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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 m^2/g, and a pore volume of 0.5–2.0 cm^3/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 (NH_2-MSNs) were collected by centrifugation, washed with ethanol, and dried. The NH_2-MSNs were then coated with PBAE by mixing 50 mg of NH_2-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 μM 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 μM) 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 μg/mL streptomycin) at 37°C in a humidified atmosphere with 5% CO_2. 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 μg/mL for 1, 2, 4, and 6 h. For competition assays, MDA-MB-231 cells were pre-incubated with 1 μM 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 μg/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× 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 μg/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 $2^{-}\Delta\Delta Ct$ 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× binding buffer. The cells were stained with 5 μL of Annexin V-FITC and 5 μL 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 μg/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 μL 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 μL 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) × 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±2°C), and humidity (50±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 μL 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 × width^2) / 2. When the tumor volume reached 100–150 mm^3, 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 μg/mL, 200 μL) 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] × 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 μm 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 ± 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 ± 4.1 mV. Coating with PBAE further increased the zeta potential to +42.3 ± 4.5 mV, as PBAE contains positively charged tertiary amines. Conjugation of AS1411 aptamer (negatively charged) reduced the zeta potential to +28.9 ± 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 ± 58 m^2/g, a pore volume of 1.85 ± 0.12 cm^3/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 m^2/g and 1.23 ± 0.09 cm^3/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 m^2/g and 1.05 ± 0.08 cm^3/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 ± 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 μM, the loading efficiency reached 92.3 ± 4.1%, with a loading capacity of 46.2 ± 2.0 μg siRNA/mg MSNs. The loading efficiency increased with increasing siRNA concentration up to 0.5 μM, 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 ± 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 ± 5.4% after 48 h, with a burst release of 45.3 ± 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 ± 5.7%, compared to 45.6 ± 4.2% for NT-MSN/siMCL-1 and 12.5 ± 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 ± 4.9% (Figure 5B). Western blot analysis confirmed that MCL-1 protein expression was reduced by 76.4 ± 5.3% in MDA-MB-231 cells and 72.8 ± 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 ± 4.8% in MDA-MB-231 cells, which was significantly higher than that of NT-MSN/siMCL-1 (38.2 ± 3.9%), free siMCL-1 (15.3 ± 2.7%), and control groups (saline: 5.2 ± 1.3%; NT-MSN/siScr: 6.8 ± 1.5%; Apt-MSN/siScr: 7.2 ± 1.6%) ($p < 0.001$) (Figure 6A). In BT-549 cells, Apt-MSN/siMCL-1 induced a total apoptotic rate of 62.3 ± 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 μg/mL (Figure 7A). For the formulation

treatments, Apt-MSN/siMCL-1 reduced the viability of MDA-MB-231 cells to 32.5 ± 3.6%, compared to 58.7 ± 4.3% for NT-MSN/siMCL-1 and 89.2 ± 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 ± 4.1% (Figure 7C). In contrast, Apt-MSN/siMCL-1 had minimal cytotoxicity in MCF-10A cells (cell viability: 82.3 ± 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 ± 23.8 mm^3, which was 71.2% smaller than that in the saline group (644.3 ± 58.7 mm^3, $p < 0.001$). The tumor growth inhibition rate (TGIR) of Apt-MSN/siMCL-1 was 73.5 ± 4.8%, compared to 42.3 ± 3.9% for NT-MSN/siMCL-1 and 12.5 ± 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 ± 4.9%, compared to 26.3 ± 3.8% for NT-MSN/siMCL-1 and 8.2 ± 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 ± 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 ± 5.7% MCL-1 mRNA silencing and 67.5 ± 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 μg/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 ± 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 ± 5.7% mRNA reduction) and high cell apoptosis (67.5 ± 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 ± 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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