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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% paraformaldehyde 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 ($\sim 10\text{--}20\%$).

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 ≈ 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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