threshold for biocompatible materials (Figure 8A). This low hemolysis rate is attributed to the PEG coating, which reduces nonspecific interaction with RBCs.

4.5.2 Cytotoxicity Assay

MDC-PEG and MDC-AMP exhibited low cytotoxicity against NIH/3T3 cells. At a concentration of $400 \mu\text{g/mL}$, the cell viability of MDC-PEG-treated cells was $89.2 \pm 4.5\%$, and that of MDC-AMP-treated cells was $85.7 \pm 5.1\%$ (Figure 8B). Even with NIR irradiation, the cell viability remained above 80% (MDC-PEG + NIR: $82.3 \pm 4.8\%$; MDC-AMP + NIR: $79.6 \pm 5.3\%$), indicating that the photothermal effect of MDC-PEG at 1.0 W/cm^2 does not cause significant damage to normal cells.

4.5.3 In Vivo Biocompatibility

Subcutaneous injection of MDC-PEG or MDC-AMP did not induce significant inflammatory responses or tissue damage. H&E staining of injection site tissues showed minimal inflammatory cell infiltration at 1, 3, and 7 days post-injection, similar to the saline group (Figure 8C). No necrosis, edema, or fibrosis was

observed, confirming the *in vivo* biocompatibility of MDC-AMP.

4.6 In Vivo MRSA-Infected Wound Healing

4.6.1 Wound Healing Rate

The MDC-AMP + NIR group showed the fastest wound healing rate. At day 7, the wound healing rate of MDC-AMP + NIR was $92.3 \pm 4.7\%$, compared to $45.6 \pm 5.2\%$ for saline, $58.7 \pm 4.9\%$ for free LL-37, $62.1 \pm 5.3\%$ for MDC-PEG, and $75.8 \pm 5.6\%$ for MDC-AMP ($p < 0.001$) (Figure 9A,B). The wounds in the MDC-AMP + NIR group were almost completely closed by day 7, with new epithelial tissue covering the wound bed, while the saline group still had large, inflamed wounds.

4.6.2 Bacterial Load in Wound Tissues

MDC-AMP + NIR significantly reduced the bacterial load in wound tissues. The bacterial count in the MDC-AMP + NIR group was $2.3 \times 10^3 \pm 0.5 \times 10^3$ CFU/g tissue, which was 4–5 orders of magnitude lower than that in the saline group ($8.7 \times 10^7 \pm 1.2 \times 10^7$ CFU/g tissue) (Figure 9C). This result confirms that the synergistic antimicrobial effect of MDC-AMP + NIR effectively eliminates MRSA *in vivo*.

4.6.3 Histopathological Analysis

H&E staining of wound tissues (Figure 10A) showed that the MDC-AMP + NIR group had complete re-epithelialization, dense granulation tissue, and minimal inflammatory cell infiltration. In contrast, the saline group had incomplete epithelial coverage, loose granulation tissue, and extensive inflammatory cell infiltration (e.g., neutrophils, macrophages). Masson's trichrome staining (Figure 10B) revealed that the MDC-AMP + NIR group had significantly higher collagen deposition (blue staining) than other groups, with well-organized collagen fibers, indicating enhanced tissue regeneration.

4.6.4 Immunohistochemical Analysis

Immunohistochemical staining showed that MDC-AMP + NIR reduced the expression of inflammatory markers (TNF- α , IL-6) and increased the expression of the angiogenesis marker (CD31) (Figure 11A–C). The positive staining area for TNF- α in the MDC-AMP + NIR group was $3.2 \pm 0.8\%$, compared to $18.5 \pm 2.3\%$ in the saline group ($p < 0.001$). Similarly, the IL-6 positive area was reduced from $16.8 \pm 2.1\%$ (saline) to $4.5 \pm 1.1\%$ (MDC-AMP + NIR, $p < 0.001$). The CD31 positive area (a measure of blood vessel density) was $12.3 \pm 1.5\%$ in the MDC-AMP + NIR group, 2.8-fold higher than that in the saline group ($4.4 \pm 0.7\%$, $p < 0.001$). These results indicate that MDC-AMP + NIR not only eliminates bacteria but also reduces inflammation and promotes angiogenesis, creating a favorable microenvironment for wound healing.

5. Discussion

5.1 Key Findings and Mechanisms of Synergistic Therapy

This study developed a multi-modal MDC-AMP nanosystem that integrates dual enzyme-mimetic activities, photothermal therapy, and AMP-based antimicrobial action for effective treatment of MRSA-infected wounds. The key findings and underlying mechanisms are:

5.1.1 Dual Enzyme-Mimetic Activity for Smart ROS Regulation

MDC-PEG exhibits pH-responsive peroxidase-like activity (maximal at pH 4.0, infected wounds) to generate $\cdot\text{OH}$ from endogenous H_2O_2 , killing bacteria without exogenous H_2O_2 addition. In normal tissues (pH 7.4), it switches to catalase-like activity to decompose excess H_2O_2 into O_2 , relieving oxidative stress. This “on-demand” ROS generation avoids off-target tissue damage and addresses the limitations of single-

enzyme nanozymes.

5.1.2 NIR-Triggered AMP Release and Photothermal Synergy

The high photothermal conversion efficiency (42.3%) of MDC-PEG enables two critical functions: (a) direct photothermal ablation of bacteria (temperature reaches 58.7°C, above the bactericidal threshold of 45°C); (b) triggered release of AMPs (90.1% release after 20 min irradiation). The heat-induced disruption of biofilm EPS further enhances AMP penetration and ROS diffusion, achieving synergistic eradication of planktonic bacteria and biofilms.

5.1.3 Enhanced Wound Healing via Anti-Inflammation and Angiogenesis

Beyond antibacterial action, MDC-AMP + NIR reduces the expression of pro-inflammatory cytokines (TNF- α , IL-6) by eliminating bacteria and scavenging ROS. The increased angiogenesis (higher CD31 expression) promotes nutrient and oxygen delivery to the wound bed, while enhanced collagen deposition accelerates tissue regeneration. This multi-faceted effect addresses the “infection-healing cycle” (bacterial infection delays healing, and delayed healing exacerbates infection) that plagues chronic wound treatment.

5.2 Comparison with Existing Wound Therapeutics

The MDC-AMP nanosystem offers significant advantages over conventional and nanomaterial-based wound therapeutics:

5.2.1 Superior Antibacterial Efficacy Against Drug-Resistant Strains

Unlike antibiotics that induce MRSA resistance, the MDC-AMP nanosystem kills bacteria via multiple mechanisms (ROS, heat, membrane disruption by AMPs), making it difficult for bacteria to develop resistance. The 99.8% and 99.2% antibacterial efficiency against *E. coli* and MRSA, respectively, outperforms most single-modal nanotherapeutics (e.g., AMP-loaded liposomes: ~85% MRSA killing; photothermal carbon NPs: ~90% MRSA killing).

5.2.2 Minimal Toxicity and High Biocompatibility

The PEG coating of MDC-PEG reduces nonspecific interactions with normal cells, resulting in low hemolysis (<5%) and cytotoxicity (>80% cell viability at 400 μ g/mL). In vivo studies show no tissue damage or inflammation, addressing the safety concerns of metal-based nanozymes (e.g., Fe₃O₄ NPs, which accumulate in the liver and cause oxidative stress).

5.2.3 Integration of Antibacterial and Wound Healing Promotion

Most existing wound therapeutics focus solely on antibacterial action, while the MDC-AMP nanosystem simultaneously eliminates bacteria, reduces inflammation, and promotes angiogenesis/collagen deposition. This holistic approach accelerates wound healing (92.3% healing rate at day 7) compared to antibiotic treatments (typically <60% healing rate at day 7 for MRSA-infected wounds).

5.3 Limitations and Future Directions

Despite its promising results, this study has several limitations:

5.3.1 In Vivo Model Limitations

The study used a murine full-thickness wound model, which has a faster healing rate than human wounds and does not fully recapitulate the chronic wound microenvironment (e.g., diabetes, ischemia). Future studies should use diabetic mouse models or porcine wound models (with skin physiology similar to humans) to evaluate the nanosystem's efficacy in chronic infected wounds.

5.3.2 Long-Term Biodistribution and Clearance

The long-term fate of MDC NPs in the body (e.g., accumulation in organs, excretion pathway) remains unknown. Although MDC NPs are carbon-based and likely biodegradable, future studies should investigate their biodistribution over 1–3 months and evaluate potential long-term toxicity.

5.3.3 Clinical Translation Challenges

Scaling up MDC-AMP synthesis with consistent properties (e.g., particle size, enzyme activity) is critical for clinical translation. Additionally, the NIR laser's penetration depth (~2 cm) limits the nanosystem's application in deep tissue infections; future work could explore second-window NIR lasers (1000–1700 nm) with deeper penetration or develop injectable MDC-AMP formulations for deep tissue applications.

5.3.4 AMP Stability in Complex Wound Environments

While NIR-triggered release improves AMP stability, the complex wound microenvironment (e.g., proteases, high salt concentrations) may still degrade AMPs. Future work could modify AMPs with protease-resistant groups (e.g., D-amino acids, polyethylene glycolylation) or encapsulate AMPs in pH-sensitive microgels within MDC NPs to further enhance stability.

6. Conclusion

In this study, we successfully developed a multi-modal nanotherapeutic platform based on MOF-derived carbon nanoparticles (MDC NPs) loaded with antimicrobial peptides (AMPs) for synergistic treatment of MRSA-infected wounds. The MDC NPs exhibited dual enzyme-mimetic activities (peroxidase-like and catalase-like) and high photothermal conversion efficiency (42.3%), enabling smart ROS regulation, photothermal ablation, and NIR-triggered AMP release.

Systematic characterization confirmed that MDC-AMP NPs had a porous structure (specific surface area: $589 \pm 25 \text{ m}^2/\text{g}$), high AMP loading efficiency ($91.2 \pm 4.3\%$), and pH-responsive enzyme activity—maximizing ROS generation at infected wounds (pH 4.0) while scavenging excess H_2O_2 in normal tissues (pH 7.4). In vitro studies showed that MDC-AMP + NIR achieved 99.8% and 99.2% antibacterial efficiency against *E. coli* and MRSA, respectively, and disrupted 87.6% of MRSA biofilms. Importantly, MDC-AMP exhibited excellent biocompatibility, with low hemolysis (<5%) and high cell viability (>80%) at 400 $\mu\text{g}/\text{mL}$.

In a MRSA-infected mouse full-thickness wound model, MDC-AMP + NIR accelerated wound healing (92.3% closure at day 7), reduced bacterial load by 4–5 orders of magnitude, and promoted tissue regeneration via anti-inflammatory (reduced $\text{TNF-}\alpha/\text{IL-6}$ expression) and pro-angiogenic (increased CD31 expression) effects. This work demonstrates the power of integrating nanomaterial engineering (MOF-derived carbon NPs) with biological principles (enzyme catalysis, AMPs) to address the unmet clinical need for drug-resistant bacterial wound infection therapy.

The MDC-AMP nanosystem not only provides a versatile strategy for treating MRSA-infected wounds but also serves as a blueprint for developing multi-modal nanotherapeutics for other infectious diseases, advancing the convergence of nanoscience and clinical microbiology.

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Article

Near-Infrared Quantum Dot-Conjugated Nanobodies for Dual-Modal Fluorescence Imaging and Photodynamic Therapy of HER2-Positive Breast Cancer

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ABSTRACT

Human epidermal growth factor receptor 2 (HER2)-positive breast cancer accounts for 15–20% of all breast cancer cases and is associated with aggressive progression and poor prognosis. Current diagnostic methods (e.g., immunohistochemistry, FISH) lack real-time imaging capabilities, while therapeutic strategies (e.g., trastuzumab) often suffer from low tumor penetration and acquired resistance. Herein, we report a dual-modal nanotherapeutic probe based on near-infrared (NIR) quantum dots (QDs) conjugated with anti-HER2 nanobodies (Nb) and photosensitizers (PS) for simultaneous fluorescence imaging and photodynamic therapy (PDT) of HER2-positive breast cancer. The NIR QDs (CdSe/ZnS core-shell, emission wavelength: 808 nm) were synthesized via a hot-injection method, surface-modified with polyethylene glycol (PEG) to enhance biocompatibility, and conjugated with anti-HER2 Nb (high-affinity, small-size targeting ligands) and chlorin e6 (Ce6, a photosensitizer) via click chemistry. The resulting Nb-QD-Ce6 probe exhibits excellent photostability, high HER2 targeting specificity, and efficient reactive oxygen species (ROS) generation under NIR laser irradiation (660 nm). In vitro studies show that Nb-QD-Ce6 achieves 4.2-fold higher cellular uptake in SK-BR-3 HER2-positive breast cancer cells than non-targeted QD-Ce6, enabling clear NIR fluorescence imaging (signal-to-noise ratio: 12.8) and efficient PDT-induced cell apoptosis (apoptotic rate: 78.5%). In vivo, Nb-QD-Ce6 provides real-time visualization of HER2-positive tumors in xenograft mice, with a tumor-to-muscle signal ratio of 8.7 at 24 h post-injection. PDT treatment with Nb-QD-Ce6 + 660 nm laser significantly inhibits tumor growth (tumor volume reduction: 76.3% vs. saline control) and prolongs mouse survival (median survival: 42 days vs. 21 days for saline). This work demonstrates the potential of NIR QD-nanobody conjugates as a versatile platform for integrated tumor imaging and targeted therapy, bridging nanomaterial optics, antibody engineering, and cancer therapeutics for precision oncology.

Keywords: Quantum Dots; Nanobodies; HER2-Positive Breast Cancer; Fluorescence Imaging; Photodynamic Therapy; Near-Infrared; Tumor Targeting; Reactive Oxygen Species

1. Introduction

1.1 Background

Breast cancer remains the most prevalent malignancy in women worldwide, with over 2.3 million new cases diagnosed annually. HER2-positive breast cancer, characterized by overexpression of the HER2 oncoprotein, is an aggressive subtype associated with rapid tumor growth, high metastatic potential, and poor response to conventional chemotherapy. Trastuzumab (Herceptin), a monoclonal antibody targeting HER2, has improved survival outcomes for HER2-positive patients, but its clinical efficacy is limited by: (1) large molecular size (~150 kDa) leading to poor tumor penetration; (2) acquired resistance due to HER2 mutations or alternative signaling pathway activation; (3) systemic toxicity (e.g., cardiotoxicity).

Accurate and real-time tumor imaging is critical for early diagnosis, treatment planning, and efficacy monitoring of HER2-positive breast cancer. Current diagnostic methods, such as immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), require invasive tissue biopsies and cannot provide dynamic information on tumor progression. Non-invasive imaging techniques, including magnetic resonance imaging (MRI) and positron emission tomography (PET), have low spatial resolution or involve radioactive tracers. Fluorescence imaging using near-infrared (NIR) probes (700–1000 nm) offers advantages of high sensitivity, real-time visualization, and minimal tissue autofluorescence, making it ideal for in vivo tumor imaging.

Photodynamic therapy (PDT) is a non-invasive therapeutic modality that uses photosensitizers (PS) activated by light to generate reactive oxygen species (ROS), which induce tumor cell death and vascular damage. PDT has shown promise for HER2-positive breast cancer, but its efficacy is limited by poor PS tumor targeting and low ROS generation efficiency. The integration of targeted imaging and PDT into a single nanoplatform (theranostics) can address these limitations by enabling precise tumor localization and synchronized therapeutic intervention.

1.2 Research Gaps

Existing theranostic platforms for HER2-positive breast cancer face several challenges. Monoclonal antibody-conjugated nanomaterials have high targeting specificity but suffer from large size and slow blood clearance. Small-molecule targeting ligands (e.g., folic acid) have low affinity for HER2 and lack specificity. Quantum dots (QDs), semiconductor nanocrystals with excellent photostability and narrow emission spectra, are ideal for NIR imaging, but their clinical application is limited by potential toxicity (e.g., Cd²⁺ leakage) and non-specific accumulation in normal organs. Additionally, few theranostic platforms combine NIR QD-based imaging with PDT using a single targeting ligand, and even fewer are validated in clinically relevant HER2-positive breast cancer models.

1.3 Research Objectives and Contributions

The primary objective of this study is to develop a dual-modal theranostic probe based on NIR QDs conjugated with anti-HER2 nanobodies and photosensitizers for integrated fluorescence imaging and PDT of HER2-positive breast cancer. Specific objectives include:

(1) Synthesize and characterize NIR QDs (CdSe/ZnS) conjugated with anti-HER2 nanobodies (Nb) and chlorin e6 (Ce6) photosensitizers.

(2) Evaluate the in vitro targeting specificity, fluorescence imaging capability, and PDT efficacy of Nb-QD-Ce6 in HER2-positive (SK-BR-3) and HER2-negative (MCF-7) breast cancer cells.

(3) Validate the in vivo NIR fluorescence imaging performance and anti-tumor efficacy of Nb-QD-Ce6 in SK-BR-3 xenograft mice.

The key contributions of this research are:

•**Nanomaterial Engineering Contribution:** The Nb-QD-Ce6 probe combines the photostability of NIR QDs, high specificity of anti-HER2 nanobodies, and ROS generation capability of Ce6, addressing the limitations of single-modal imaging or therapy platforms.

•**Targeting Innovation Contribution:** Anti-HER2 nanobodies (15 kDa) offer advantages over monoclonal antibodies (150 kDa) in tumor penetration and blood clearance, enhancing targeting efficiency and reducing off-target accumulation.

•**Theranostic Integration Contribution:** The dual-modal probe enables real-time tumor imaging to guide PDT, ensuring precise therapeutic delivery and minimizing damage to normal tissues—advancing the field of precision oncology.

2. Literature Review

2.1 Quantum Dots in Tumor Imaging

Quantum dots are semiconductor nanocrystals (2–10 nm) with unique optical properties, including size-tunable emission, high quantum yield, and excellent photostability. NIR-emitting QDs (700–1000 nm) are particularly suitable for in vivo imaging due to reduced tissue absorption and autofluorescence, enabling deeper tissue penetration (up to 1 cm) compared to visible-light QDs. CdSe/ZnS core-shell QDs are the most widely used NIR QDs, with ZnS shells reducing Cd²⁺ leakage and improving biocompatibility.

QDs have been conjugated with targeting ligands (e.g., antibodies, peptides, aptamers) for tumor-specific imaging. For example, anti-HER2 monoclonal antibody-conjugated QDs have been used for fluorescence imaging of HER2-positive breast cancer, but their large size limits tumor penetration. Nanobody-conjugated QDs offer a solution, as nanobodies are small (15 kDa), single-domain antibodies derived from camelid heavy-chain-only antibodies, with high affinity and specificity for target antigens. However, few studies have explored NIR QD-nanobody conjugates for in vivo tumor imaging of HER2-positive breast cancer.

2.2 Nanobodies as Targeting Ligands in Cancer Therapy

Nanobodies possess several advantages over traditional monoclonal antibodies for cancer targeting: (1) small size (15 kDa vs. 150 kDa) enabling efficient tumor penetration and rapid blood clearance; (2) high solubility and stability under harsh conditions (e.g., low pH, high temperature); (3) easy genetic engineering and conjugation to nanomaterials; (4) low immunogenicity due to high sequence homology with human antibodies.

Anti-HER2 nanobodies have been developed with dissociation constants (K_d) in the nanomolar range, comparable to monoclonal antibodies. They have been conjugated to drug carriers (e.g., liposomes, polymersomes) for targeted drug delivery to HER2-positive tumors, improving therapeutic efficacy and reducing systemic toxicity. However, the integration of anti-HER2 nanobodies with QDs for dual-modal imaging and PDT has not been fully explored.

2.3 Photodynamic Therapy for HER2-Positive Breast Cancer

Photodynamic therapy relies on three components: a photosensitizer (PS), light of a specific

wavelength, and oxygen. Upon light activation, the PS transitions from the ground state to an excited state, transferring energy to oxygen to generate ROS (e.g., singlet oxygen, $\cdot\text{OH}$), which induce tumor cell apoptosis and necrosis. Chlorin e6 (Ce6) is a widely used PS with strong absorption at 660 nm (compatible with NIR light) and high singlet oxygen quantum yield.

Ce6 has been conjugated to targeting ligands for HER2-positive breast cancer PDT. For example, anti-HER2 monoclonal antibody-conjugated Ce6 shows enhanced tumor accumulation, but its large size limits penetration into deep tumor layers. Nanoparticle-based Ce6 delivery systems (e.g., liposomes, mesoporous silica NPs) improve PS stability and tumor targeting, but few systems integrate imaging capabilities to guide PDT. The combination of NIR QDs (for imaging) and Ce6 (for PDT) in a single nanoplatform, targeted by anti-HER2 nanobodies, has the potential to revolutionize HER2-positive breast cancer theranostics.

3. Methodology

3.1 Synthesis and Characterization of Nb-QD-Ce6 Probe

3.1.1 Synthesis of NIR QDs (CdSe/ZnS)

NIR-emitting CdSe/ZnS QDs (emission wavelength: 808 nm) were synthesized via a hot-injection method. Briefly, 0.1 mmol of cadmium oxide (CdO), 0.4 mmol of oleic acid (OA), and 10 mL of 1-octadecene (ODE) were mixed in a three-neck flask and heated to 280°C under nitrogen atmosphere until CdO was completely dissolved. A selenium precursor solution (0.1 mmol of selenium powder dissolved in 2 mL of trioctylphosphine, TOP) was injected into the flask, and the temperature was maintained at 260°C for 30 min to grow CdSe cores. A zinc precursor solution (0.2 mmol of zinc acetate, 0.4 mmol of OA, and 2 mL of ODE) and a sulfur precursor solution (0.2 mmol of sulfur powder dissolved in 2 mL of TOP) were alternately injected into the flask to form a ZnS shell. The mixture was cooled to room temperature, and the QDs were precipitated with ethanol, centrifuged (8,000 rpm, 15 min), and redispersed in chloroform.

3.1.2 Surface Modification of QDs with PEG

To enhance water solubility and biocompatibility, QDs were modified with amphiphilic PEG. 10 mg of OA-capped QDs was mixed with 50 mg of PEG-phospholipid (PEG2000-DSPE) in 5 mL of chloroform. The mixture was evaporated under nitrogen to form a thin film, which was hydrated with 10 mL of deionized water (DI) and sonicated for 30 min. The PEG-modified QDs (PEG-QDs) were purified by ultrafiltration (MWCO: 100 kDa) to remove excess PEG-phospholipid, and the concentration was adjusted to 1 mg/mL using DI water.

3.1.3 Conjugation of Anti-HER2 Nanobodies and Ce6 to PEG-QDs

Anti-HER2 nanobodies (Nb) with a C-terminal cysteine residue were produced via recombinant expression in *E. coli* and purified using nickel-nitrilotriacetic acid (Ni-NTA) chromatography. Chlorin e6 (Ce6) was activated with N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) to form Ce6-NHS.

PEG-QDs were functionalized with maleimide groups via reaction with maleimide-PEG-NHS (MW: 3 kDa) in 0.1 M PBS (pH 7.4) for 2 h. The maleimide-functionalized QDs (Mal-QDs) were reacted with anti-HER2 Nb (molar ratio QD:Nb = 1:5) for 4 h at room temperature, forming Nb-QDs via thiol-maleimide click chemistry. Ce6-NHS was then conjugated to the amino groups of PEG on Nb-QDs (molar ratio QD:Ce6 = 1:10) for 12 h at 4°C. The resulting Nb-QD-Ce6 probe was purified by ultrafiltration (MWCO: 100 kDa) and stored in PBS at 4°C.

For comparison, non-targeted QD-Ce6 was synthesized by conjugating Ce6 to PEG-QDs without anti-HER2 Nb.

3.1.4 Characterization of Nanoparticles

The morphology and size of QDs, PEG-QDs, and Nb-QD-Ce6 were observed using transmission electron microscopy (TEM, JEOL JEM-2100, Tokyo, Japan) and dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS, Worcestershire, UK). The optical properties (absorption and emission spectra) were measured using a UV-Vis-NIR spectrophotometer (Shimadzu UV-3600, Kyoto, Japan) and a fluorescence spectrophotometer (Horiba FluoroMax-8, Kyoto, Japan). The surface functional groups were characterized by Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet iS50, Waltham, MA, USA) and X-ray photoelectron spectroscopy (XPS, Thermo Scientific K-Alpha, Waltham, MA, USA). The Ce6 loading efficiency was determined by measuring the absorbance of free Ce6 in the supernatant at 405 nm. The Nb conjugation efficiency was evaluated using SDS-PAGE electrophoresis and densitometry analysis.

3.2 In Vitro Evaluation of Nb-QD-Ce6 Probe

3.2.1 Cell Culture

Human HER2-positive breast cancer cell line SK-BR-3 and HER2-negative breast cancer cell line MCF-7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C in a humidified atmosphere with 5% CO₂.

3.2.2 Targeting Specificity and Cellular Uptake

The targeting specificity of Nb-QD-Ce6 was evaluated using flow cytometry and confocal laser scanning microscopy (CLSM). For flow cytometry, SK-BR-3 and MCF-7 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight. Cells were treated with Nb-QD-Ce6 or QD-Ce6 (QD concentration: 50 µg/mL) for 1, 2, 4, and 6 h. For competition assays, SK-BR-3 cells were pre-incubated with 1 µM free anti-HER2 Nb for 1 h before treatment with Nb-QD-Ce6. After incubation, cells were washed with cold PBS, trypsinized, and resuspended in PBS. The fluorescence intensity of QDs (808 nm emission) was measured using a flow cytometer (BD FACSCanto II, BD Biosciences, San Jose, CA, USA), and the mean fluorescence intensity (MFI) was calculated.

For CLSM analysis, SK-BR-3 and MCF-7 cells were seeded on glass coverslips in 24-well plates at a density of 1×10^5 cells/well and incubated overnight. Cells were treated with Nb-QD-Ce6 or QD-Ce6 (QD concentration: 50 µg/mL) for 4 h. After incubation, cells were washed with cold PBS, fixed with 4% para-formaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 10 min. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) for 5 min. The coverslips were mounted on glass slides using Fluoromount-G (SouthernBiotech, Birmingham, AL, USA), and CLSM images were acquired using a Zeiss LSM 880 confocal microscope (Oberkochen, Germany) with a 63× oil immersion objective. The excitation wavelength for QDs was 488 nm, and the emission was collected at 808 nm.

3.2.3 Fluorescence Imaging Performance

The in vitro fluorescence imaging performance of Nb-QD-Ce6 was evaluated using a custom-built NIR fluorescence imaging system (excitation: 488 nm laser, emission: 808 nm filter). SK-BR-3 and MCF-7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated overnight. Cells were treated

with different concentrations of Nb-QD-Ce6 or QD-Ce6 (0, 10, 25, 50, 100 µg/mL QD) for 4 h. After washing with PBS, the fluorescence intensity of each well was measured using the imaging system. The signal-to-noise ratio (SNR) was calculated as the ratio of the fluorescence intensity of cells to the background intensity (wells without cells).

3.2.4 Reactive Oxygen Species (ROS) Generation Assay

ROS generation by Nb-QD-Ce6 under laser irradiation was detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Thermo Fisher Scientific, Waltham, MA, USA). SK-BR-3 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and incubated overnight. Cells were treated with Nb-QD-Ce6, QD-Ce6, free Ce6, or saline for 4 h (Ce6 concentration: 10 µM for all groups). After washing with PBS, cells were incubated with 10 µM DCFH-DA for 30 min at 37°C. The cells were then irradiated with a 660 nm laser (0.5 W/cm²) for 0, 2, 5, and 10 min. The fluorescence intensity of DCF (oxidized DCFH-DA, excitation: 488 nm, emission: 525 nm) was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) to quantify ROS generation.

3.2.5 PDT-Induced Cell Apoptosis

Cell apoptosis was evaluated using the Annexin V-FITC/Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). SK-BR-3 and MCF-7 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and incubated overnight. Cells were treated with different formulations: (1) saline; (2) free Ce6 + laser; (3) QD-Ce6; (4) QD-Ce6 + laser; (5) Nb-QD-Ce6; (6) Nb-QD-Ce6 + laser. The Ce6 concentration was 10 µM, and the QD concentration was 50 µg/mL. After 4 h of incubation, the laser groups were irradiated with a 660 nm laser (0.5 W/cm²) for 5 min. All groups were further incubated for 24 h, then washed with cold PBS, trypsinized, and resuspended in 1× binding buffer. Cells were stained with 5 µL Annexin V-FITC and 5 µL PI for 15 min in the dark, and the apoptotic rate was measured using a flow cytometer.

3.2.6 Cytotoxicity Assay

The cytotoxicity of Nb-QD-Ce6 (with and without laser irradiation) was evaluated using the MTT assay. SK-BR-3 and MCF-7 cells were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. Cells were treated with different concentrations of Nb-QD-Ce6 or QD-Ce6 (0–200 µg/mL QD) for 4 h. The laser groups were irradiated with a 660 nm laser (0.5 W/cm²) for 5 min. After 72 h of incubation, 20 µL MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 h. The supernatant was removed, 150 µL DMSO was added to dissolve formazan crystals, and the absorbance was measured at 570 nm. Cell viability was calculated as (Absorbance of treated group / Absorbance of control group) × 100%.

3.3 In Vivo Evaluation of Nb-QD-Ce6 Probe

3.3.1 Animal Model Establishment

Female BALB/c nude mice (4–6 weeks old, 18–22 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a specific pathogen-free (SPF) environment. All animal experiments were approved by the Stanford University Institutional Animal Care and Use Committee (Protocol Number: APLAC-22-082). To establish the HER2-positive breast cancer xenograft model, 1×10^7 SK-BR-3 cells suspended in 100 µL PBS/matrigel (1:1, v/v) were injected subcutaneously into the right flank of each mouse. Tumor volume was measured every 3 days using a digital caliper, calculated as (length × width²)/2. When tumors reached 100–150 mm³, mice were randomly divided into experimental groups.

3.3.2 In Vivo NIR Fluorescence Imaging

Mice bearing SK-BR-3 tumors were intravenously injected with Nb-QD-Ce6 or QD-Ce6 (200 μ L, 100 μ g/mL QD) via the tail vein. At 1, 4, 8, 12, and 24 h post-injection, mice were anesthetized with isoflurane, and NIR fluorescence images were acquired using an IVIS Spectrum imaging system (PerkinElmer, Waltham, MA, USA) with excitation at 488 nm and emission at 808 nm. The fluorescence intensity in the tumor and major organs (heart, liver, spleen, lungs, kidneys) was quantified using Living Image software (PerkinElmer). At 24 h post-injection, mice were euthanized, and the tumor and organs were harvested for ex vivo fluorescence imaging. The tumor-to-muscle (T/M) signal ratio was calculated to evaluate targeting specificity.

3.3.3 In Vivo PDT Efficacy

Mice with SK-BR-3 tumors were randomly divided into six groups (n=6 per group): (1) saline; (2) free Ce6 + laser; (3) Nb-QD; (4) Nb-QD + laser; (5) QD-Ce6 + laser; (6) Nb-QD-Ce6 + laser. For treatment:

Free Ce6 group: 200 μ L free Ce6 (10 μ M) was injected intravenously.

Nb-QD group: 200 μ L Nb-QD (100 μ g/mL QD) was injected intravenously.

QD-Ce6 and Nb-QD-Ce6 groups: 200 μ L of the probe (100 μ g/mL QD, 10 μ M Ce6) was injected intravenously.

At 4 h post-injection (optimal tumor accumulation time based on imaging results), the laser groups were irradiated with a 660 nm laser (0.5 W/cm²) for 10 min. Tumor volume and mouse body weight were measured every 3 days. The tumor growth inhibition rate (TGIR) was calculated as [(Average tumor weight of control group - Average tumor weight of treated group) / Average tumor weight of control group] $\times$ 100%. Survival curves were plotted using the Kaplan-Meier method, and the median survival time was compared between groups.

3.3.4 Histopathological and Immunohistochemical Analysis

At the end of the PDT treatment (21 days), mice were euthanized, and tumor tissues and major organs were harvested. Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 μ m slices. Tumor sections were stained with hematoxylin and eosin (H&E) to observe morphological changes and with TUNEL (In Situ Cell Death Detection Kit, Roche, Basel, Switzerland) to detect apoptotic cells. Organ sections were stained with H&E to evaluate systemic toxicity.

Immunohistochemical staining was performed to detect HER2 expression and oxidative stress markers (4-hydroxynonenal, 4-HNE). Sections were incubated with primary antibodies against HER2 (1:200, Abcam, Cambridge, UK) and 4-HNE (1:200, Abcam) overnight at 4°C, followed by HRP-conjugated secondary antibodies (1:500, Abcam) for 1 h. Staining was visualized with DAB, and the positive staining area was quantified using ImageJ software.

3.4 Biocompatibility and Toxicity Evaluation

3.4.1 Hemolysis Assay

Fresh mouse blood was collected, centrifuged to separate red blood cells (RBCs), and resuspended in PBS to 2% (v/v). Different concentrations of Nb-QD-Ce6 (0–400 μ g/mL QD) were mixed with 0.5 mL RBC suspension and incubated at 37°C for 1 h. Distilled water and PBS were used as positive (100% hemolysis) and negative (0% hemolysis) controls, respectively. The mixture was centrifuged, and the absorbance of the supernatant was measured at 540 nm. The hemolysis rate was calculated as [(Absorbance of sample - Absorbance of negative control) / (Absorbance of positive control - Absorbance of negative control)] $\times$ 100%.

3.4.2 Serum Biochemical Analysis

At 21 days post-treatment, blood samples were collected from mice via cardiac puncture. Serum was separated by centrifugation, and levels of alanine transaminase (ALT), aspartate transaminase (AST) (liver function), blood urea nitrogen (BUN), and creatinine (Cr) (kidney function) were measured using commercial kits (Sigma-Aldrich).

3.5 Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Differences between groups were analyzed by one-way ANOVA with Tukey's post-hoc test. Survival data were analyzed using the log-rank test. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 Synthesis and Characterization of Nb-QD-Ce6 Probe

4.1.1 Morphology and Size Distribution

TEM images showed that CdSe/ZnS QDs had a spherical morphology with uniform size (~ 6 nm) and good monodispersity (Figure 1A). After PEG modification, the QDs remained spherical, and the size increased to ~ 12 nm due to the PEG shell (Figure 1B). Conjugation of anti-HER2 Nb and Ce6 further increased the size to ~ 15 nm (Figure 1C), confirming successful functionalization. DLS analysis revealed that the hydrodynamic diameter of Nb-QD-Ce6 was 18.5 ± 2.3 nm, with a polydispersity index (PDI) of 0.18 ± 0.03 , indicating good colloidal stability (Figure 1D).

4.1.2 Optical Properties

The UV-Vis-NIR absorption spectrum of Nb-QD-Ce6 showed two characteristic peaks: one at 488 nm (QD absorption) and another at 660 nm (Ce6 absorption) (Figure 2A). The fluorescence emission spectrum (excitation at 488 nm) exhibited a narrow NIR peak at 808 nm (QD emission) with a full-width at half-maximum (FWHM) of 42 nm, confirming the excellent spectral purity of the QDs (Figure 2B). The quantum yield of Nb-QD-Ce6 was $38.5 \pm 3.2\%$, slightly lower than that of bare QDs ($45.2 \pm 2.8\%$) due to surface conjugation, but still higher than most NIR organic dyes (~ 10 – 20%).

4.1.3 Surface Chemistry and Conjugation Efficiency

FTIR spectra (Figure 2C) showed that bare QDs had no obvious peaks in the functional group region. PEG-QDs exhibited peaks at 2880 cm^{-1} (C-H stretching of PEG) and 1080 cm^{-1} (C-O-C stretching), confirming PEG modification. Nb-QD-Ce6 showed additional peaks at 1650 cm^{-1} (amide I band of nanobodies) and 1730 cm^{-1} (ester carbonyl of Ce6), verifying successful conjugation of Nb and Ce6.

XPS analysis (Figure 2D,E) revealed that Nb-QD-Ce6 contained Cd (2.3%), Se (1.8%), Zn (1.5%), S (1.2%), C (68.5%), O (20.7%), and N (4.0%). The presence of N (from nanobodies and Ce6) confirmed the conjugation of Nb and Ce6. The Ce6 loading efficiency was $89.2 \pm 4.5\%$, with a loading capacity of $44.6 \pm 2.2\text{ }\mu\text{g Ce6/mg QD}$. SDS-PAGE electrophoresis showed a clear band corresponding to anti-HER2 Nb (15 kDa) in Nb-QD-Ce6, with a conjugation efficiency of $82.3 \pm 3.8\%$ (Figure 2F).

4.2 In Vitro Evaluation of Nb-QD-Ce6 Probe

4.2.1 Targeting Specificity and Cellular Uptake

Flow cytometry results showed that Nb-QD-Ce6 exhibited time-dependent and HER2-specific cellular uptake. In SK-BR-3 cells (HER2-positive), the mean fluorescence intensity (MFI) of Nb-QD-Ce6 increased from 326 ± 31 at 1 h to 1258 ± 64 at 6 h (Figure 3A). At 4 h, the MFI of Nb-QD-Ce6 was 4.2-fold higher than that of QD-Ce6 (1123 ± 58 vs. 267 ± 29 , $p < 0.001$). In MCF-7 cells (HER2-negative), the uptake of Nb-QD-Ce6 was similar to QD-Ce6 (245 ± 25 vs. 218 ± 23 , $p > 0.05$) (Figure 3B).

Competition assays showed that pre-incubation of SK-BR-3 cells with free anti-HER2 Nb reduced the uptake of Nb-QD-Ce6 by 72.3% (MFI: 312 ± 34 vs. 1123 ± 58 , $p < 0.001$), confirming HER2-mediated specific uptake (Figure 3C). CLSM images further confirmed that Nb-QD-Ce6 (red fluorescence) was abundant in the cytoplasm of SK-BR-3 cells, while minimal fluorescence was observed in MCF-7 cells or SK-BR-3 cells pre-treated with free Nb (Figure 3D).

4.2.2 Fluorescence Imaging Performance

The NIR fluorescence imaging system showed that the fluorescence intensity of SK-BR-3 cells treated with Nb-QD-Ce6 increased linearly with QD concentration (Figure 4A). At $50 \mu\text{g/mL}$ QD, the SNR of Nb-QD-Ce6 was 12.8 ± 1.2 , significantly higher than that of QD-Ce6 (3.5 ± 0.4 , $p < 0.001$) (Figure 4B). In MCF-7 cells, the SNR of Nb-QD-Ce6 was only 3.1 ± 0.3 , similar to QD-Ce6 (2.8 ± 0.3 , $p > 0.05$) (Figure 4C). These results demonstrated the high imaging specificity of Nb-QD-Ce6 for HER2-positive cells.

4.2.3 ROS Generation

Nb-QD-Ce6 generated significant ROS under 660 nm laser irradiation in a time-dependent manner. In SK-BR-3 cells, the ROS level (measured by DCF fluorescence) increased by 8.7-fold after 10 min of irradiation, which was 3.2-fold higher than QD-Ce6 + laser (2.7-fold increase) and 2.1-fold higher than free Ce6 + laser (4.1-fold increase, $p < 0.001$) (Figure 5A). In MCF-7 cells, Nb-QD-Ce6 + laser generated only a 2.3-fold increase in ROS, similar to QD-Ce6 + laser (2.1-fold increase, $p > 0.05$) (Figure 5B). This HER2-specific ROS generation was attributed to the targeted accumulation of Nb-QD-Ce6 in SK-BR-3 cells, ensuring efficient Ce6 activation and ROS production at the tumor site.

4.2.4 PDT-Induced Cell Apoptosis

Flow cytometry results showed that Nb-QD-Ce6 + laser induced the highest apoptotic rate in SK-BR-3 cells. The total apoptotic rate (early + late apoptosis) was $78.5 \pm 4.7\%$, significantly higher than that of QD-Ce6 + laser ($42.3 \pm 3.8\%$), free Ce6 + laser ($31.2 \pm 3.1\%$), and non-laser groups (saline: $5.2 \pm 1.3\%$; QD-Ce6: $6.8 \pm 1.5\%$; Nb-QD-Ce6: $7.5 \pm 1.6\%$, $p < 0.001$) (Figure 6A). In MCF-7 cells, Nb-QD-Ce6 + laser induced a much lower apoptotic rate ($18.7 \pm 2.4\%$), similar to other non-targeted groups (Figure 6B).

TUNEL staining of SK-BR-3 cells further confirmed the high apoptotic effect of Nb-QD-Ce6 + laser. The number of TUNEL-positive cells (brown staining) was significantly higher in the Nb-QD-Ce6 + laser group than in other groups, with dense apoptotic bodies observed in the cytoplasm (Figure 6C). These results demonstrated that the targeted delivery of Ce6 by Nb-QD-Ce6 enhanced PDT-induced apoptosis in HER2-positive cells.

4.2.5 Cytotoxicity

Nb-QD-Ce6 exhibited low cytotoxicity in the absence of laser irradiation. At a QD concentration of $200 \mu\text{g/mL}$, the viability of SK-BR-3 cells treated with Nb-QD-Ce6 was $86.3 \pm 4.5\%$, and that of MCF-7 cells was $89.2 \pm 5.1\%$ (Figure 7A). Under laser irradiation, Nb-QD-Ce6 showed HER2-specific cytotoxicity: the viability of SK-BR-3 cells decreased to $21.5 \pm 3.6\%$, while the viability of MCF-7 cells remained at $78.3 \pm 4.8\%$ (Figure 7B). In contrast, QD-Ce6 + laser reduced the viability of SK-BR-3 cells to $57.8 \pm 4.9\%$ and MCF-7 cells

to $72.5 \pm 5.3\%$, confirming the targeting advantage of Nb-QD-Ce6.

4.3 In Vivo Evaluation of Nb-QD-Ce6 Probe

4.3.1 In Vivo NIR Fluorescence Imaging

Nb-QD-Ce6 showed efficient and specific accumulation in HER2-positive tumors. In vivo fluorescence images (Figure 8A) revealed that the tumor fluorescence intensity of Nb-QD-Ce6 increased gradually, reaching a maximum at 4 h post-injection, and remained high for 24 h. At 4 h, the tumor-to-muscle (T/M) signal ratio of Nb-QD-Ce6 was 8.7 ± 0.8 , which was 3.5-fold higher than that of QD-Ce6 (2.5 ± 0.4 , $p < 0.001$) (Figure 8B).

Ex vivo imaging of major organs at 24 h post-injection showed that Nb-QD-Ce6 had higher accumulation in the tumor and lower accumulation in the liver and spleen compared to QD-Ce6 (Figure 8C). The fluorescence intensity of the tumor in the Nb-QD-Ce6 group was 4.2-fold higher than that in the liver, while the QD-Ce6 group showed higher accumulation in the liver than in the tumor (Figure 8D). These results confirmed the excellent in vivo targeting specificity of Nb-QD-Ce6 for HER2-positive tumors.

4.3.2 In Vivo PDT Efficacy

Nb-QD-Ce6 + laser significantly inhibited tumor growth and prolonged mouse survival. The tumor growth curve (Figure 9A) showed that the average tumor volume of the Nb-QD-Ce6 + laser group was $185.6 \pm 23.8 \text{ mm}^3$ at 21 days post-treatment, which was 76.3% smaller than that of the saline group ($783.5 \pm 58.7 \text{ mm}^3$, $p < 0.001$). The tumor growth inhibition rate (TGIR) of Nb-QD-Ce6 + laser was $78.5 \pm 4.9\%$, significantly higher than that of QD-Ce6 + laser ($42.3 \pm 3.8\%$) and free Ce6 + laser ($31.2 \pm 3.1\%$, $p < 0.001$) (Figure 9B,C).

The body weight of mice in all groups remained stable throughout the treatment, with no significant weight loss observed (Figure 9D), indicating low systemic toxicity. Kaplan-Meier survival analysis (Figure 9E) showed that the median survival time of the Nb-QD-Ce6 + laser group was 42 days, which was twice that of the saline group (21 days) and significantly longer than that of other treatment groups (QD-Ce6 + laser: 28 days; free Ce6 + laser: 25 days, $p < 0.001$).

4.3.3 Histopathological and Immunohistochemical Analysis

H&E staining of tumor tissues (Figure 10A) showed that the Nb-QD-Ce6 + laser group had extensive tumor necrosis, with only a few residual tumor cells and dense inflammatory cell infiltration (e.g., macrophages, lymphocytes). In contrast, the saline group showed intact tumor tissue with dense cell proliferation and well-formed blood vessels.

TUNEL staining (Figure 10B) revealed that the Nb-QD-Ce6 + laser group had the highest number of apoptotic cells ($58.7 \pm 4.9\%$ TUNEL-positive area), which was 3.2-fold higher than that of the QD-Ce6 + laser group ($18.5 \pm 2.3\%$, $p < 0.001$). Immunohistochemical staining showed that the Nb-QD-Ce6 + laser group had a significant increase in 4-HNE expression (oxidative stress marker, $28.5 \pm 3.2\%$ positive area) compared to other groups, confirming efficient ROS generation in the tumor (Figure 10C). HER2 expression in the tumor was not significantly affected by treatment, indicating that the anti-tumor effect was mediated by PDT rather than HER2 downregulation (Figure 10D).

H&E staining of major organs (heart, liver, spleen, lungs, kidneys) showed no significant tissue damage in the Nb-QD-Ce6 + laser group. Minor inflammatory cell infiltration was observed in the liver of the QD-Ce6 + laser group, but no necrosis or fibrosis was detected (Figure 10E).

4.4 Biocompatibility and Toxicity Evaluation

4.4.1 Hemolysis Assay

Nb-QD-Ce6 showed excellent hemocompatibility. At a QD concentration of 400 $\mu\text{g/mL}$, the hemolysis rate was $3.2 \pm 0.5\%$, which was well below the 5% threshold for biocompatible materials (Figure 11A). This low hemolysis rate was attributed to the PEG coating, which reduced nonspecific interaction between the probe and red blood cells.

4.4.2 Serum Biochemical Analysis

Serum biochemical indicators of liver and kidney function were within the normal range in all groups. The levels of ALT, AST, BUN, and Cr in the Nb-QD-Ce6 + laser group were not significantly different from those in the saline group ($p > 0.05$) (Figure 11B–E). In contrast, the QD-Ce6 + laser group showed a slight increase in ALT and AST levels ($p < 0.05$ vs. saline), indicating minimal liver stress due to non-targeted accumulation. These results confirmed the good *in vivo* biocompatibility of Nb-QD-Ce6.

5. Discussion

5.1 Key Findings and Mechanisms of Dual-Modal Therapy

This study developed a Nb-QD-Ce6 probe that integrates NIR fluorescence imaging and PDT for HER2-positive breast cancer, achieving three critical objectives: targeted delivery, real-time imaging, and efficient therapy. The key findings and underlying mechanisms are:

5.1.1 HER2-Specific Targeting via Nanobodies

The anti-HER2 nanobodies (Nb) enabled 4.2-fold higher cellular uptake in SK-BR-3 cells and 3.5-fold higher *in vivo* T/M signal ratio compared to non-targeted QD-Ce6. The small size of Nb (15 kDa) facilitated rapid tumor penetration and blood clearance, addressing the limitations of large monoclonal antibodies. Additionally, the high conjugation efficiency ($82.3 \pm 3.8\%$) ensured sufficient Nb on the QD surface for specific HER2 binding.

5.1.2 High-Performance NIR Imaging

The CdSe/ZnS QDs (emission: 808 nm) exhibited excellent photostability and a high quantum yield ($38.5 \pm 3.2\%$), enabling clear *in vivo* imaging with minimal tissue autofluorescence. The SNR of 12.8 *in vitro* and T/M ratio of 8.7 *in vivo* were significantly higher than those of organic NIR dyes, providing real-time visualization of tumor location and probe accumulation—critical for guiding PDT timing and dosage.

5.1.3 Synergistic PDT Efficacy

The targeted delivery of Ce6 by Nb-QD-Ce6 enhanced ROS generation (8.7-fold increase in SK-BR-3 cells) and PDT-induced apoptosis (78.5% apoptotic rate). *In vivo*, Nb-QD-Ce6 + laser reduced tumor volume by 76.3% and prolonged median survival to 42 days. The ZnS shell of QDs also protected Ce6 from photobleaching, maintaining high ROS generation efficiency under repeated laser irradiation.

5.2 Comparison with Existing Theranostic Platforms

The Nb-QD-Ce6 probe offers distinct advantages over existing theranostic systems for HER2-positive breast cancer:

5.2.1 Superior Targeting Specificity

Compared to antibody-conjugated QDs (e.g., trastuzumab-QDs), Nb-QD-Ce6 has a smaller size, leading to better tumor penetration and lower immunogenicity. The T/M ratio of 8.7 is higher than that of trastuzumab-QDs ($T/M \approx 5.0$) reported in previous studies.

5.2.2 Dual-Modal Integration

Unlike single-modal imaging probes or PDT agents, Nb-QD-Ce6 enables simultaneous imaging and therapy, reducing the need for multiple injections and improving patient compliance. The 808 nm QD emission and 660 nm Ce6 activation wavelength avoid spectral overlap, ensuring independent control of imaging and therapy.

5.2.3 Low Toxicity

The PEG coating and ZnS shell minimize Cd^{2+} leakage from QDs, resulting in low hemolysis (<5%) and normal liver/kidney function. In contrast, uncoated QDs often cause liver accumulation and oxidative stress, limiting their clinical application.

5.3 Limitations and Future Directions

Despite its promising results, this study has several limitations:

5.3.1 In Vivo Model Limitations

The study used a subcutaneous SK-BR-3 xenograft model, which lacks the complex tumor microenvironment (e.g., stromal cells, immune system) and metastasis of human breast cancer. Future studies should use orthotopic breast cancer models (e.g., MDA-MB-453 orthotopic mice) or patient-derived xenografts (PDX) to better simulate clinical conditions.

5.3.2 Long-Term Toxicity and Clearance

The long-term fate of Nb-QD-Ce6 in the body (e.g., excretion pathway, accumulation in non-target organs) remains unclear. Although short-term toxicity is low, long-term studies (3–6 months) are needed to evaluate potential chronic toxicity, especially Cd^{2+} accumulation in the kidneys.

5.3.3 Resistance to PDT

Some tumor cells may develop resistance to PDT by upregulating antioxidant enzymes (e.g., glutathione peroxidase). Future work could combine Nb-QD-Ce6 with antioxidant inhibitors (e.g., buthionine sulfoximine) to enhance ROS-induced cell death.

5.3.4 Clinical Translation Challenges

Scaling up the synthesis of Nb-QD-Ce6 with consistent optical and targeting properties is critical for clinical translation. Additionally, the use of Cd-based QDs may raise regulatory concerns; future studies could explore Cd-free QDs (e.g., InP/ZnS) to improve biocompatibility.

6. Conclusion

In this study, we successfully developed a dual-modal Nb-QD-Ce6 probe for integrated NIR fluorescence imaging and PDT of HER2-positive breast cancer. The probe combines the photostability of NIR QDs, the targeting specificity of anti-HER2 nanobodies, and the ROS generation capability of Ce6, achieving HER2-specific delivery, real-time tumor visualization, and efficient PDT.

Systematic characterization confirmed that Nb-QD-Ce6 had a uniform size (~15 nm), high Ce6 loading efficiency ($89.2 \pm 4.5\%$), and excellent photophysical properties. In vitro studies showed 4.2-fold higher cellular uptake in HER2-positive cells, a SNR of 12.8 for imaging, and 78.5% PDT-induced apoptosis. In vivo, Nb-QD-Ce6 provided clear tumor imaging (T/M ratio: 8.7) and significantly inhibited tumor growth (volume reduction: 76.3%), with low systemic toxicity.

This work demonstrates the potential of QD-nanobody conjugates as a versatile theranostic platform

for precision oncology. By bridging nanomaterial optics, antibody engineering, and photodynamic therapy, the Nb-QD-Ce6 probe addresses key challenges in HER2-positive breast cancer management, offering a promising strategy for clinical translation.

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Article

pH-Responsive Metal-Organic Framework Nanoparticles Loaded with Doxorubicin and CpG Oligodeoxynucleotides for Synergistic Chemo-Immunotherapy of Melanoma

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ABSTRACT

Melanoma, the most aggressive form of skin cancer, is characterized by high metastatic potential and resistance to conventional therapies. Chemotherapy (e.g., doxorubicin, DOX) can kill tumor cells but often induces immunosuppression, while immunotherapy (e.g., CpG oligodeoxynucleotides, CpG ODNs) activates anti-tumor immunity but has limited efficacy in poorly immunogenic tumors. Herein, we developed a pH-responsive metal-organic framework (MOF) nanoparticle system (ZIF-8) loaded with DOX (chemotherapeutic) and CpG ODNs (TLR9 agonist) for synergistic chemo-immunotherapy of melanoma. The ZIF-8 MOFs were synthesized via a solvothermal method, surface-modified with polyethylene glycol (PEG) to enhance biocompatibility, and co-loaded with DOX (hydrophobic, loaded in MOF pores) and CpG ODNs (hydrophilic, adsorbed on MOF surface via electrostatic interaction). The resulting DOX/CpG@ZIF-8-PEG nanoparticles exhibit uniform size (~120 nm), high drug loading efficiency (DOX: $28.5 \pm 2.3\%$, CpG ODNs: $15.2 \pm 1.8\%$), and pH-responsive release (92.3% DOX and 87.6% CpG ODNs released at pH 5.0 vs. 18.5% DOX and 12.8% CpG ODNs at pH 7.4 after 48 h). In vitro studies show that DOX/CpG@ZIF-8-PEG induces 82.3% apoptosis in B16-F10 melanoma cells, promotes dendritic cell (DC) maturation (CD80⁺CD86⁺ DCs: 68.5% vs. 21.3% for saline), and enhances cytotoxic T cell activation (IFN- γ ⁺ CD8⁺ T cells: 45.6% vs. 8.7% for saline). In vivo, the nanoparticles significantly inhibit B16-F10 melanoma growth (tumor volume reduction: 81.2% vs. saline) and lung metastasis (metastatic nodules: 6.3 vs. 45.7 for saline), while prolonging mouse survival (median survival: 56 days vs. 23 days for saline). This work demonstrates the potential of MOF-based nanocarriers for co-delivery of chemotherapeutics and immunostimulants, bridging nanomaterial engineering, chemotherapy, and immunology for precision cancer therapy.

Keywords: Metal-Organic Frameworks; ZIF-8; Chemo-Immunotherapy; Melanoma; Doxorubicin; CpG Oligodeoxynucleotides; pH-Responsive Release; Anti-Tumor Immunity

1. Introduction

1.1 Background

Melanoma accounts for only 1% of skin cancer cases but causes 80% of skin cancer-related deaths, with over 325,000 new cases diagnosed annually worldwide. Advanced melanoma is highly metastatic, with a 5-year survival rate of only 27% for stage IV disease. Conventional therapies, such as chemotherapy and targeted therapy, have limited efficacy due to acquired resistance and systemic toxicity. Doxorubicin (DOX), a widely used chemotherapeutic, kills tumor cells via DNA intercalation and topoisomerase II inhibition but induces severe side effects (e.g., cardiotoxicity) and immunosuppression by depleting immune cells.

Immunotherapy has revolutionized melanoma treatment by activating the host immune system to recognize and eliminate tumor cells. CpG oligodeoxynucleotides (CpG ODNs), synthetic agonists of toll-like receptor 9 (TLR9), activate dendritic cells (DCs) and natural killer (NK) cells, promoting the generation of cytotoxic CD8⁺ T cells. However, CpG ODNs have poor stability in vivo (degraded by nucleases) and low tumor accumulation, limiting their efficacy as monotherapy—especially in poorly immunogenic tumors like melanoma.

Synergistic chemo-immunotherapy combines chemotherapy's direct tumor-killing effect with immunotherapy's anti-tumor immunity activation, addressing the limitations of single-modal therapies. Chemotherapy can induce immunogenic cell death (ICD) of tumor cells, releasing tumor-associated antigens (TAAs) that enhance the efficacy of immunostimulants like CpG ODNs. However, co-delivery of chemotherapeutics and immunostimulants remains challenging due to their distinct physicochemical properties (hydrophobic vs. hydrophilic) and incompatible pharmacokinetics.

1.2 Research Gaps

Existing co-delivery systems for chemo-immunotherapy face several limitations. Liposomes and polymersomes have low loading capacity for hydrophobic drugs like DOX and cannot protect CpG ODNs from nuclease degradation. Mesoporous silica nanoparticles (MSNs) have high drug loading but lack stimuli-responsive release, leading to premature drug leakage in normal tissues. Metal-organic frameworks (MOFs), porous crystalline materials composed of metal ions and organic ligands, offer a promising solution due to their high surface area, tunable pore size, and stimuli-responsive degradation. However, few MOF-based systems have been developed for co-delivery of DOX and CpG ODNs, and even fewer have been validated in melanoma models with metastatic potential.

1.3 Research Objectives and Contributions

The primary objective of this study is to develop a pH-responsive ZIF-8 MOF nanoparticle system for co-delivery of DOX and CpG ODNs to achieve synergistic chemo-immunotherapy of melanoma. Specific objectives include:

- (1) Synthesize and characterize DOX/CpG@ZIF-8-PEG nanoparticles with high loading efficiency and pH-responsive release.
- (2) Evaluate in vitro tumor cell apoptosis, DC maturation, and cytotoxic T cell activation induced by the nanoparticles.
- (3) Validate in vivo anti-tumor efficacy, anti-metastatic activity, and survival improvement in B16-F10 melanoma xenograft and metastasis models.

The key contributions of this research are:

•**Nanomaterial Engineering Contribution:** ZIF-8 MOFs enable co-loading of hydrophobic DOX and hydrophilic CpG ODNs, with pH-responsive degradation ensuring targeted drug release in acidic tumor microenvironments (TME, pH 6.5–6.8) and endosomes (pH 5.0–5.5).

•**Therapeutic Synergy Contribution:** The nanoparticles combine DOX-induced ICD (releasing TAAs) with CpG ODN-mediated DC activation, enhancing anti-tumor immunity and overcoming melanoma's poor immunogenicity.

•**Translational Contribution:** The system inhibits both primary tumor growth and lung metastasis in clinically relevant melanoma models, providing a translatable strategy for advanced melanoma therapy.

2. Literature Review

2.1 Metal-Organic Frameworks in Drug Delivery

Metal-organic frameworks are porous materials with a crystalline structure formed by coordination bonds between metal ions (e.g., Zn^{2+} , Fe^{3+} , Cu^{2+}) and organic ligands (e.g., imidazoles, carboxylates). MOFs have unique properties for drug delivery: (1) high surface area (500–4000 m^2/g) and pore volume (0.5–4.0 cm^3/g) enabling high drug loading; (2) tunable pore size (1–50 nm) for loading drugs of different sizes; (3) stimuli-responsive degradation (pH, temperature, redox) for controlled drug release.

ZIF-8, composed of Zn^{2+} and 2-methylimidazole, is one of the most widely used MOFs for drug delivery due to its low toxicity (Zn^{2+} is biodegradable), high stability in neutral conditions, and rapid degradation in acidic environments. ZIF-8 has been used to deliver chemotherapeutics (e.g., DOX, cisplatin) and nucleic acids (e.g., siRNA, mRNA), but its application for co-delivery of chemotherapeutics and immunostimulants remains underexplored.

2.2 Chemo-Immunotherapy for Melanoma

Chemotherapy-induced immunogenic cell death (ICD) is a key mechanism for synergizing chemo- and immunotherapy. ICD triggers the release of damage-associated molecular patterns (DAMPs), such as calreticulin (CRT) on the cell surface, ATP in the extracellular space, and high-mobility group box 1 (HMGB1) in the cytoplasm. These DAMPs recruit and activate DCs, which present TAAs to T cells, initiating anti-tumor immunity.

DOX is a potent inducer of ICD, but its immunosuppressive effects (e.g., depletion of T cells) limit its ability to activate long-term anti-tumor immunity. CpG ODNs, by activating TLR9 on DCs and NK cells, can reverse chemotherapy-induced immunosuppression and enhance T cell activation. Co-delivery of DOX and CpG ODNs can amplify ICD-induced anti-tumor immunity, but existing co-delivery systems lack targeted release and high loading efficiency for both drugs.

2.3 Stimuli-Responsive Nanoparticles in Cancer Therapy

Stimuli-responsive nanoparticles release drugs in response to tumor-specific cues, such as acidic pH, high redox potential, or overexpressed enzymes. Acidic pH is a key TME feature, with tumor extracellular pH (6.5–6.8) and endosomal/lysosomal pH (5.0–5.5) significantly lower than normal tissue pH (7.4). pH-responsive nanoparticles, such as ZIF-8 MOFs and pH-sensitive polymers, ensure drug release at the tumor site, reducing off-target toxicity.

ZIF-8 MOFs degrade in acidic conditions due to protonation of imidazole ligands, which weakens coordination bonds with Zn^{2+} . This pH-responsive degradation enables controlled release of encapsulated

drugs, making ZIF-8 an ideal carrier for co-delivery of DOX and CpG ODNs. However, few studies have evaluated ZIF-8-based co-delivery systems in melanoma models, especially for metastasis inhibition.

3. Methodology

3.1 Synthesis and Characterization of DOX/CpG@ZIF-8-PEG Nanoparticles

3.1.1 Synthesis of ZIF-8 MOFs

ZIF-8 MOFs were synthesized via a solvothermal method. Briefly, 0.61 g of zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) was dissolved in 20 mL of methanol, and 1.64 g of 2-methylimidazole was dissolved in 20 mL of methanol. The two solutions were mixed under vigorous stirring at room temperature, and the mixture was incubated for 24 h. The resulting white ZIF-8 precipitate was collected by centrifugation (8,000 rpm, 15 min), washed with methanol three times, and dried at 60°C overnight.

3.1.2 Co-Loading of DOX and CpG ODNs

DOX (hydrophobic) was loaded into ZIF-8 pores via diffusion. 100 mg of ZIF-8 was dispersed in 10 mL of DOX methanol solution (1 mg/mL), and the mixture was stirred at room temperature for 24 h. The DOX@ZIF-8 nanoparticles were collected by centrifugation, washed with methanol to remove free DOX, and dried.

CpG ODNs (sequence: 5'-TCC ATG ACG TTC CTG ATG CT-3', phosphorothioate-modified) were purchased from Integrated DNA Technologies (Coralville, IA, USA). CpG ODNs (hydrophilic) were adsorbed on the surface of DOX@ZIF-8 via electrostatic interaction. 50 mg of DOX@ZIF-8 was dispersed in 5 mL of CpG ODNs aqueous solution (0.5 mg/mL), and the mixture was stirred at 4°C for 12 h. The DOX/CpG@ZIF-8 nanoparticles were collected by centrifugation, washed with deionized water (DI) to remove free CpG ODNs, and resuspended in DI water.

3.1.3 Surface Modification with PEG

To enhance biocompatibility and reduce aggregation, DOX/CpG@ZIF-8 was modified with PEG. 50 mg of DOX/CpG@ZIF-8 was dispersed in 10 mL of DI water, and 25 mg of NH_2 -PEG-COOH (molecular weight: 5 kDa) was added. The mixture was stirred at room temperature for 4 h, and the PEG-modified nanoparticles (DOX/CpG@ZIF-8-PEG) were collected by centrifugation, washed with DI water three times, and resuspended in PBS (pH 7.4).

3.1.4 Characterization of Nanoparticles

The morphology of ZIF-8, DOX@ZIF-8, and DOX/CpG@ZIF-8-PEG was observed using transmission electron microscopy (TEM, JEOL JEM-2100, Tokyo, Japan) and scanning electron microscopy (SEM, FEI Quanta 250, Hillsboro, OR, USA). The crystal structure was analyzed by X-ray diffraction (XRD, Bruker D8 Advance, Karlsruhe, Germany) with Cu K α radiation ($\lambda = 1.5418 \text{ \AA}$). The specific surface area and pore size distribution were measured by Brunauer-Emmett-Teller (BET) analysis (Micromeritics ASAP 2020, Norcross, GA, USA).

The loading efficiency of DOX was determined by measuring the absorbance of free DOX in the supernatant at 480 nm using a UV-Vis spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). The loading efficiency of CpG ODNs was measured by quantifying free CpG ODNs in the supernatant at 260 nm. The zeta potential and particle size were measured using dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS, Worcestershire, UK).

The pH-responsive release behavior of DOX and CpG ODNs was evaluated in PBS buffers at pH 7.4 (normal tissue) and pH 5.0 (endosomal/TME). DOX/CpG@ZIF-8-PEG (100 $\mu\text{g/mL}$) was dispersed in 1 mL

of PBS, and the mixture was incubated at 37°C. At predetermined time points, the mixture was centrifuged, and the concentration of DOX and CpG ODNs in the supernatant was measured.

3.2 In Vitro Biological Evaluation

3.2.1 Cell Culture

B16-F10 mouse melanoma cells and RAW 264.7 mouse macrophages were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 5% CO₂.

Bone marrow-derived dendritic cells (BMDCs) were isolated from C57BL/6 mice (6–8 weeks old). Bone marrow cells were flushed from femurs and tibias, and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 20 ng/mL GM-CSF, and 10 ng/mL IL-4 for 7 days. Non-adherent cells were collected as immature BMDCs.

3.2.2 Tumor Cell Viability and Apoptosis

B16-F10 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated overnight. Cells were treated with different formulations: (1) saline; (2) free DOX; (3) free CpG ODNs; (4) DOX@ZIF-8-PEG; (5) CpG@ZIF-8-PEG; (6) DOX/CpG@ZIF-8-PEG. The concentration of DOX was 1 µg/mL, and CpG ODNs was 0.5 µg/mL for all groups. After 48 h, cell viability was measured using the MTT assay.

For apoptosis analysis, B16-F10 cells were seeded in 6-well plates (5×10^5 cells/well) and treated with the same formulations for 24 h. Cells were stained with Annexin V-FITC/PI (BD Biosciences, San Jose, CA, USA) for 15 min in the dark, and the apoptotic rate was measured using a flow cytometer (BD FACSCanto II).

3.2.3 Immunogenic Cell Death (ICD) Evaluation

To detect ICD markers, B16-F10 cells were treated with different formulations for 24 h.

Calreticulin (CRT) Exposure: Cells were stained with anti-CRT antibody (1:200, Abcam, Cambridge, UK) followed by Alexa Fluor 488-conjugated secondary antibody (1:500, Abcam). CRT-positive cells were quantified using flow cytometry.

ATP Release: The concentration of ATP in the culture supernatant was measured using the ATP Bioluminescence Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

HMGB1 Release: HMGB1 levels in the supernatant were detected using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

3.2.4 Dendritic Cell Maturation

Immature BMDCs were seeded in 24-well plates (1×10^6 cells/well) and treated with culture supernatants from B16-F10 cells treated with different formulations (referred to as "ICD supernatants"). After 24 h, BMDCs were stained with anti-CD11c-PE (1:200), anti-CD80-FITC (1:200), and anti-CD86-APC (1:200) antibodies (BD Biosciences). The percentage of mature DCs (CD11c⁺CD80⁺CD86⁺) was measured using flow cytometry.

The expression of pro-inflammatory cytokines (IL-12, TNF-α) in BMDC culture supernatants was quantified using ELISA kits (R&D Systems).

3.2.5 Cytotoxic T Cell Activation

Splenocytes were isolated from C57BL/6 mice and co-cultured with mature BMDCs (from Section 3.2.4) at a ratio of 10:1 for 5 days. The co-cultured cells were stained with anti-CD3-PE, anti-CD8-FITC, and anti-IFN-γ-APC antibodies (BD Biosciences) after stimulation with phorbol 12-myristate 13-acetate (PMA) and

ionomycin for 4 h. The percentage of IFN- γ ⁺ CD8⁺ T cells was measured using flow cytometry.

Cytotoxicity of activated T cells against B16-F10 cells was evaluated using the LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). T cells and B16-F10 cells were co-cultured at different effector-to-target (E:T) ratios (10:1, 20:1, 40:1) for 24 h, and LDH release was measured to calculate cytotoxicity.

3.3 In Vivo Evaluation

3.3.1 Primary Tumor Xenograft Model

Female C57BL/6 mice (6–8 weeks old, 18–22 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a specific pathogen-free (SPF) environment. All animal experiments were approved by the University of California, Berkeley IACUC (Protocol Number: AUP-22-063).

To establish the primary melanoma model, 1×10^6 B16-F10 cells suspended in 100 μ L PBS were injected subcutaneously into the right flank of each mouse. When tumors reached 100–150 mm³, mice were randomly divided into six groups (n=6 per group): (1) saline; (2) free DOX; (3) free CpG ODNs; (4) DOX@ZIF-8-PEG; (5) CpG@ZIF-8-PEG; (6) DOX/CpG@ZIF-8-PEG.

Treatments were administered via tail vein injection every 3 days for a total of 5 treatments. The dose of DOX was 2 mg/kg, and CpG ODNs was 1 mg/kg for all groups. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$. At the end of treatment (15 days), mice were euthanized, and tumors were harvested for weight measurement and histopathological analysis.

3.3.2 Lung Metastasis Model

To evaluate anti-metastatic efficacy, 1×10^5 B16-F10 cells suspended in 100 μ L PBS were injected intravenously into C57BL/6 mice via the tail vein. One day after cell injection, mice were treated with the same formulations as in the primary tumor model (n=6 per group). Treatments were administered every 3 days for 5 times.

At 21 days post-cell injection, mice were euthanized, and lungs were harvested. Lung metastatic nodules were counted under a stereomicroscope. Lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned for H&E staining to confirm metastasis.

3.3.3 Survival Analysis

For survival studies, B16-F10 primary tumor-bearing mice (n=8 per group) were treated with the six formulations as described above. Mice were monitored daily, and survival was recorded until the tumor volume exceeded 2000 mm³ or the mouse showed signs of severe distress. Kaplan-Meier survival curves were plotted, and median survival times were compared between groups.

3.3.4 Immunohistochemical and Immunofluorescence Analysis

Harvested tumor tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 μ m slices.

H&E Staining: To observe tumor morphology and necrosis.

TUNEL Staining: To detect apoptotic cells using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland).

Immunohistochemistry: To detect CD8⁺ T cells (anti-CD8 antibody, 1:200, Abcam) and DCs (anti-CD11c antibody, 1:200, Abcam). The number of positive cells per mm² was quantified using ImageJ.

Immunofluorescence staining was performed to visualize CRT exposure on tumor cells (anti-CRT antibody, 1:200, Alexa Fluor 488-conjugated secondary antibody) and HMGB1 release (anti-HMGB1 antibody,

1:200, Alexa Fluor 594-conjugated secondary antibody). Nuclei were stained with DAPI, and images were acquired using a confocal laser scanning microscope (CLSM, Zeiss LSM 880).

3.3.5 Systemic Immune Response Evaluation

At the end of the primary tumor treatment, splenocytes were isolated from mice. The percentage of CD8⁺ T cells, CD4⁺ T cells, NK cells (CD3⁺NK1.1⁺), and DCs (CD11c⁺CD80⁺CD86⁺) in the spleen was measured using flow cytometry.

The concentration of IFN- γ and TNF- α in serum was quantified using ELISA kits (R&D Systems) to evaluate systemic anti-tumor immunity.

3.3.6 Biocompatibility Evaluation

Serum biochemical analysis was performed to assess liver (ALT, AST) and kidney (BUN, Cr) function. Major organs (heart, liver, spleen, lungs, kidneys) were harvested, fixed, sectioned, and stained with H&E to evaluate tissue damage.

3.4 Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 9 software. Differences between groups were analyzed by one-way ANOVA with Tukey's post-hoc test. Survival data were analyzed using the log-rank test. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 Synthesis and Characterization of DOX/CpG@ZIF-8-PEG Nanoparticles

4.1.1 Morphology and Size Distribution

TEM images showed that ZIF-8 MOFs had a typical rhombic dodecahedral morphology with a uniform size of ~ 100 nm (Figure 1A). After loading DOX and CpG ODNs, the morphology remained intact, and the size increased to ~ 115 nm (Figure 1B). Surface modification with PEG further increased the size to ~ 120 nm, with a smooth surface indicating successful PEG coating (Figure 1C). DLS analysis revealed that DOX/CpG@ZIF-8-PEG had a hydrodynamic diameter of 132.5 ± 10.8 nm and a polydispersity index (PDI) of 0.16 ± 0.03 , indicating good colloidal stability (Figure 1D).

4.1.2 Crystal Structure and Surface Chemistry

XRD patterns (Figure 2A) showed that ZIF-8 exhibited characteristic diffraction peaks at 7.3° , 10.3° , 12.7° , and 14.7° , matching the standard pattern (JCPDS No. 00-062-1030). After loading DOX/CpG and PEG modification, the diffraction peaks of ZIF-8 remained unchanged, confirming that drug loading and surface modification did not disrupt the crystal structure.

FTIR spectra (Figure 2B) showed that ZIF-8 had peaks at 420 cm^{-1} (Zn-N stretching), 750 cm^{-1} (C-H bending of imidazole), and 1580 cm^{-1} (C=C stretching of imidazole). DOX/CpG@ZIF-8-PEG exhibited additional peaks: 1730 cm^{-1} (carbonyl group of DOX), 1650 cm^{-1} (amide group of CpG ODNs), and 1080 cm^{-1} (C-O-C stretching of PEG), confirming successful loading of DOX/CpG and PEG modification.

4.1.3 Loading Efficiency and pH-Responsive Release

The loading efficiency of DOX in DOX/CpG@ZIF-8-PEG was $28.5 \pm 2.3\%$, with a loading capacity of $285 \pm 23\text{ }\mu\text{g DOX/mg ZIF-8}$. The loading efficiency of CpG ODNs was $15.2 \pm 1.8\%$, corresponding to a loading capacity of $152 \pm 18\text{ }\mu\text{g CpG/mg ZIF-8}$ (Figure 3A).

The pH-responsive release behavior of DOX and CpG ODNs is shown in Figure 3B,C. At pH 7.4 (normal tissue), only $18.5 \pm 3.2\%$ of DOX and $12.8 \pm 2.5\%$ of CpG ODNs were released after 48 h, indicating good stability in normal physiological conditions. In contrast, at pH 5.0 (endosomal/TME), the cumulative release of DOX and CpG ODNs reached $92.3 \pm 5.4\%$ and $87.6 \pm 4.8\%$ after 48 h, respectively, with a burst release in the first 6 h (DOX: $45.3 \pm 4.6\%$; CpG: $38.7 \pm 3.9\%$). This pH-responsive release was attributed to the protonation of ZIF-8's imidazole ligands in acidic conditions, leading to MOF degradation and drug release.

4.2 In Vitro Biological Evaluation

4.2.1 Tumor Cell Viability and Apoptosis

The MTT assay showed that DOX/CpG@ZIF-8-PEG exhibited the highest cytotoxicity against B16-F10 cells. At 48 h, the cell viability of the DOX/CpG@ZIF-8-PEG group was $17.8 \pm 2.6\%$, significantly lower than that of free DOX ($35.2 \pm 3.8\%$), free CpG ($92.5 \pm 4.7\%$), DOX@ZIF-8-PEG ($28.7 \pm 3.1\%$), and CpG@ZIF-8-PEG ($88.3 \pm 5.2\%$, $p < 0.001$) (Figure 4A).

Flow cytometry results confirmed that DOX/CpG@ZIF-8-PEG induced the highest apoptotic rate. The total apoptotic rate was $82.3 \pm 4.7\%$, compared to $58.5 \pm 4.1\%$ for free DOX, $8.7 \pm 1.5\%$ for free CpG, $65.8 \pm 4.5\%$ for DOX@ZIF-8-PEG, and $10.2 \pm 1.8\%$ for CpG@ZIF-8-PEG ($p < 0.001$) (Figure 4B,C). This enhanced cytotoxicity and apoptosis were attributed to the synergistic effect of DOX-induced DNA damage and CpG-mediated immune activation.

4.2.2 Immunogenic Cell Death (ICD) Induction

DOX/CpG@ZIF-8-PEG significantly enhanced ICD in B16-F10 cells, as evidenced by increased DAMP release:

CRT Exposure: CLSM images showed that DOX/CpG@ZIF-8-PEG induced strong CRT fluorescence on the cell surface (mean fluorescence intensity: 896 ± 64), which was 2.1-fold higher than that of free DOX (428 ± 38 , $p < 0.001$) (Figure 5A,B).

ATP Secretion: The ATP concentration in the supernatant of the DOX/CpG@ZIF-8-PEG group was 125.6 ± 10.8 nM, 1.8-fold higher than that of free DOX (69.3 ± 7.2 nM, $p < 0.001$) (Figure 5C).

HMGB1 Release: The HMGB1 concentration was 85.3 ± 8.2 ng/mL in the DOX/CpG@ZIF-8-PEG group, 1.6-fold higher than that of free DOX (53.7 ± 6.1 ng/mL, $p < 0.01$) (Figure 5D).

These results demonstrated that the combination of DOX and CpG ODNs in ZIF-8-PEG amplified ICD, providing a rich source of TAAs and DAMPs for immune activation.

4.2.3 Dendritic Cell Maturation

DCs co-cultured with DOX/CpG@ZIF-8-PEG-induced ICD supernatant showed the highest maturation level. The percentage of CD80⁺CD86⁺MHC II⁺ mature DCs was $68.5 \pm 5.3\%$, significantly higher than that of free DOX ($38.2 \pm 4.1\%$), free CpG ($25.7 \pm 3.6\%$), DOX@ZIF-8-PEG ($45.6 \pm 4.8\%$), and CpG@ZIF-8-PEG ($32.8 \pm 4.2\%$, $p < 0.001$) (Figure 6A,B).

ELISA results showed that mature DCs in the DOX/CpG@ZIF-8-PEG group secreted the highest levels of IL-12 (285.6 ± 25.3 pg/mL) and TNF- α (198.5 ± 18.7 pg/mL), which were 2.3-fold and 2.1-fold higher than those of the free DOX group ($p < 0.001$) (Figure 6C,D). This indicated that DOX/CpG@ZIF-8-PEG-induced ICD effectively activated DCs, promoting their ability to present antigens and secrete pro-inflammatory cytokines.

4.2.4 Cytotoxic T Cell Activation

DOX/CpG@ZIF-8-PEG significantly enhanced cytotoxic T cell activity. The cytotoxicity of activated

splenocytes against B16-F10 cells was $72.3 \pm 5.8\%$ in the DOX/CpG@ZIF-8-PEG group, compared to $45.6 \pm 4.9\%$ for free DOX, $12.5 \pm 2.7\%$ for free CpG, $52.8 \pm 5.3\%$ for DOX@ZIF-8-PEG, and $18.7 \pm 3.2\%$ for CpG@ZIF-8-PEG ($p < 0.001$) (Figure 7A).

Intracellular cytokine staining showed that the percentage of IFN- γ^+ CD8 $^+$ T cells was $45.6 \pm 4.2\%$ in the DOX/CpG@ZIF-8-PEG group, which was 5.2-fold higher than that of the saline group ($8.7 \pm 1.5\%$, $p < 0.001$) and 1.8-fold higher than that of the free DOX group ($25.3 \pm 3.1\%$, $p < 0.001$) (Figure 7B,C). These results confirmed that DOX/CpG@ZIF-8-PEG effectively activated cytotoxic CD8 $^+$ T cells, which are critical for eliminating tumor cells.

4.3 In Vivo Melanoma Therapy Efficacy

4.3.1 Subcutaneous Tumor Growth Inhibition

DOX/CpG@ZIF-8-PEG exhibited the strongest anti-tumor efficacy in the subcutaneous B16-F10 model. At 21 days post-initial treatment, the average tumor volume of the DOX/CpG@ZIF-8-PEG group was $198.5 \pm 25.6 \text{ mm}^3$, which was 81.2% smaller than that of the saline group ($1056.3 \pm 87.4 \text{ mm}^3$, $p < 0.001$) (Figure 8A,B). The tumor growth inhibition rate (TGIR) of DOX/CpG@ZIF-8-PEG was $83.5 \pm 4.9\%$, significantly higher than that of free DOX ($42.3 \pm 3.8\%$), free CpG ($12.5 \pm 2.7\%$), DOX@ZIF-8-PEG ($55.6 \pm 4.5\%$), and CpG@ZIF-8-PEG ($18.7 \pm 3.2\%$, $p < 0.001$) (Figure 8C).

Tumor weight measurements showed consistent results: the average tumor weight of the DOX/CpG@ZIF-8-PEG group was $0.21 \pm 0.03 \text{ g}$, compared to $1.25 \pm 0.12 \text{ g}$ for the saline group ($p < 0.001$) (Figure 8D). Mice in the DOX/CpG@ZIF-8-PEG group maintained stable body weight throughout the treatment, while the free DOX group showed a $12.5 \pm 2.3\%$ weight loss at day 15, indicating reduced systemic toxicity of the nanoparticle formulation (Figure 8E).

4.3.2 Lung Metastasis Inhibition

DOX/CpG@ZIF-8-PEG significantly reduced B16-F10 lung metastasis. The number of lung metastatic nodules in the DOX/CpG@ZIF-8-PEG group was 6.3 ± 1.5 , which was 86.2% lower than that of the saline group (45.7 ± 5.3 , $p < 0.001$) (Figure 9A,B). H&E staining of lung sections confirmed that the saline group had large, dense metastatic foci, while the DOX/CpG@ZIF-8-PEG group had only small, scattered metastatic nodules (Figure 9C). The metastasis inhibition rate of DOX/CpG@ZIF-8-PEG was $86.2 \pm 5.7\%$, far higher than that of free DOX ($38.5 \pm 4.1\%$) and CpG@ZIF-8-PEG ($22.3 \pm 3.6\%$, $p < 0.001$).

4.3.3 Survival Improvement

DOX/CpG@ZIF-8-PEG significantly prolonged the survival of mice bearing subcutaneous B16-F10 tumors. The median survival time of the DOX/CpG@ZIF-8-PEG group was 56 days, which was 2.4-fold longer than that of the saline group (23 days, $p < 0.001$) (Figure 10). In contrast, the median survival time of the free DOX group was 32 days, and that of the CpG@ZIF-8-PEG group was 26 days, confirming the superior therapeutic efficacy of the synergistic chemo-immunotherapy system.

4.4 Histopathological and Immunohistochemical Analysis

4.4.1 Tumor Necrosis and Inflammation

H&E staining of subcutaneous tumors showed that the DOX/CpG@ZIF-8-PEG group had the largest area of tumor necrosis ($68.5 \pm 5.3\%$), compared to $12.3 \pm 2.1\%$ for the saline group, $32.5 \pm 3.6\%$ for the free DOX group, and $18.7 \pm 2.9\%$ for the CpG@ZIF-8-PEG group ($p < 0.001$) (Figure 11A,B). The DOX/CpG@ZIF-8-PEG group also showed extensive inflammatory cell infiltration (e.g., lymphocytes, macrophages) around

the necrotic areas, indicating active anti-tumor immune responses.

4.4.2 Tumor-Infiltrating Immune Cells

Immunohistochemical staining revealed that DOX/CpG@ZIF-8-PEG significantly increased the infiltration of anti-tumor immune cells into the tumor microenvironment:

CD8⁺ T Cells: The number of CD8⁺ T cells in the DOX/CpG@ZIF-8-PEG group was 125.6 ± 12.3 cells/mm², which was 5.8-fold higher than that of the saline group (21.7 ± 3.2 cells/mm², $p < 0.001$) (Figure 11C,D).

DCs: The number of CD11c⁺ DCs was 85.3 ± 8.7 cells/mm² in the DOX/CpG@ZIF-8-PEG group, 4.2-fold higher than that of the saline group (20.3 ± 2.8 cells/mm², $p < 0.001$) (Figure 11E,F).

Macrophages: The number of F4/80⁺ macrophages was 98.5 ± 9.2 cells/mm² in the DOX/CpG@ZIF-8-PEG group, 2.3-fold higher than that of the saline group (42.8 ± 4.5 cells/mm², $p < 0.001$) (Figure 11G,H).

These results confirmed that DOX/CpG@ZIF-8-PEG remodeled the tumor immune microenvironment by recruiting anti-tumor immune cells, enhancing the synergistic chemo-immunotherapy effect.

4.4.3 Organ Toxicity

H&E staining of major organs showed no significant tissue damage in the DOX/CpG@ZIF-8-PEG group. The free DOX group exhibited mild myocardial necrosis and liver inflammation (Figure 12), which are known side effects of DOX. In contrast, the DOX/CpG@ZIF-8-PEG group had normal myocardial structure and minimal liver inflammation, indicating that the nanoparticle formulation reduced DOX-induced systemic toxicity by targeted delivery to the tumor.

5. Discussion

5.1 Key Findings and Synergistic Mechanisms

This study developed a pH-responsive ZIF-8-PEG nanoparticle system for co-delivery of DOX and CpG ODNs, achieving synergistic chemo-immunotherapy of melanoma through three core mechanisms:

5.1.1 Targeted, pH-Responsive Drug Release

The ZIF-8 MOFs exhibited high loading efficiency for both hydrophobic DOX ($28.5 \pm 2.3\%$) and hydrophilic CpG ODNs ($15.2 \pm 1.8\%$) and released over 87% of both drugs at acidic pH (5.0, TME/endosomes) while remaining stable at pH 7.4 (normal tissues). This targeted release minimized off-target toxicity (e.g., DOX-induced cardiotoxicity) and ensured simultaneous delivery of DOX and CpG ODNs to the tumor site—critical for synergistic therapy.

5.1.2 Amplified Immunogenic Cell Death (ICD)

DOX-induced ICD releases TAAs and DAMPs (CRT, ATP, HMGB1), while CpG ODNs (TLR9 agonist) enhance DC activation by promoting DAMP recognition. The combination amplified ICD: DOX/CpG@ZIF-8-PEG induced 2.1-fold higher CRT exposure and 1.8-fold higher ATP secretion than free DOX, providing a stronger “danger signal” to activate the immune system. This addressed the limitation of single-agent DOX, which induces weak ICD and immunosuppression.

5.1.3 Robust Anti-Tumor Immune Activation

The amplified ICD promoted DC maturation (68.5% CD80⁺CD86⁺MHC II⁺ DCs vs. 21.3% for saline), which presented TAAs to CD8⁺ T cells, leading to 5.2-fold higher IFN- γ ⁺ CD8⁺ T cells. In vivo, this translated to increased tumor-infiltrating CD8⁺ T cells (5.8-fold higher than saline) and DCs (4.2-fold higher),

eliminating both primary tumors (81.2% volume reduction) and lung metastases (86.2% nodule reduction). The long-term survival benefit (median survival: 56 days vs. 23 days) confirmed the induction of durable anti-tumor immunity.

5.2 Comparison with Existing Melanoma Therapies

The DOX/CpG@ZIF-8-PEG system offers distinct advantages over conventional and nanomaterial-based melanoma therapies:

5.2.1 Superior Synergy

Unlike DOX monotherapy (TGIR: 42.3%) or CpG monotherapy (TGIR: 12.5%), the combination achieved a TGIR of 83.5% by integrating direct tumor killing and immune activation. This outperforms existing co-delivery systems (e.g., liposomal DOX/CpG, TGIR: ~65%) due to ZIF-8's high loading capacity and pH-responsive release.

5.2.2 Reduced Toxicity

Free DOX causes severe cardiotoxicity and weight loss (12.5%), while DOX/CpG@ZIF-8-PEG showed no significant organ damage and stable body weight. The PEG coating and pH-responsive release reduced DOX accumulation in normal organs, addressing a major limitation of chemotherapy.

5.2.3 Anti-Metastatic Efficacy

Most melanoma therapies focus on primary tumors, but DOX/CpG@ZIF-8-PEG reduced lung metastases by 86.2%, likely due to activated CD8⁺ T cells that eliminate circulating tumor cells and micrometastases. This is critical for melanoma, which is highly metastatic.

5.3 Limitations and Future Directions

Despite promising results, this study has several limitations:

5.3.1 Immune Memory Evaluation

The study did not evaluate long-term immune memory (e.g., memory T cells), which is critical for preventing tumor recurrence. Future studies should assess tumor rechallenge models to determine if the system induces durable immune memory.

5.3.2 Clinical Translation Challenges

ZIF-8's degradation product (Zn²⁺) is biodegradable, but long-term biodistribution (e.g., Zn²⁺ accumulation in kidneys) needs evaluation. Additionally, scaling up ZIF-8 synthesis with consistent size and drug loading is required for clinical translation.

5.3.3 Combination with Checkpoint Inhibitors

Immune checkpoint inhibitors (e.g., anti-PD-1) can reverse T cell exhaustion. Combining DOX/CpG@ZIF-8-PEG with anti-PD-1 may further enhance efficacy, especially in advanced melanoma. Future studies should explore this combination.

5.3.4 Patient-Derived Models

The study used B16-F10 cells, which are poorly immunogenic. Patient-derived xenografts (PDX) or genetically engineered mouse models (GEMM) that better mimic human melanoma should be used to validate efficacy in clinically relevant settings.

6. Conclusion

In this study, we developed a pH-responsive ZIF-8-PEG nanoparticle system for co-delivery of DOX and CpG ODNs, achieving synergistic chemo-immunotherapy of melanoma. The system exhibited uniform size (~120 nm), high drug loading, and targeted release in acidic tumor microenvironments. In vitro, it induced robust ICD, DC maturation, and cytotoxic T cell activation. In vivo, it significantly inhibited primary tumor growth (volume reduction: 81.2%), reduced lung metastases (86.2%), and prolonged survival (median survival: 56 days), with minimal systemic toxicity.

This work demonstrates the potential of MOF-based nanocarriers to bridge chemotherapy and immunology, addressing the limitations of single-modal therapies for melanoma. The synergistic mechanism—amplified ICD + immune activation—provides a blueprint for developing similar co-delivery systems for other poorly immunogenic cancers, advancing the field of precision cancer therapy.

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Author Guide for Nano-Bio Convergence Letters

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