

Nanobody-Based Drug Delivery: Emerging Strategies for Targeted Cancer Therapy

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Abstract: Traditional cancer treatments such as chemotherapy and radiotherapy remain effective but lack specificity, often causing collateral damage to healthy tissues. Antibody-drug conjugates (ADCs) using monoclonal antibodies (mAbs) have been developed to achieve advanced targeted delivery; however, preclinical and pharmacokinetic studies have indicated that factors such as large size, complex conjugation processes, high production cost, and immunogenicity can limit tumor penetration, pharmacokinetics, and broader translational applicability. Nanobodies (Nbs), or single-domain antibodies (sdAbs) derived from camelid heavy-chain-only antibodies (HCAbs), represent a promising alternative with smaller size, high aqueous solubility, stability, refolding capacity, and low immunogenicity. Preclinical studies have shown that Nbs retain high affinity and specificity while providing improved access to hidden epitopes on target antigens compared to conventional antibodies. These unique features have supported the development of Nb-drug conjugates (NDCs), which have been evaluated for the selective delivery of cytotoxic drugs to antigen-expressing cancer cells in vitro and in animal models, demonstrating improved target specificity. Furthermore, Nb-attached drug delivery vehicles (NDVs) functionalized with nanoscale carriers, such as liposomes, dendrimer-based nanoparticles, upconversion nanoparticles, and polymeric micelles, have expanded the scope of Nb-based drug delivery systems. This review summarizes the current progress in Nb-mediated drug delivery, compares different strategies, and discusses their translational potential in cancer therapy, highlighting opportunities and limitations based on available experimental data.

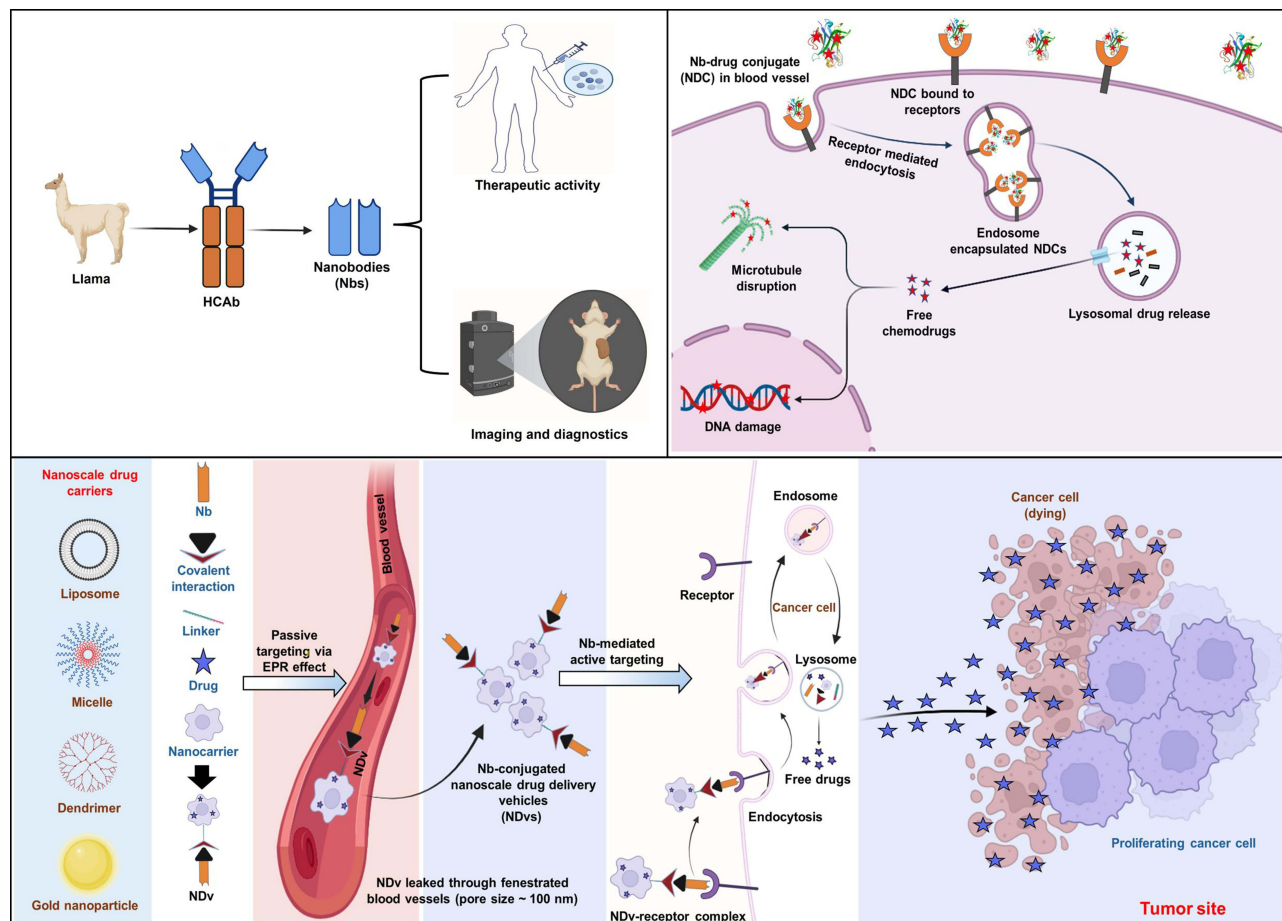
Keywords: nanobody, single domain antibody, targeted cancer therapy, drug delivery, nanobody drug conjugate

Introduction

Cancer is a group of diseases distinguished by a variety of molecular modifications. These changes involve gene mutations and amplifications, alterations in the number of gene copies, modifications in genes associated with tumor suppression and DNA repair, and epigenetic modifications that affect the activation of associated genes.^{1–4} Although conventional chemotherapy and radiotherapy are effective in eliminating tumor cells, they also result in the inadvertent destruction of healthy cells.⁵ The development of targeted therapeutic strategies has been a major focus in cancer treatment because of the lack of drug specificity and unwanted interactions of therapeutic drugs with healthy tissues.⁶ The landscape of cancer therapy is undergoing a transformative shift with the emergence of innovative strategies aimed at enhancing treatment efficacy while minimizing damage to the surrounding healthy cells. Over the years, antibody–drug conjugates (ADCs) have been used as a promising approach for cancer treatment, delivering cytotoxic drugs selectively to tumor cells with specific antigens.^{7–9} By conjugating monoclonal antibodies (mAbs) with cytotoxic agents through a linker, ADCs can combine mAb selectivity with diverse chemotherapeutics for potent interventions against various cancers.^{8,10} However, the large size of mAbs poses challenges in accessing tumor tissues, which limits their potential as effective drug carriers in ADCs for cancer treatment.^{11–13}

A novel class of antibodies known as single-domain antibodies (sdAbs) or nanobodies (Nbs) was identified in camelids, offering a compact alternative to mAbs.¹⁴ Their small size, high aqueous solubility, stability, low immunogenicity, deep tumor-penetration capability, and excellent specificity and affinity toward cancer biomarkers make them ideal targeting

Graphical Abstract



ligands.^{15–18} To select targets for site-specific chemodrug delivery, strategies involve identifying cancer biomarkers or antigens that are overexpressed in cancer cells, but not in healthy cells. Nbs exhibit intrinsic therapeutic activities by specifically targeting disease-related receptors and inhibiting key biological processes. For instance, anti-HER2 and anti-VEGFR2 Nbs in breast cancer block signaling pathways and angiogenesis, respectively, leading to reduced tumor growth and blood vessel formation.^{19,20} Furthermore, anti-EGFR Nbs inhibit tumor growth and induce apoptosis in glioblastoma.²¹ These examples illustrate the diverse applications of Nbs in cancer treatment, showcasing their potential as effective therapeutic agents across various types of cancer owing to their small size and high specificity. On the other hand, Nbs have found applications in *in vivo* molecular imaging for diagnosing diseased tissues and monitoring the effects of the treatment.^{22–24} The small size and unique properties of Nbs support them as candidates for obtaining high-quality images and conducting comprehensive disease evaluations. Nbs not only possess intrinsic therapeutic activity but can also be conjugated with toxins,^{25,26} inhibitors^{27,28} and utilized to decorate viral vectors^{29,30} for clinical therapeutic use.

Several versatile strategies have been developed to utilize Nbs in drug delivery.³¹ Nb-drug conjugates (NDCs) are created by conjugating Nbs directly to cytotoxic drugs through chemical linkers, whereas bispecific and multivalent Nbs have been developed to showcase the versatility of these molecular tools.^{31,32} NDCs deliver drugs through direct active targeting using Nbs as targeting ligands, which specifically bind to cancer cell antigens, ensuring precise drug delivery to the tumor. The design of NDCs is influenced by various factors, including the improved specificity or affinity of Nbs, the type of chemotherapeutic drugs, site-specific attachment strategy, chemodrug location, and the ratio of chemodrugs to Nbs. The general mechanism of action of NDCs is as follows (Figure 1). After administration, the NDC, guided by its

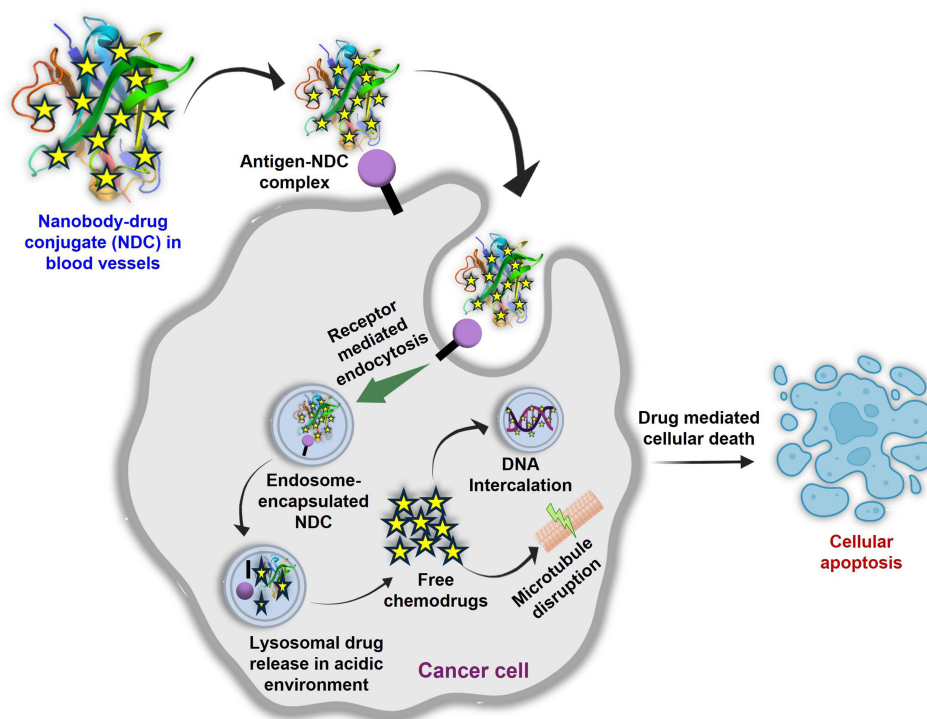


Figure 1 Schematic representation of the general drug delivery mechanism of NDCs to cause cancer cell death.

targeting moiety, travels to the cancer site. Nbs, which are linked to chemodrugs, recognize overexpressed cancer antigens on the surface of cancer cells. Subsequently, the NDC-antigen complex undergoes internalization often via receptor-mediated endocytosis. In endosomes and lysosomes, the complex is processed to release activated chemodrugs from lysosome into the cytoplasm in response to the acidic environment or lysosomal enzymes. This released payload inhibits the rapid proliferation of cancer cells, inducing apoptosis by disturbing DNA strands or inhibiting RNA polymerase, topoisomerase, or microtubule activity.^{31–34} The uptake of NDC-antigen complex by cancer cells occurs through two pathways: clathrin-dependent lysosomal internalization and clathrin-independent endosomal internalization. In clathrin-dependent internalization, lysosomal degradation can affect drug molecules through enzymatic breakdown. In addition, after clathrin-independent internalization, NDC exists in the acidic environment within endosomes.³⁵ However, Nb-drug conjugates that enter the cells can be protected from lysosomal degradation by conjugation with human serum albumin (HSA).³⁶

NDCs offer a crucial advantage over ADCs by enabling homogeneous formulations that address the limitations associated with the heterogeneous nature of traditional ADCs. Heterogeneous ADCs suffer from variable drug-to-antibody ratios (DARs) and random conjugation at multiple sites on the mAb.^{37–41} The smaller size of NDCs, complemented by advances in biotechnology and site-specific conjugation strategies, holds promise for the development of homogenous formulations with cancer cell toxicity at picomolar-femtomolar levels. Homogeneous formulations of NDCs provide consistent DAR. Unlike heterogeneous ADCs, homogeneous formulations of NDCs improve stability, reliability, and batch-to-batch consistency.^{42–44} Homogeneous NDCs also reduce immunogenicity risks, facilitate smoother clinical development, and optimize dosage schedules. These advantages contribute to safer and more effective cancer therapies. Enhanced blood retention capabilities and potent anticancer effects of NDCs against drug-resistant tumors further underscore their advantages. Additionally, Nbs attached to the surface of nanoscale drug delivery vehicles, such as liposomes, nanoparticles, or micelles, form Nb-attached drug delivery vehicles (NDVs). These vehicles offer an effective option to enhance the uptake of chemotherapeutic drugs by cancer cells, ensuring optimal anticancer effectiveness with minimal side effects.³¹ NDVs use passive targeting to cross leaky tumor blood vessels through the enhanced permeability and retention (EPR) effect. Once they escape the blood vessels, NDVs diffuse through the dense

extracellular matrix and employ active targeting via Nbs to attach specifically to tumor cells. Once internalized through receptor-mediated endocytosis, they release chemotherapeutic drugs into the cytoplasm to inhibit cancer growth.^{31,32,45} NDVs show greater effectiveness over NDCs due to their higher drug-loading capacity, improved stability, and their ability to respond to external and internal stimuli for controlled drug release.^{46,47} All these approaches represent a promising avenue to overcome the limitations associated with traditional chemotherapy, aiming to achieve targeted transport of cytotoxic agents directly to cancer cells and mitigate collateral damage to healthy cells.

However, while Nbs offer substantial advantages in terms of specificity and therapeutic versatility (Figure 2), they are often compared with other targeting agents, such as cell-penetrating peptides (CPPs) and cell-targeting peptides.^{48,49} Due to their high specificity in targeting cancer cells with minimal off-target effects, Nbs may be superior to other drug delivery vehicles like CPPs, which include peptides such as TAT and polyarginine,⁵⁰ or cell-targeting peptides like iRGD^{51,52} or RGD peptides (arginine-glycine-aspartic acid). Although all of these have demonstrated similar therapeutic outcomes, direct comparisons between these targeting systems are challenging owing to their different mechanisms of action. For example, Nbs rely on receptor-mediated endocytosis for highly specific targeting of tumor markers.^{53,54} In contrast, CPPs utilize processes such as direct membrane translocation and endocytosis to facilitate the delivery of larger payloads into cells.^{55,56} CPPs are often employed to carry larger molecules, such as proteins or nucleic acids,^{57–59} whereas Nbs can be conjugated with comparatively smaller agents, including drugs, toxins, or imaging agents.^{31,60} Notably, some researchers have successfully employed CPPs to enhance the delivery of Nbs into cells, improving their therapeutic efficacy.^{61–63}

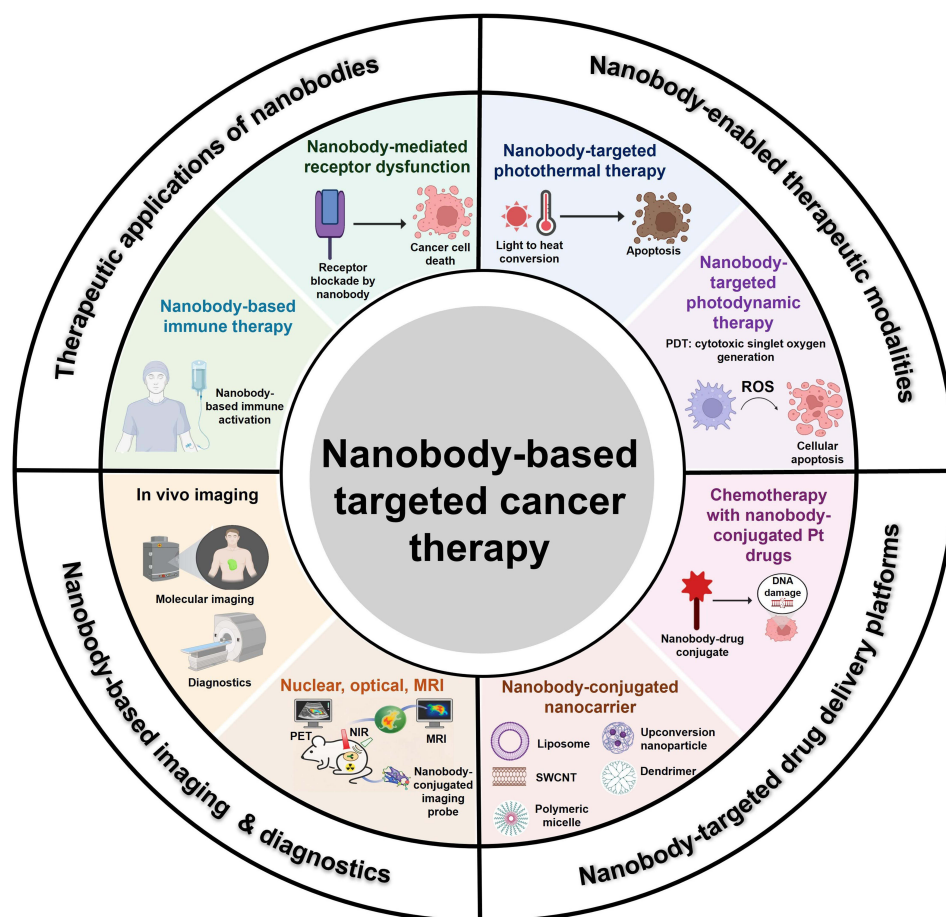


Figure 2 Schematic overview of Nb-based targeted cancer therapy.

This review provides an overview of the latest advancements in Nb research with a focus on medical applications, particularly targeted drug delivery for cancer treatment. This review emphasizes the substantial efforts dedicated to NDC and NDv development and subsequently explores the obstacles and approaches to advancing their clinical application. The success observed in various preclinical animal models of different cancers reflects the potential of Nb-based chemodrug delivery strategies to overcome the drawbacks associated with conventional antibody-based approaches. With their versatility and ongoing progress, Nbs have emerged as potential tools for clinically relevant applications, particularly in the development of drug delivery systems for targeted cancer therapy.

Method

A systematic literature search was conducted using PubMed, Scopus, and Web of Science to identify relevant studies on Nb-based targeted cancer drug delivery. Searches were performed using combinations of the following keywords: nanobody, single-domain antibody, targeted cancer therapy, drug delivery, nanobody–drug conjugate, and antibody–drug conjugate. The primary search covered the period from 2010 to 2025 and was restricted to English language original research articles and review papers. Earlier seminal studies published prior to 2010 were selectively included where historically relevant and are cited in the descriptive sections of the Introduction and research background. After the removal of duplicate records, titles and abstracts were screened independently by two authors to assess relevance. Full-text articles were then evaluated according to predefined inclusion and exclusion criteria. Studies were included if they described Nb-based drug delivery mechanisms, targeting strategies, and therapeutic outcomes in animal models of cancer, whereas articles without direct relevance to Nb-mediated targeted drug delivery were excluded. This review focuses primarily on Nb-based therapeutic drug delivery platforms; therefore, detailed discussions of intrinsic Nb therapeutic activity, Nb conjugated with toxins, inhibitors, imaging agents, or Nb-decorated viral vectors are beyond the main scope and are only briefly mentioned for contextual completeness. Any discrepancies during the screening and selection processes were resolved by consensus.

Monoclonal Antibodies and Smaller Antibody Fragments in Conventional Drug Delivery

Antibodies have demonstrated promising treatment outcomes compared to traditional chemotherapy.⁶⁴ Traditional immunoglobulin G (IgG)-type monoclonal antibodies, found in humans, consist of two heavy chains and two light chains, resulting in a collective molecular mass of approximately 150 kDa. The heavy chain comprises three constant domains (C_H1 , C_H2 , and C_H3) and a variable domain (V_H), whereas the light chain includes a constant domain (C_L) and a variable domain (V_L) (Figure 3a).^{65–68} However, mAbs struggle to penetrate tumor tissue due to their large size.⁶⁹ To address this issue, smaller alternatives have been developed over the last 50 years, including naturally derived or artificially created antigen-binding fragments such as Fab (approximately 50 kDa), variable fragments such as Fv (around 15 kDa), and single-chain variable fragments such as scFv

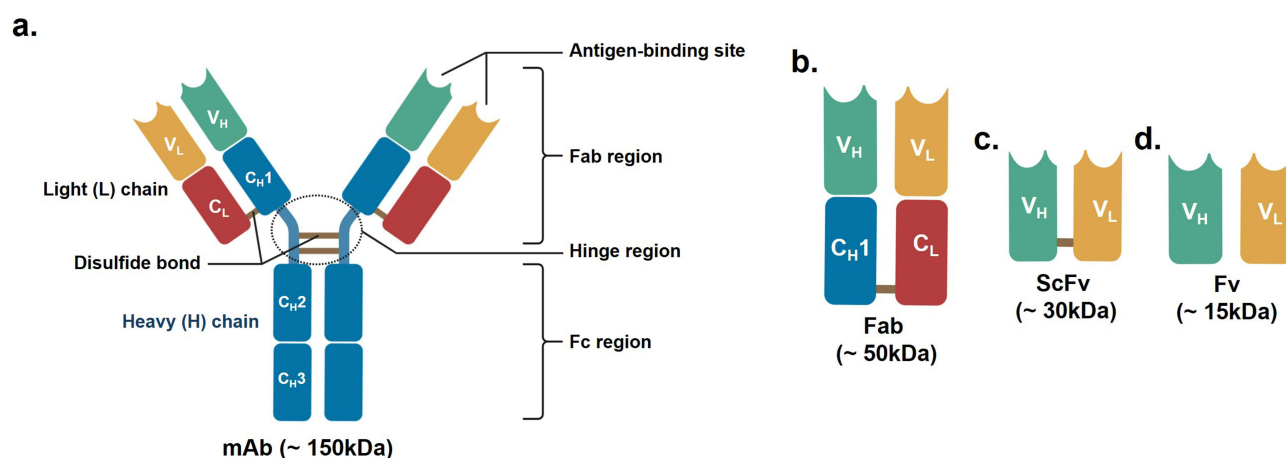


Figure 3 Structure of (a) a conventional monoclonal antibody and various smaller antibody fragments including (b) antigen-binding fragments (Fab), (c) single-chain variable fragments (scFv), and (d) variable fragments (Fv).

(approximately 30 kDa), with antigen-binding capability comparable to that of the original antibody^{70–73} (Figure 3b–d). mAbs play a pivotal role in inhibiting tumor growth by binding to a specific cell surface receptor and targeting one type of epitope present on multivalent antigens. This binding disrupts the signaling pathways that promote tumor cell growth and the formation of new blood vessels.^{74,75} Furthermore, the fragment crystallizable (Fc) domain of antibodies plays a pivotal role in initiating antibody-dependent cell-mediated cytotoxicity, a crucial immune response that facilitates the targeted destruction of cancer cells.^{76,77} When a mAb binds to a specific target on a cancer cell, it forms a complex that signals the immune system to destroy the cancer cell. This binding involves the Fc region, which is the tail region of the antibody that interacts with cell surface receptors on immune cells. Immune cells have Fc receptors on their surface that can recognize and bind to the Fc region of the antibody. Once the immune cells recognize the bound antibody at its Fc region, they become activated and initiate an attack on the cancer cell.⁷⁸ This coordinated immune response enhances the ability of the immune system to effectively eliminate cancer cells.

Furthermore, mAbs can be engineered as targeting ligands to selectively deliver various cytotoxic loads to diseased cells, such as in ADCs, immunotoxins, nanomedicines, or nanoparticles encapsulating cytotoxic agents. They have found success in cancer therapy, with more than 100 having received FDA approval for clinical use, among which trastuzumab and cetuximab can efficiently target solid tumors.^{64,69,79–81} ADCs, which are complex molecules made of an antibody linked to a biologically active cytotoxic drug, show promising effectiveness in targeted cancer treatment. The general mechanism of action of an ADC involves several key steps (Figure 4). First, the ADC, consisting of a targeting moiety

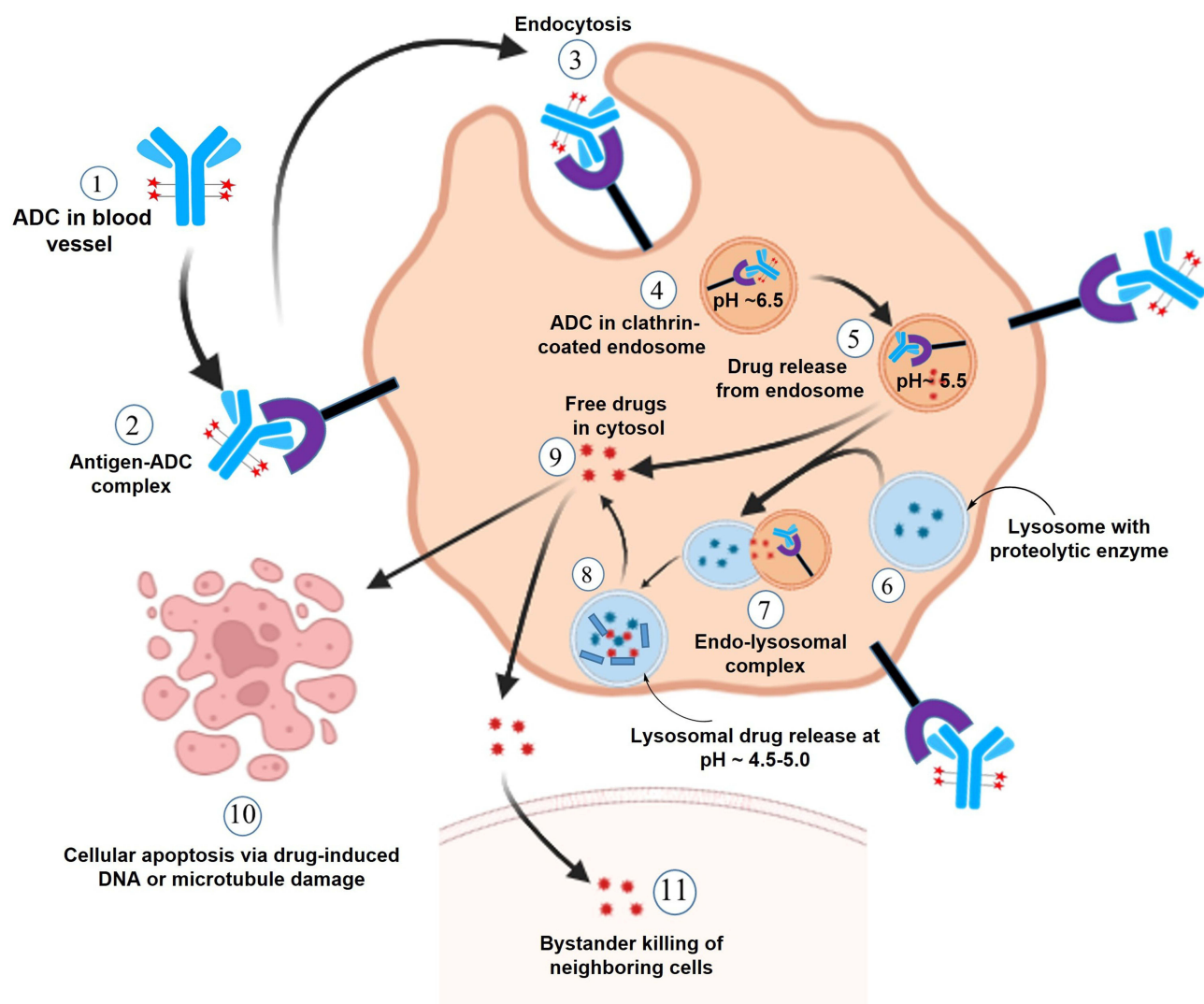


Figure 4 The general mechanism of action of an ADC begins with its binding to specific receptors on the surface of cancer cells. This triggers endocytosis, internalizing the ADC into the cancer cell, where it is processed in lysosomes, releasing its cytotoxic drug, which kills the cancer cell. The numbers 1–11 indicate the sequence of events during ADC binding, internalization, intracellular trafficking, payload release, and subsequent cancer cell death, including the bystander killing effect.

(usually an antibody), a cytotoxic payload, and a chemical linker, binds to specific cell-surface receptors on cancer cells. This binding triggers receptor-mediated endocytosis, leading to internalization of the ADC into the cancer cell.^{82–84} Once inside, the ADC is trafficked through endosomes and ultimately to lysosomes,^{85,86} where the chemical linker is cleaved, releasing the cytotoxic payload. The released payload then exerts its cytotoxic effects, typically disrupting microtubule function or DNA replication, leading to cancer cell death.^{7,64,83,87} The precise mechanism of payload release depends on the design of the linker-drug moiety. Overall, the ADC selectively delivers its cytotoxic payload to cancer cells, sparing healthy cells and minimizing systemic toxicity in targeted therapy for cancer treatment. Notably, the “bystander effect” in ADCs involves the spread of cytotoxic effects beyond targeted cells to neighboring cells lacking the antigen, facilitated by release of the cytotoxic payload into the vicinity of the targeted cell.⁸⁸ While ADC therapy directs the cytotoxic payload specifically to cancer cells by binding to overexpressed surface antigens, the nature of payload release can lead to collateral damage to adjacent cells with heterogeneous expression of antigens, enhancing therapeutic efficacy.⁸⁹ However, the extent of this effect varies based on factors such as payload release mechanism, payload nature, and tumor microenvironment, prompting ongoing research to optimize ADC design for maximum bystander effects with minimal off-target toxicity.

Some ADCs have progressed to clinical trials.⁸¹ However, the large size of mAbs, which is further increased by conjugation with nanoparticles or cytotoxic payloads, poses challenges for targeted drug delivery. This enlarged size leads to the inaccessibility of some epitopes of antigens that are abundantly expressed on cancer cell surfaces.⁹⁰ The stability of mAbs is characterized within narrow pH and temperature ranges.^{91,92} Their production should be specific to the antigen to which they bind. Minor changes in antigen epitope structure can deteriorate the mAb.⁹³ In addition, monospecificity limits their application as they target a single antigen, reducing their effectiveness in complex diseases involving multiple targets.

Reducing the size of antibodies to smaller fragments has benefits, such as increased tissue penetration. However, small antibody fragments have drawbacks, such as low serum half-life and the risk of immunogenicity induced by aggregation. In particular, scFv molecules, composed of V_H and V_L domains linked by a chain of artificial amino acids or disulfides, may be non-functional because the linking orientation can affect the specificity of antigen binding. In addition, the stability of small antibody fragments is greatly decreased compared to mAbs due to the exposure of crucial hydrophobic amino acids within the V_H/V_L domain of the fragments. This results in increased antibody susceptibility to polymer formation, especially in the case of scFv. For mAbs, the hydrophobic amino acids located on the surface of the V_H domain play an important role as binding sites for the V_L domains. Thus, exposure of the hydrophobic area to small antibody fragments can reduce stability, leading to aggregation, limited solubility in physiological conditions, and inconsistent binding specificity and affinity. Furthermore, the large-scale production of these smaller antibody fragments faces obstacles.⁶⁹ All of these factors can hinder the effectiveness of a therapeutic and restrict the drug delivery applications of small antibody fragments.⁹⁴ Therefore, an innovative solution to overcome the limitations of mAb and small antibody fragments is required, and a new type of antibody should be developed.

Exploring the Promise: Single-Domain Antibodies as a Versatile Toolkit

In 1993, Professor Raymond Hamers-Casterman at Vrije University Brussel discovered antibodies originating from dromedary camels infected with *Trypanosoma evansi*. The antibodies, known as heavy-chain-only antibodies (HCAbs), lack the typical light chains found in conventional antibodies.¹⁵ Despite the absence of light chains, HCAbs remain functional, offering unique advantages in terms of robustness and stability compared to conventional antibodies. HCAbs were found to exist in camels, llamas, and cartilaginous fish species like sharks, and they emerged as a promising alternative to mAbs.⁹⁵ These HCAbs each have a molecular mass of approximately 95 kDa and retain full antigen specificity and selectivity for cancer targets (Figure 5a).⁹⁵ The antigen-binding site of these unique HCAbs contains a single domain, known as the variable heavy domain of the heavy chain (VHH).^{96,97} These HCAbs can be truncated to include only their isolated variable domains, VHHs, which typically weigh between 12 and 14 kDa, without losing their antigen recognition capabilities (Figure 5b).^{95,98} HCAbs consist of C_H2 and C_H3 domains, hinge regions, and antigen-binding regions, which are specifically the VHH domains that allow antigen recognition and binding.⁹⁵ These unique VHHs use a single domain, measured in the single-digit nanometer range and are referred to as single-domain

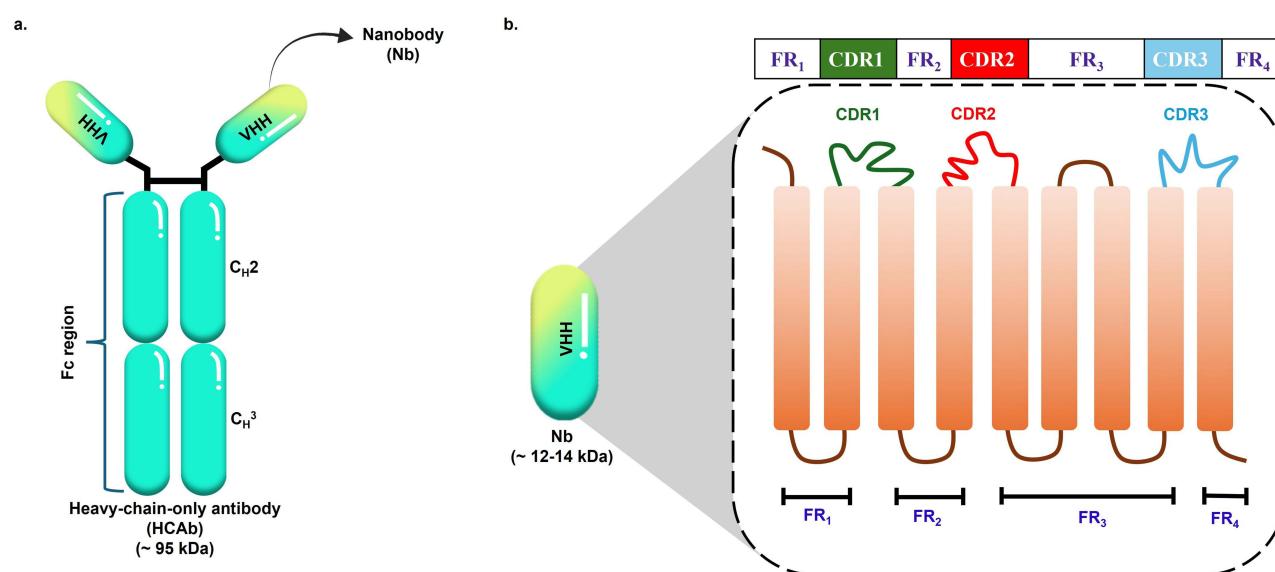


Figure 5 (a) Schematic representation of an HCAb. (b) The domain structure of an Nb consists of four conserved framework regions (FR_{1/2/3/4}) surrounding three long, hypervariable complementarity determining regions (CDR1, CDR2, CDR3).

antibodies⁹⁹ or nanobodies, a term coined by Ablynx.¹⁰⁰ VHH can be easily made as recombinant proteins.^{99,101–104} The streamlined process of Nb generation typically involves camelid immunization with the desired antigen, followed by the isolation of antibody-producing cells, often from peripheral blood lymphocytes or bone marrow. A cDNA library is generated from these cells, encoding the genetic information for the HCABs. Display technologies, such as phage display or yeast display, are employed to screen and select Nbs with high specificity and affinity for the target antigen. The selected Nbs undergo rigorous screening and characterization, including assessments of binding affinity, stability, and immunogenicity.^{99,105} Further engineering may be conducted to optimize pharmacokinetic properties, such as extending the serum half-life or reducing immunogenicity.

A unique feature of these Nbs is the presence of four conserved framework regions (FR_{1/2/3/4}) in the protein structure. These regions surround three long and hypervariable complementarity determining regions (CDR1, CDR2, and CDR3) in the antigen-binding domain (Figure 5b).^{106–108} CDR3 is responsible for approximately 60–80% of the specificity of antigen recognition among all CDRs, whereas CDR1 and CDR2 are responsible for boosting the binding strength.^{109,110} These CDRs exhibit an exceptional ability to form long, finger-like structures that can reach into cavities and hidden epitopes of antigens, allowing Nbs to access areas that are inaccessible to conventional antibodies due to their larger size.^{95,111} CDR3 in Nbs shares similarities with or is potentially longer than the corresponding region in the human variable domain of the heavy immunoglobulin chain (V_H). In Nbs, CDR3 consists of 3–28 amino acids, whereas that in the human V_H domain is limited to 8–15.^{106,112–115} These characteristics not only improve the strength and specificity of Nb binding to antigens^{116–118} but also facilitate the identification of new pharmacological targets. For instance, receptor-binding pockets or enzymatic active sites can be identified more effectively, paving the way for precise drug delivery using NDCs.^{119,120} Nbs exhibit hydrophilicity and higher water solubility due to the presence of hydrophilic residues in the VHH, specifically on the FR₂, where four highly conserved and hydrophobic amino acids in human V_H (V42, G49, L50, and W52) are replaced by smaller, more hydrophilic ones (F42 or Y42, E49, R50, and G52) [the international ImMunoGeneTics (IMGT) information system has been used for amino acid numbering], enhancing solubility in polar solvents like water.^{33,118,121–123} In addition, Nbs are less likely to be immunotoxic.¹²⁴ The primary cause of low immunogenicity in Nbs is their high sequence identity with the human type 3 V_H domain.^{95,111,125,126} The non-human origin of Nbs may trigger unwanted immune responses and stimulate the production of anti-drug antibodies, especially when they form misfolded aggregates. Kibria et al discovered that native Nbs were minimally immunogenic, whereas VHH-65 (VHH incubated at 65 °C) showed slight immunogenicity, and VHH-95 (VHH incubated at 95 °C) was highly

immunogenic.¹²⁷ This indicates that the immunogenicity of Nbs is significantly influenced by their aggregation state and physicochemical properties. Native Nbs exhibit minimal immunogenicity, but aggregated or misfolded Nbs, especially those exposed to high temperatures, are more likely to trigger an immune response. When Nbs are exposed to elevated temperatures (like 65 or 95 °C), they can undergo denaturation, leading to structural changes, misfolding, or aggregation. These altered forms are recognized as foreign substances by the immune system, increasing the risk of an immune response and anti-drug antibody production. This underscores the importance of maintaining proper Nb conformation to reduce adverse immune reactions. Phase I clinical trials conducted by Ablynx confirm the low immunogenicity of Nbs, supporting their use for sustained administration.¹²⁸ Furthermore, Nbs have an extended shelf life at +4 °C and –20 °C and can withstand high temperatures (60–80 °C).^{129,130} In addition, they demonstrate stability over several weeks at 37 °C.¹³¹ Nbs exhibit remarkable resistance to proteolytic degradation, tolerance to non-physiological pH levels ranging from 3.0 to 9.0, and resilience to elevated pressure (500–750 MPa) and chemical denaturants (2–3 M guanidinium chloride and 6–8 M urea).^{132,133} These features, which contribute to their exceptional stability under physiological conditions encountered in the body, provide Nbs with significant clinical advantages over mAbs. Moreover, Nbs exhibit reversible refolding capacity after chemical or thermal denaturation, with lower aggregation tendencies.^{132,134–137} They are cost-effective to manufacture and exhibit superior binding affinity and specificity against various targets, including tumor markers.^{138,139} These characteristics suggest Nb as an appealing choice for targeted drug delivery. In addition, the absence of light chains and certain constant domains streamlines their structure, facilitating their deep tissue penetration in vivo.⁹⁷

Due to their smaller size compared to mAbs, Nbs have a limited ability to be conjugated to multiple chemodrug molecules, which may affect the effectiveness of NDCs in cancer treatment. However, one study reported that only 1.56% of the administered ADCs efficiently reached the target sites, indicating inefficiency.¹³⁰ Therefore, although Nbs are smaller, they compensate for this size limitation with better cellular uptake, long-term stability, and greater potential for inhibiting tumor growth, demonstrating NDCs as more competent than ADCs in cancer therapy.^{123,140,141} The compact size, robust structure, and monomeric nature of Nbs present an opportunity for the creation of NDCs with unique pharmacological benefits.¹⁴² The essential optimization step before progressing to clinical applications includes humanization of Nbs, as reported by Vincke et al in 2009.¹⁴³ This stage involves altering the amino acid sequences in the framework region of Nbs to match those found in the human heavy chain variable domain. Notably, this modification does not compromise antigen specificity or physiological properties such as solubility and stability. Nbs, which are encoded by a single gene of approximately 360 base pairs, are more straightforward to develop, humanize, and conjugate to chemotherapeutic drugs compared to traditional mAbs.¹⁴⁰

Nbs can be classified based on numbers and types of antigen binding sites. Monomeric Nbs can be engineered into bispecific, bivalent, and multivalent constructs (Figure 6a–d).^{97,144} Bivalent constructs can either specifically bind to a single target antigen or be designed as bispecific entities through genetic fusion with peptide linkers. By combining multiple monomeric copies of Nb, multivalent constructs are formed, leading to increased avidity, prolonged circulation, and improved drug-to-Nb ratios.^{145,146} Coppieters et al (2006) observed a significant potency increase when transitioning from monomeric to trimeric forms of bivalent anti-TNF α Nbs, enhancing their affinity toward the RSV F protein.^{147,148} Bradley et al (2015) identified monomeric Nbs binding to distinct epitopes on the CXCR2 receptor, and they subsequently engineered bivalent and biparatopic Nbs, demonstrating superior pharmacological properties.¹⁴⁹ Conrath et al (2001) showed that fusing two Nbs results in bispecific Nbs capable of binding to multiple enzymes.¹⁵⁰ This suggests that bispecific Nbs, compared to monomeric Nbs, may be more valuable for delivering chemotherapeutic drugs to cancer sites that overexpress specific antigens, as they can simultaneously bind to two distinct targets. Bispecific Nbs show improved therapeutic efficacy over monomeric Nbs through enhanced targeting precision, affinity, and avidity. Therefore, multimeric Nbs can concurrently block or downregulate cancer antigens, while facilitating chemodrug delivery, potentially leading to synergistic efficacy.

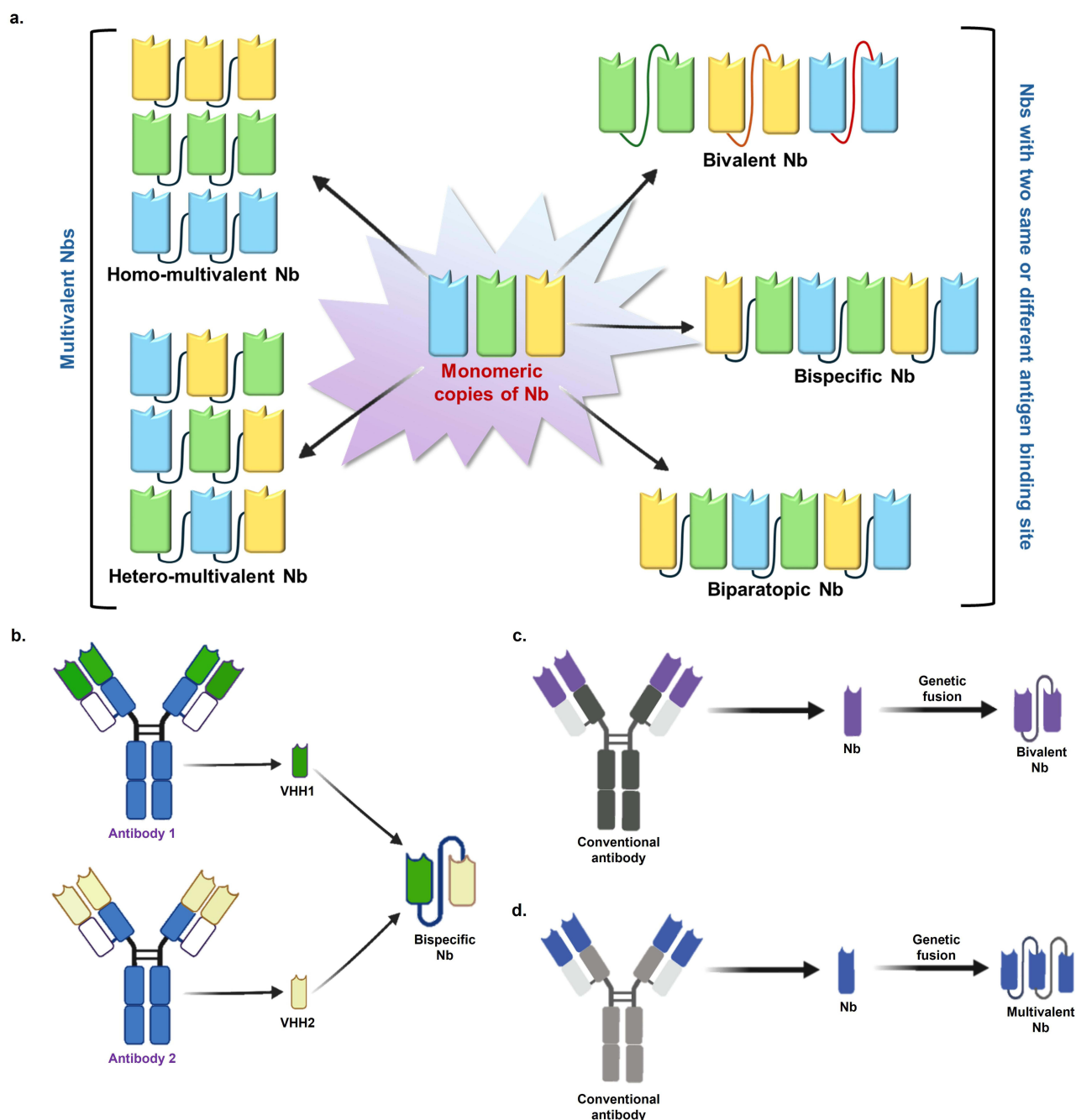


Figure 6 (a) Schematic representation of various engineered Nb-constructs for efficient drug delivery, including Nbs with the same or different antigen binding sites. (b) Formation of a bispecific Nb via genetic fusion of two different VHH domains. Generation of (c) bivalent and (d) multivalent Nb from conventional antibodies.

Challenges and Solutions in Nb-Based Drug Delivery

Linker Properties for Conjugation of Nbs with Chemotherapeutic Drugs

The role of the linker is crucial in maintaining the conjugation between Nb and the chemodrug, which contributes to improved stability and extended blood circulation in NDCs. The molecular design and chemical properties of the linker significantly influence the therapeutic outcomes and pharmacokinetics. The linker for NDC development must exhibit high stability in human blood plasma to ensure prolonged circulation for efficient tumor-site targeting. Inadequate linkers may result in premature drug release, leading to undesirable side effects.¹⁵¹ The hydrophobicity of the linker is an important consideration, as hydrophobic linkers with hydrophobic chemodrugs may induce low aqueous solubility

leading to aggregation, rapid renal clearance, and immunogenic reactions. Conversely, hydrophilic alternatives, such as pyrophosphate diester groups,¹⁵² polyethylene glycol (PEG) groups,¹⁵³ and sulfonate groups,¹⁵⁴ can mitigate these issues and increase the chemodrug-to-Nb ratio. Since hydrophilic linkers can contribute to enhanced stability and reduced aggregation of NDCs, higher drug loading on the Nb is practicable. Moreover, selection between cleavable and non-cleavable linkers is essential for NDC circulation throughout the system, facilitating payload release upon reaching the target site, and enabling site-specific conjugation.¹⁵⁵ For effective drug release, the linker in the NDCs should be capable of releasing chemodrugs bound to Nbs upon internalization within the target cell. Ideally, a cleavable linker should undergo self-cleavage in specific environments exclusive to cancer cells.^{151,155}

PEG is a hydrophilic polymer that, when attached to proteins or peptides, enhances their solubility and stability and reduces immunogenicity, a process referred to as PEGylation.¹⁵⁶ PEGylation is a widely used technique to enhance the in vivo stability and extend the serum half-life of Nbs.^{54,157,158} A molecule of PEG can be attached to the Nb surface using various methods, including chemical modifications and enzyme-catalyzed reactions. However, nonspecific PEGylation of amino acids on the Nb surface can reduce its affinity for target receptors compared to the native form. Therefore, site-specific PEGylation is preferred to minimize alterations in Nb activity.¹⁵⁹ Enzyme-catalyzed reactions, especially under physiological conditions, have emerged as promising methods for efficient Nb modification without a decrease in bioactivity.¹⁵⁸ In a study by Li et al (2021), the drug monomethyl auristatin E (MMAE) was conjugated to the affibody, Z_{HER2:2891} (engineered small protein scaffold, similar to Nb) through the specific crosslinker SMCC to form Z_{HER2}-SMCC-MMAE, whereas other affibody-based drug conjugates were prepared with PEG insertions, specifically Z_{HER2}-PEG10K-MMAE and Z_{HER2}-PEG4K-MMAE. The use of PEG chains as linkers in Nb conjugates significantly extended the circulation half-life but reduced the cytotoxicity of the conjugates. The results revealed that, compared to the Z_{HER2}-SMCC-MMAE conjugate without a PEG insert, Z_{HER2}-PEG10K-MMAE and Z_{HER2}-PEG4K-MMAE with 10 or 4 kDa PEG insertions had 11.2- and 2.5-times longer half-lives and 22- and 4.5-times lower cytotoxicity, respectively. Although in vitro cytotoxicity is reduced, the combined effect enables Z_{HER2}-PEG10K-MMAE to deliver superior tumor therapy at the same dose in animal models, accompanied by a greater than 4-fold reduction in off-target toxicity relative to Z_{HER2}-SMCC-MMAE.¹⁶⁰ In addition, unmodified Nbs may trigger an immune response, leading to their rapid removal from the bloodstream.¹⁶¹ PEGylation helps mitigate this immunogenic response, decreasing the immune system's sensitivity to the Nb and increasing its longevity in circulation.

Various Strategies for Site-Specific Conjugation of Nbs with Chemodrugs

Modifications to Nbs primarily focus on harnessing the natural chemical activity of amino acids within the Nb protein backbone.⁴³ In recent decades, homogeneous ADC mixtures have been achieved using new tools developed in protein chemistry. These tools include controlled chemodrug conjugation methods such as lysine-directed amide coupling, insertion of cysteine residues, use of unnatural amino acids, and enzymatic conjugation (Figure 7a).^{43,162} These methods can significantly improve the efficiency of NDC production and lead to higher drug-to-Nb ratios.

Lysine, which is abundant in Nbs,¹⁶³ can be efficiently utilized for amide coupling using NHS-EDC linkage chemistry to attach chemotherapy drugs, resulting in a high yield of Nb conjugates (Figure 7b).^{164,165} However, since lysine is prevalent on the Nb surface, this approach can produce a heterogeneous mixture of NDCs with varied chemodrug-Nb ratios that can negatively influence therapeutic outcomes. Additionally, unintentional coupling of a chemodrug with a lysine within the antigen binding region may lead to a reduced binding affinity for targeted cancer antigens.¹⁶⁶ Nevertheless, the FDA-approved and clinically tested ADC Kadcyla®, which combines the targeted antibody trastuzumab and chemotherapy drug emtansine for HER2+ breast cancer treatment, demonstrates that careful lysine amide coupling can yield a reproducible and successful ADC.^{167,168} This indicates that lysine amide coupling with Nbs can also produce effective NDCs.

Cysteine, which is highly nucleophilic amino acid, offers an efficient site for selective chemodrug conjugation to Nbs.¹⁶⁹ A maleimide-based linker is commonly used for its specificity to cysteine. The two conserved cysteine bridges in Nbs offer efficient sites for attaching chemodrugs selectively, usually with maleimide-based linkers for thiol groups (Figure 7c). Inserting cysteine residues at the C-terminus of the Nbs away from the antigen-binding interface allows controlled conjugation. This method has been efficiently applied to introduce defined cysteine residues on Nbs for

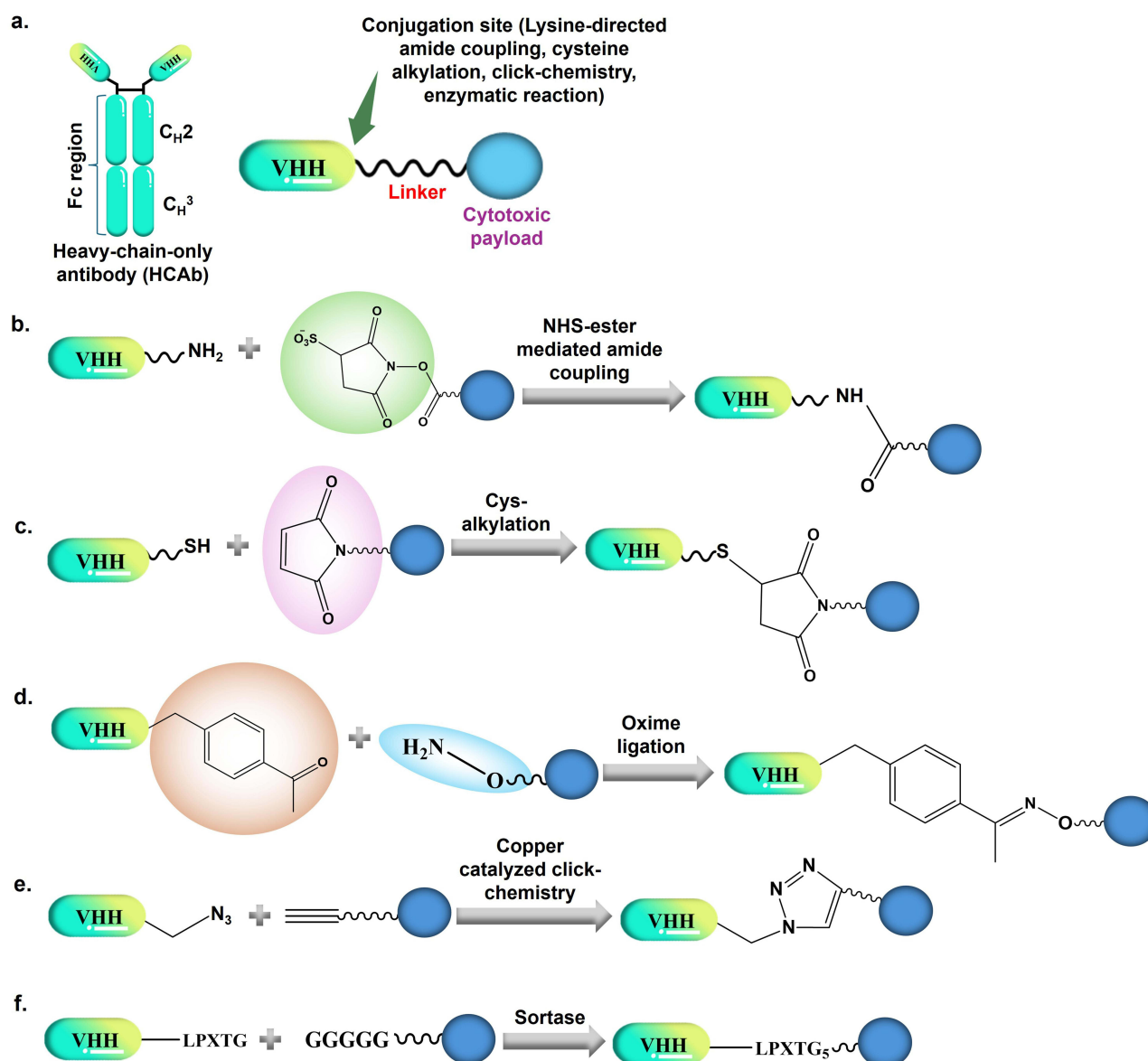


Figure 7 (a) General structure of an NDC containing a humanized Nb, a cleavable/non-cleavable chemical linker, and a cytotoxic payload. The linker is covalently bound to the Nb at the conjugation site. (b) An activated carboxylic acid moiety reacts with a lysine residue, which results in amide bond linkage between Nb and the payload. (c) A maleimide moiety reacts with a cysteine residue of an Nb. (d) and (e) Non-natural amino acid incorporation by genetic engineering into Nbs and subsequent chemical conjugation (oxime ligation) and copper-catalyzed click-chemistry, respectively. (f) Site-specific (chemo)enzymatic sortase-mediated conjugation. Sortase attaches oligocysteine-G functionalized linkers to LPXTG tags on the Nb.

targeting cancer biomarkers such as carbonic anhydrase IX receptor (CAIX)¹⁷⁰ and prostate-specific membrane antigen (PSMA9).¹⁷¹ However, this strategy presents potential challenges, including reduced expression yields, dimerization, and capping of unpaired cysteine residues by glutathione.¹⁷² Additionally, supplementary reduction steps are required to activate cysteine residues. Despite these challenges, this approach has shown promise in improving the accumulation of NDCs at tumor sites.

The introduction of unnatural amino acids into Nbs can enable site-specific conjugation.^{42,173,174} For example, *p*-acetylphenylalanine (pAcF) can be genetically encoded and employed for efficient conjugation through oxime ligation (Figure 7d).⁴² This approach has been effectively used to develop anti-HER2 Fab antibody fragments, demonstrating enhanced tumor regression in vivo. Other unnatural amino acids, such as *p*-azidomethyl-*L*-phenylalanine, allow conjugation through azide-alkyne cycloaddition (Figure 7e).¹⁷⁵ Although this approach is highly effective for generating

ADCs, it has been previously reported that the incorporation of unnatural amino acids may elicit immunogenic responses.¹⁷⁶

Enzymes can play a critical role in drug conjugation to Nbs and the formation of specific moieties for subsequent conjugation. One such example is the Sortase A enzyme from *Staphylococcus aureus*, which recognizes its LPXTG (Leu-Pro-X-Thr-Gly) motif, where X can be any amino acid. This enzyme cleaves the threonine-glycine bond, facilitating the attachment of molecules containing an oligoglycine (Oligo-G) sequence, including therapeutic agents, imaging probes, and fluorescent dyes (Figure 7f).^{177,178} Enzymatic techniques ensure a controlled drug-to-antibody ratio, contributing to the creation of homogeneous NDCs. Therefore, fusion of the LPXTG motif at the C-terminus of Nbs serves as a potential conjugation site. Similarly, formylglycine-generating enzymes, upon encountering the CXPXR motif (where X is typically serine, threonine, alanine, or glycine), catalyze the transformation of cysteine into an aldehyde. The aldehyde produced can then be modified with hydrazine- or amino-containing molecules. Modifying reactive aldehydes with hydrazine- or amino-containing molecules enables site-specific bioorthogonal conjugation, which is fast, and the resulting conjugates are stable under physiological conditions. This approach allows precise functionalization of biomolecules for applications such as targeted drug delivery, protein labeling, and enhanced therapeutic development without interference from cellular components.¹⁷⁹ However, this conjugation method has a drawback as hydration of the aldehyde occurs in the aqueous phase, resulting in an inactive form and reducing the overall conjugation yield.¹⁸⁰ Overall, these enzymes selectively react to specific amino acid sequences, enabling precise control of the drug-to-Nb ratio.

Prolonged Blood Circulation and Enhanced Stability of Nbs by Albumin Binding

Because Nbs are smaller than the renal filtration threshold (50 to 60 kDa) of the glomerular membrane of the kidney, they can be excreted in the urine, like peptides or small proteins. Frequent administration is required to achieve optimal efficacy because only a small portion of administered Nbs accumulates in the affected areas. On the other hand, HSA is an abundant serum protein in the bloodstream and is characterized by an exceptionally prolonged half-life of approximately three weeks.^{181,182} Unlike small biomolecules (<50 kDa), which are rapidly removed through glomerular filtration within minutes to a few hours, the larger HSA (66.5 kDa) exhibits prolonged retention in the bloodstream. HSA interacts with the neonatal fragment crystallizable receptor (FcRn) at acidic pH and undergoes continuous recycling through FcRn-mediated transcytosis.^{183,184} The FcRn receptor plays a crucial role in prolonging the half-life of IgG antibodies and albumin by protecting them from lysosomal degradation. Favorable binding affinity between HSA and FcRn protects HSA from the endo-lysosomal degradation pathway, ensuring remarkable stability in the serum.^{184,185} A study published in 2011 used a bivalent Nb that targeted both EGFR and albumin to create a trivalent bispecific Nb. The half-life of the trivalent Nbs in mice was prolonged to 2–3 days due to the presence of albumin.¹⁸⁵ This tandem formation has several advantages. First, the prolonged circulation time of HSA in the bloodstream contributes to the extended presence of HSA-Nb conjugates.^{181,186,187} Second, conjugation with HSA provides a shield against enzymatic degradation or rapid elimination, increasing the overall stability of the Nbs. Therefore, linking Nbs with HSA is a strategic approach for enhancing the durability and persistence of Nbs in the bloodstream to improve their localization at the tumor site.^{188–192}

Tumor Microenvironment as a Determinant of Cellular Targeting Efficiency in Nb-Based Therapeutics

Efficient cellular targeting in solid tumors is not solely determined by target biomarker expression but is profoundly influenced by the tumor microenvironment (TME). The dense extracellular matrix (ECM), abnormal vasculature, hypoxia, acidic pH, immune suppression, and spatially heterogeneous receptor distribution collectively limit the penetration, distribution, and cellular accessibility of targeted therapeutics.^{193,194} As highlighted in recent immunological analyses, these microenvironmental factors are major determinants of therapeutic response and delivery efficiency, underscoring the need to integrate TME considerations into targeted drug design.^{195–198}

Nbs possess intrinsic properties that are particularly advantageous in hostile tumor microenvironments. Their small molecular size enables enhanced diffusion through the dense ECM and facilitates effective recognition of tumor-

associated biomarkers within spatially heterogeneous and poorly accessible tumor regions.¹⁹⁹ Unlike conventional antibodies, Nbs exhibit reduced Fc-mediated sequestration by immune cells due to the absence of an Fc domain, thereby improving their effective bioavailability at the tumor site under immune-suppressive conditions.²⁰⁰ Several studies have demonstrated that Nb-based systems are explicitly engineered to bypass stromal exclusion. For example, bispecific Nbs targeting PD-L1 and CXCR4 have been shown to disrupt cancer-associated fibroblast (CAF)-mediated stromal signaling, reduce extracellular matrix density, and enhance intratumoral penetration and immune infiltration.²⁰¹

Tumors exhibit pronounced intratumoral receptor heterogeneity, with spatially variable antigen densities across tumor regions and individual cells. In the context of the TME, this heterogeneity is further amplified by uneven perfusion, hypoxia-driven phenotypic shifts, and stromal shielding, which further influence biomarker accessibility and targeting efficiency. Nbs are particularly suited for targeting such heterogeneous environments because of their high affinity, fast on-rates, and ability to recognize low-abundance antigens.¹²³ Within spatially heterogeneous tumors, the fast-binding kinetics and small size of Nbs enable the rational design of dual-targeting or bispecific formats, allowing the simultaneous engagement of multiple tumor-associated markers to compensate for heterogeneous expression and improve cellular targeting robustness.²⁰²

In parallel with advances in molecular targeting, stimuli-responsive nanocarrier systems have emerged as a general strategy to address TME-imposed barriers.⁴⁷ These platforms exploit tumor-associated physicochemical cues, such as acidic pH, elevated glutathione (GSH) levels, abnormal redox balance, and externally applied triggers (eg, near-infrared irradiation), to enable spatiotemporally controlled drug activation within tumors, rather than relying solely on passive accumulation.^{203–205} Within this broader paradigm, Nb-based drug delivery systems can be viewed as modular extensions of stimuli-responsive nanocarrier strategies, in which targeting and activation are functionally decoupled. In these systems, Nbs primarily serve as high-affinity targeting modules that confer cellular, stromal, or microenvironmental specificity, whereas stimulus-responsive linkers or carrier materials regulate the spatiotemporal release of the therapeutic payload in response to intracellular enzymes, acidic compartments, or redox conditions characteristic of the tumor microenvironment.²⁰⁶ Preclinical studies have provided mechanistic support for this integration, as NDCs incorporating protease-cleavable or pH-sensitive linkers undergo intracellular payload release following endocytosis into enzymatically active or acidic compartments.²⁰⁷ Similarly, NDVs engineered with redox-responsive architectures enable controlled drug release in response to elevated intracellular GSH levels after cellular internalization.^{203,208} Collectively, these studies position NDCs and NDVs as platforms that may enable improved payload access within the TME, rather than relying solely on antigen abundance.

Therapeutic Nb Delivery Platforms Through Intracellular Tumor Targeting

To date, research on Nb-based drug delivery has focused on targeting extracellular biomarkers, such as cytokines, signaling receptors, and cell surface proteins, to enhance treatment effectiveness and minimize side effects.²⁰⁹ However, a large proportion of the signaling that controls the growth and proliferation of tumor cells occurs inside the cells. Therefore, intracellular biomarkers are promising targets for therapeutic interventions.²¹⁰ The difficulty lies in the cell membrane, which serves as a biological barrier to prevent passage of Nbs into the intracellular space. Therefore, researchers are actively developing an effective delivery approach to facilitate intracellular transport of intact or functional therapeutic Nb into the cells. One promising approach is to engineer lentiviral vectors (LVs) to specifically target tumor cells by decorating them with Nbs that recognize tumor surface markers.³² Furthermore, the incorporation of Nbs within LVs has the potential to transport Nbs into cells to interact with intracellular tumor markers, inhibiting cell growth, and proliferation. Alternatively, bacteria have developed sophisticated systems, such as the type III secretion system (T3SS) delivering exogenous proteins into eukaryotic host cells.²¹¹ Researchers have explored the use of non-invasive *E. coli* and *Y. enterocolitica* equipped with T3SS to transport Nbs into mammalian cells.^{212,213} In *E. coli*, T3SS successfully translocated Nbs into HeLa cells by targeting the intracellular components.¹⁶³ Similarly, *Y. enterocolitica*, utilizing T3SS, delivered various functional Nb proteins into HeLa cells, demonstrating efficient, homogeneous, and controllable delivery.²¹³ However, the main challenge lies in the non-specific targeting of bacteria between normal and tumor cells. Further engineering, possibly by attaching tumor-specific Nb to the bacterial surface, is necessary for exclusive tumor-specific Nb delivery. Therefore, modified NDCs can be engineered and delivered inside cells using these

intracellular delivery platforms targeting intracellular biomarkers or proteins. After internalization, the modified NDCs can mediate a dual mechanism of action, with the Nb component specifically blocking intracellular signaling pathways and the conjugated drug exerting its cytotoxic effect.

NDCs Targeting Various Biomarkers for Cancer Therapy

ADCs have shown therapeutic effects with various targets such as nectin 4 in urothelial carcinoma,²¹⁴ HER2 in breast cancer,²¹⁵ and TROP2 in triple-negative breast cancer.²¹⁶ These cancer antigens, while also present to some extent in healthy tissues, are significantly overexpressed in tumor cells. Additionally, CD30 (hematological cancers), CD79b (B-cell lymphoma), B-cell maturation antigen (multiple myeloma), and CD20 (lymphoblastic leukemia) are widely used antigens for ADC treatment.²¹⁷ All these targets hold significant potential for NDC- or NDv-based cancer therapies. For the development of new NDCs and NDvs, biomarkers, such as HER2, EGFR, VEGFR2, MHC-II, MUC1, CD147, TROP2, and VCAM-1, are the most studied antigens for Nb interactions. Table 1 presents various Nb-constructs utilizing some important cancer biomarkers and antigens extensively used for targeted chemotherapeutic drug delivery.

Table 1 Various Nb-Constructs Targeting Key Cancer Biomarkers for Chemotherapeutic Delivery

Nb-Construct	Biomarker	Conjugated Drug	Application	Ref.
2Rb17c-16	HER2	Doxorubicin	Nb-targeted controlled doxorubicin release improves drug delivery.	[218]
11A4-ABD-Lx-AF	HER2	Auristatin F	Sustained tumor remission with a single dose of Nb-conjugated auristatin F.	[189]
Doxo/VHHs-PEG-Lip	HER2	Doxorubicin	Oligoclonal Nb-targeted liposomal doxorubicin effectively treats breast cancer.	[219]
Anti-HER2 AuNP	HER2	AuNP	Nb-conjugated gold nanoparticles enable targeted photothermal therapy for breast and ovarian cancer.	[220]
11A4-CIS-NIT	HER2	Nitroxoline and Cisplatin	Nb-conjugated liposomes loaded with cisplatin and nitroxoline show enhanced efficacy in breast cancer treatment.	[221]
oVHH-Lip- Methotrexate	HER2	Methotrexate	Tetra-specific VHH-PEG-liposome delivers methotrexate selectively to breast cancer cells.	[222]
RPDC-L	HER2/ Integrin $\alpha\beta3$	Doxorubicin	An anti-HER2 Nb functionalized with an integrin receptor $\alpha\beta3$ -binding ligand (RGD) forms a dual-targeted Nb–drug conjugate that enhances doxorubicin delivery to tumors and improves antitumor efficacy.	[223]
Nb-G3 _{MMAE}	HER2	Monomethyl auristatin E (MMAE)	Anti-HER2 Nb conjugated monomethyl auristatin E loaded PEGylated polylysine dendrimer (Nb-G3 _{MMAE}) enhances intracellular uptake and antitumor efficacy in HER2-positive tumor cells.	[224]
ID5-18A12-PS	HER2	IRDye700DX	Nb-photosensitizer conjugate shows significant regression of tumors.	[225]
Anti-EGFR-iRGD + DOX	EGFR	Doxorubicin	The fusion of iRGD peptide and anti-EGFR Nb improves drug delivery in gastric cancer cells.	[226]
EV-DAF-EGaI	EGFR	NA	Nbs enhances the targeting of extracellular vesicles to cancer cells.	[227]
MnPc@Nb-Ftn	EGFR	Manganese phthalocyanine	Nb-ferritin conjugates selectively deliver manganese phthalocyanine to cancer cells, facilitating effective photodynamic cancer therapy.	[228]
Pt-NGCA	EGFR	Cisplatin	Nb-targeted platinum-based anticancer drug delivery to cancer cells.	[229]
Pt@NbP	EGFR	Oxaliplatin	PEGylated Nb enables targeted delivery of Pt (IV) oxaliplatin prodrug to cancer cells.	[54]
Nb-TET56MESS	EGFR	56MESS	Nb-conjugated DNA duplex selectively delivers the Pt-based DNA intercalator 56MESS to cancer cells.	[169]
cNDC@PEG	EGFR	Cisplatin	PEGylated dendritic nanoparticles conjugated to Nb efficiently deliver cisplatin prodrug to cancer cells.	[53]
Nb _(PEG) /DOX/HSA-UCNPs	EGFR	Doxorubicin	Targeted cellular uptake of Nb-conjugated doxorubicin to cancer cells, with imaging by UCNPs.	[158]
EGaI-DOX-PM	EGFR	Doxorubicin	Targeted cancer cell killing using Nb-modified micelles with entrapped doxorubicin.	[230]
DOX-pAcF-ELP _{BC} -EgA1	EGFR	Doxorubicin	Nb-decorated nanoparticles conjugated to doxorubicin effectively target and kill cancer cells.	[231]
MaAbNA-PEG2000- ADM	EGFR and HER2	Adriamycin (DOX)	Targeted delivery of Nb-conjugated doxorubicin to cancer cells.	[232]
7D12-QD-AF	EGFR	Aminoflavone	Nb-targeted micelles loaded with aminoflavone show higher uptake and greater cytotoxicity in triple-negative breast cancer cells.	[233]

(Continued)

Table I (Continued).

Nb-Construct	Biomarker	Conjugated Drug	Application	Ref.
Nb-VEGFR2-IRDye700DX	VEGFR2	IRDye700DX	Nb-targeted photosensitizer enables targeted photodynamic therapy on tumor vasculature.	[234]
VHH7-DM1	MHC-II	Mertansine	Nb-mertansine conjugate targets MHC-II to inhibit metastasis, offering effective B-cell lymphoma treatment.	[141]
DOX-11-1	CD147	Doxorubicin	Selective delivery of doxorubicin by Nb to CD147-positive cancer cells, inhibiting cell proliferation.	[235]
A5-LNP-DOX	CD155	Doxorubicin	A5-LNP-DOX enhances selective cytotoxicity in CD155+ lung adenocarcinoma and suppresses focal adhesion signaling and migratory potential.	[236]
HuNb ^{TROP2} -HSA-MMAE	TROP2	Monomethyl auristatin E	Nb-targeted delivery of monomethyl auristatin E for effective killing of pancreatic cancer cells.	[207]
TNF- α Nb	TNF- α	Paclitaxel	TNF α -specific Nb with paclitaxel inhibits breast cancer metastasis effectively.	[237]
Anti-MucI-VHH	MUCI	Chitosan	Anti-MUCI Nb-conjugated chitosan nanoparticles effectively target and inhibit the proliferation of breast cancer cells.	[238]

Recent Advances in Therapeutic NDCs

Nb as a Targeting Ligand for Tumor-Specific Drug Delivery

Sha et al (2015) designed a novel recombinant protein called anti-EGFR-iRGD that leveraged the advantages of Nbs to deliver anticancer drugs such as doxorubicin (Figure 8a), bevacizumab, and nanoparticles in a targeted manner.²²⁶ This

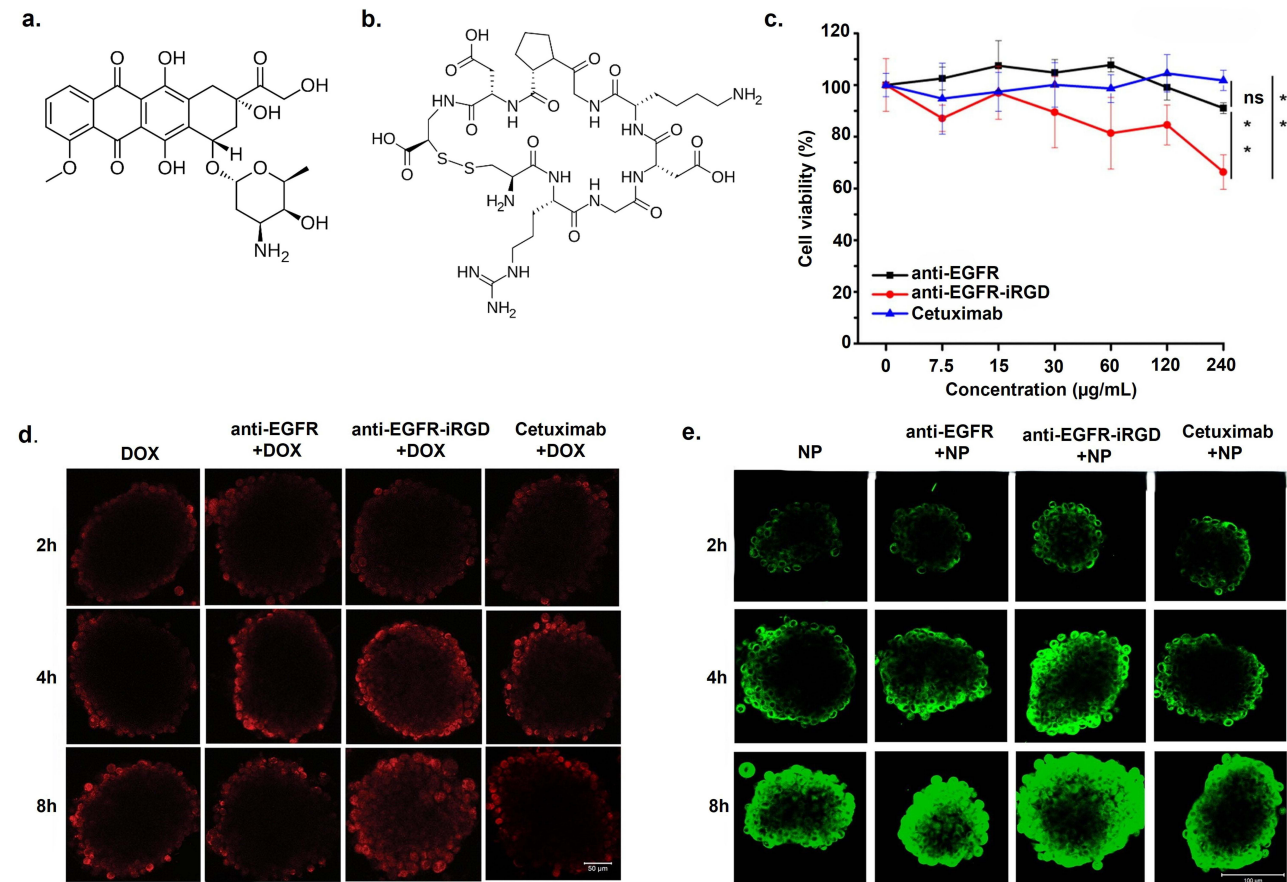


Figure 8 (a) Structure of doxorubicin. (b) Structure of the iRGD peptide. (c) Cell viability of gastric cancer (BGC-823) cells after treatment with anti-EGFR, anti-EGFR-iRGD, or cetuximab at the indicated concentrations for 48 h, as determined by MTT assay. Data are expressed as mean $\pm$ SD (n = 4). *p < 0.05; ns, not significant. (d) Laser scanning confocal microscopy (LSCM) images of BGC-823 multicellular spheroids (MCS) incubated with doxorubicin, doxorubicin combined with anti-EGFR, doxorubicin combined with anti-EGFR-iRGD, and doxorubicin combined with cetuximab for 2, 4, and 8 h, respectively. Scale bar: 50 µm. (e) LSCM images when BGC-823 MCS was incubated with PEG-PCL-coumarin-6-NP, PEG-PCL-coumarin-6-NP combined with anti-EGFR, PEG-PCL-coumarin-6-NP combined with anti-EGFR-iRGD, or PEG-PCL-coumarin-6-NP combined with cetuximab for 2, 4, and 8 h, respectively. Scale bar: 100 µm. Figure reproduced with permission from Elsevier, *Journal of Controlled Release*, 2015.²²⁶

protein is a fusion of anti-EGFR VHH Nb and iRGD (Figure 8b), a tumor-homing peptide with high cell membrane permeability,^{239–243} aimed at targeted therapy for gastric cancer.^{244,245} Gastric cancer is known to overexpress EGFR, which is associated with a poor prognosis for patients.²⁴⁶ The results demonstrated that the constructed protein was able to spread extensively in multicellular spheroids and tumor masses and showed antitumor activity in cell lines, spheroids, and mice. Anti-EGFR-iRGD enhanced the permeability and specificity of anticancer drugs, including doxorubicin, bevacizumab, and nanoparticles, in multicellular spheroids. This construct targets both EGFR and integrin $\alpha\beta3$, a receptor extensively expressed in cancer cells, and that is involved in tumor angiogenesis and metastasis.²⁴⁷ The dual specificity of anti-EGFR-iRGD, which binds to both EGFR and $\alpha\beta3$, highlights its potential as a valuable bispecific entity for targeted cancer therapy. The iRGD motif enhanced the cytotoxicity of anti-EGFR Nb, reducing cell viability (Figure 8c). The results showed that anti-EGFR-iRGD improved the penetration of anticancer drugs, including nanoparticles, and enhanced their targeting specificity and cellular uptake, especially in EGFR-expressing cells (Figure 8d and e). Therefore, this study highlights how penetration-enhancing motifs can substantially improve intratumoral distribution beyond receptor binding alone, although the observed benefits remain contingent on high EGFR and integrin expression and the lack of pharmacokinetic or toxicity benchmarking.

Yuan et al (2024) developed a long-circulating dual-targeted recombinant Nb-doxorubicin conjugate, RPDC-L, comprising an anti-HER2 Nb, integrin receptor $\alpha_v\beta_3$ -binding ligand RGD ligand, albumin-binding domain, and doxorubicin linked via an acid-sensitive PEG-hydrazone (Figure 9a and b).²²³ They also prepared non-long-circulating RPDC-S, lacking the albumin-binding domain (Figure 9b). RPDC-L and RPDC-S showed concentration-dependent cytotoxicity in HeLa cells, with a higher cell-killing effect than free doxorubicin with extended co-incubation to 48 h, especially at low concentrations, due to enhanced Nb and RGD dual-targeting (Figure 9c). Additionally, the cytotoxicity of RPDC-L is stronger than that of RPDC-S, which may be due to its superior cellular internalization because of the presence of the albumin-binding domain. In HeLa xenograft models, both RPDC-L and RPDC-S demonstrated higher tumor growth inhibition compared with free doxorubicin (Figure 9d). This study exemplifies how circulation engineering can be systematically evaluated in the design of NDCs. However, renal clearance, systemic toxicity beyond body weight, and inter-animal variability were not quantitatively resolved, limiting the conclusions on translational robustness.

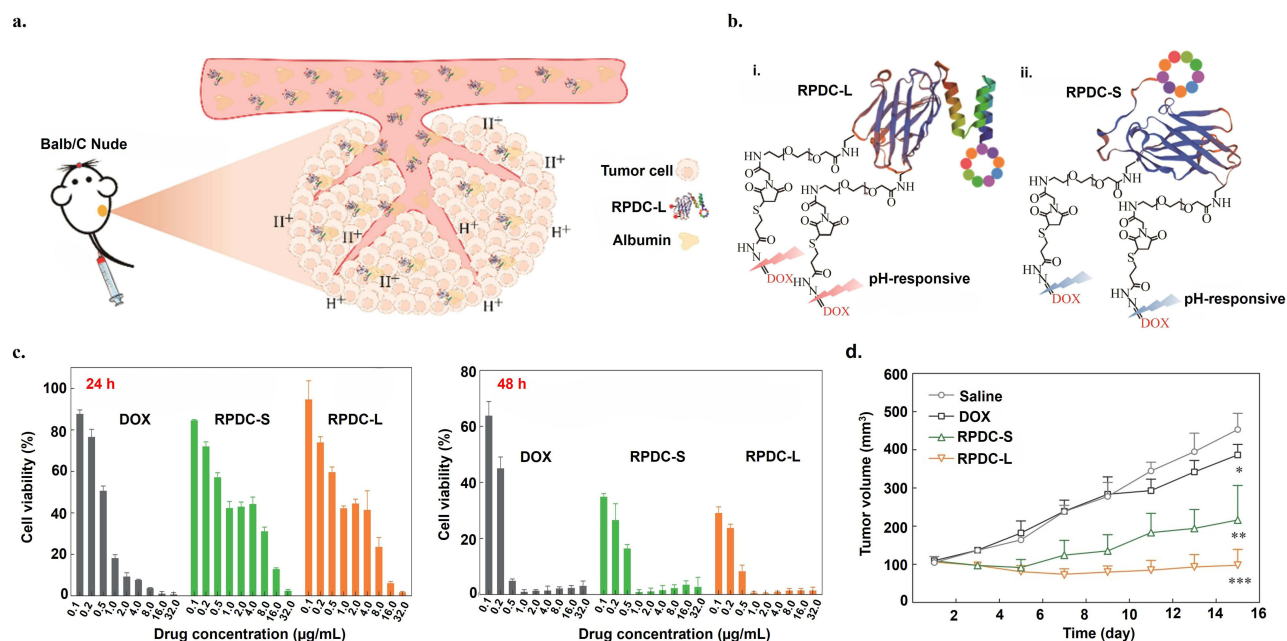


Figure 9 (a) Schematic representation of enhanced tumor targeting and antitumor efficacy of a long-circulating dual-targeted recombinant Nb-doxorubicin conjugate in BALB/C nude mice. (b) (i) The structure of long-circulating dual-targeted recombinant protein drug conjugate (RPDC-L). (ii) The structure of non-long-circulating dual-targeted recombinant protein drug conjugate (RPDC-S). (c) The cell viability of HeLa cells measured by MTT assay after incubation with DOX, RPDC-S, or RPDC-L for 24 h and 48 h. (d) The antitumor efficiency against a HeLa xenograft tumor. The changes of tumor volumes in mice treated with saline, DOX, RPDC-S, or RPDC-L from day 1 to day 15, n=4. Data are presented as mean $\pm$ SD. *p < 0.05, **p < 0.01, ***p < 0.001. Figure has been adapted with permission from *Acta Polymerica Sinica*, 2025.²²³

Similarly, Ji et al (2017) developed a TNF α -specific Nb, Nb-TNF α , that, in combination with paclitaxel, enhances therapeutic efficacy and inhibits breast cancer cell migration and invasion *in vitro*.²³⁷ Notably, unlike conventional NDCs, this approach relies on the modulation of cytokine signaling within the tumor microenvironment rather than direct drug conjugation, providing insight into combination approaches that complement Nb-based targeting rather than functioning as a drug delivery platform. Furthermore, in combination with paclitaxel, a microtubule-stabilizing chemotherapeutic drug, Nb-TNF α significantly enhanced therapeutic efficacy and reduced metastasis in breast cancer by targeting TNF α in the tumor microenvironment. Ding et al (2015) developed a small-sized (29 kDa) MaAbNA consisting of one anti-EGFR1 Nb and two anti-HER2 affibodies, and that exhibited high affinity for EGFR1 and HER2. Then MaAbNA was conjugated with adriamycin (ADM) using a PEG2000 linker, forming an MaAbNA-PEG2000-ADM construct to enhance antitumor efficacy.²³² The conjugate showed improved tumor inhibition and reduced cytotoxicity in cells with low EGFR1 and HER2 expression.

In a related approach, Fang et al (2016) identified VHH7, an Nb with low nanomolar affinity specifically targeting murine class II major histocompatibility complex (MHC-II) expressed on non-Hodgkin B-cell lymphoma cells. They developed a precisely structured NDC (VHH7-DM1) specific for MHC-II by conjugating VHH7 Nb and mertansine (DM1), a potent cytotoxic payload that inhibits microtubule polymerization.¹⁴¹ *In vivo* testing revealed that the VHH7-DM1 conjugate effectively treated highly invasive A20 lymphoma by targeting the spleen, liver, and lymph nodes, leading to significantly smaller tumors than those in the PBS-treated controls. The VHH7-DM1 conjugate selectively killed MHC-II-positive A20 cells with a lower IC₅₀ than MHC-II-negative HeLa cells, suggesting reduced toxicity and higher cell viability in MHC-II-negative HeLa cells or normal cells lacking MHC-II expression. The unconjugated DM1 drug, however, showed similar cytotoxicity across all three cell lines, indicating that the VHH7-DM1 fusion enhances selectivity toward MHC-II-positive cells. *In vivo* imaging demonstrated that Alexa Fluor 647-labeled VHH7 Nbs (VHH-AF647) selectively accumulated in MHC-II-expressing A20 lymphoma cells, reinforcing the importance of receptor internalization and expression specificity in determining the therapeutic index.

Recently, Stenton et al (2018) developed an NDC (2Rb17c-16) with a bifunctional propargyl carbamate linker that enables site-specific protein modification and controlled drug delivery through palladium-assisted bioorthogonal decaging (Figure 10a).²¹⁸ They synthesized a PEGylated anti-HER2 Nb doxorubicin prodrug (2Rb17c-16), where anti-HER2 Nb was

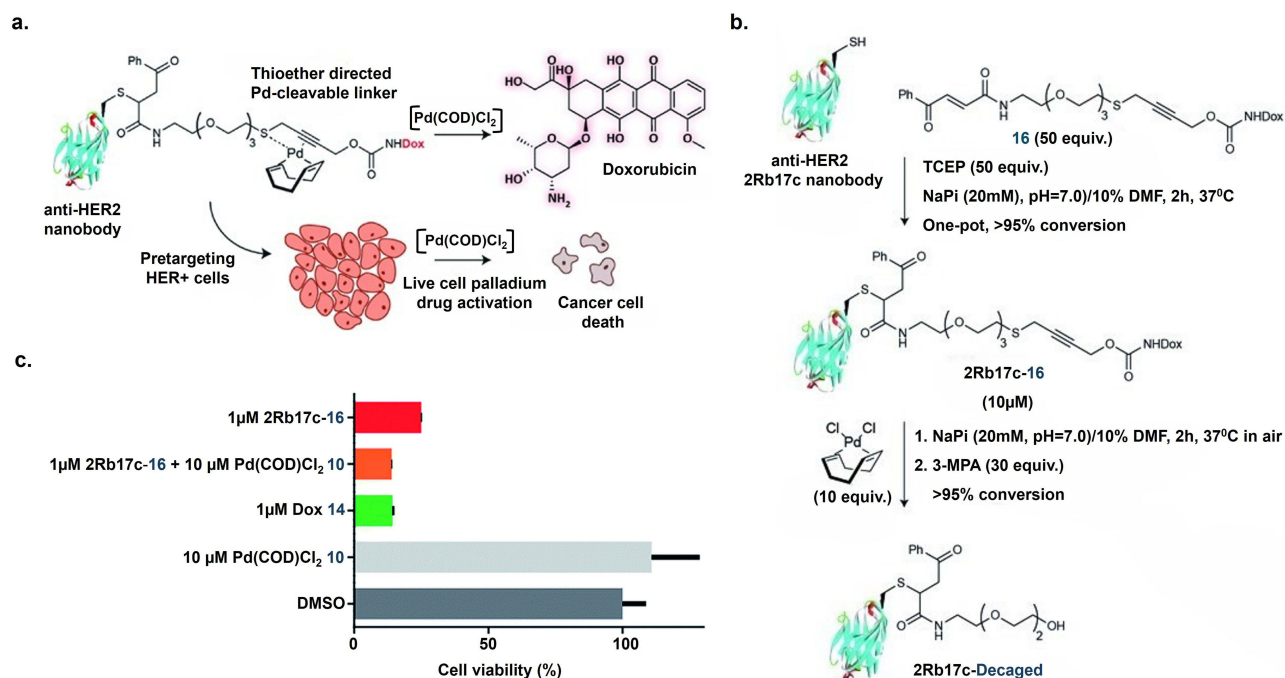


Figure 10 (a) Schematic description of the development of NDC with a bifunctional propargyl carbamate linker that simultaneously allows site-specific protein modification and palladium-mediated bioorthogonal decaging for controlled and targeted drug delivery. (b) Schematic representation of cellular decaging of a bifunctional propargyl carbamate linker by Pd-catalyst along with the synthetic scheme of 2Rb17c-16. (c) Cell viability of HER2+ MCF-7 cells when treated with a combination of 2Rb17c-16 and Pd(COD)Cl₂ 10, only 2Rb17c-16, or free doxorubicin at the same concentration (1 μ M). Figure reproduced from ref.,²¹⁸ Chemical Science, with permission from the Royal Society of Chemistry, 2018.

conjugated to one side of the propargyl carbamate linker, and the anticancer drug doxorubicin was conjugated to the other side (Figure 10b). The cell viability study showed a two-fold improvement in anticancer efficiency for NDC 2Rb17c–16 in the presence of Pd(COD)Cl₂ 10 compared to using NDC 2Rb17c–16 alone at the same concentration (1 μM) (Figure 10c). The result suggests that constructing bifunctional propargyl carbamate conjugates with Nb could lead to efficient palladium decaging for targeted drug release, exhibiting cytotoxicity similar to that of free doxorubicin in cancer cells and may hold promise for controlled metal-mediated small-molecule activation in cancer therapy. However, in this study, despite precise spatiotemporal control over drug activation, the reliance on an external metal catalyst positions it primarily as a chemical proof-of-concept rather than a clinically mature delivery strategy for drug delivery.

A study conducted by Kooijmans et al (2016) explores the feasibility of extracellular vesicles (EVs) as drug delivery systems by enhancing their targeting capabilities through attachment to Nbs.²²⁷ EVs, which are lipid bilayer-surrounded vesicles released by various cell types, are composed of proteins and nucleic acids.^{248–250} The researchers proposed a method for efficiently attaching an anti-EGFR Nb to EVs, specifically EGa1 Nbs linked to glycosylphosphatidylinositol (GPI) anchors.^{227,251} This study demonstrated the successful display of GPI-linked EGa1 Nbs on EV surfaces, enhancing their binding to tumor cells, particularly those expressing EGFR, without altering the general EV characteristics, such as shape, size distribution, and expression of protein markers. This novel approach may improve the therapeutic potential of EVs in drug delivery systems, as it establishes the targeting feasibility and modularity of Nb decoration on biological carriers at the preclinical level.

Li et al (2022) developed an Nb, VHH-11-1, against CD147 using the phage display technique and successfully conjugated this Nb with doxorubicin (DOX) to create DOX-11-1 (Figure 11a).²³⁵ CD147 is a transmembrane

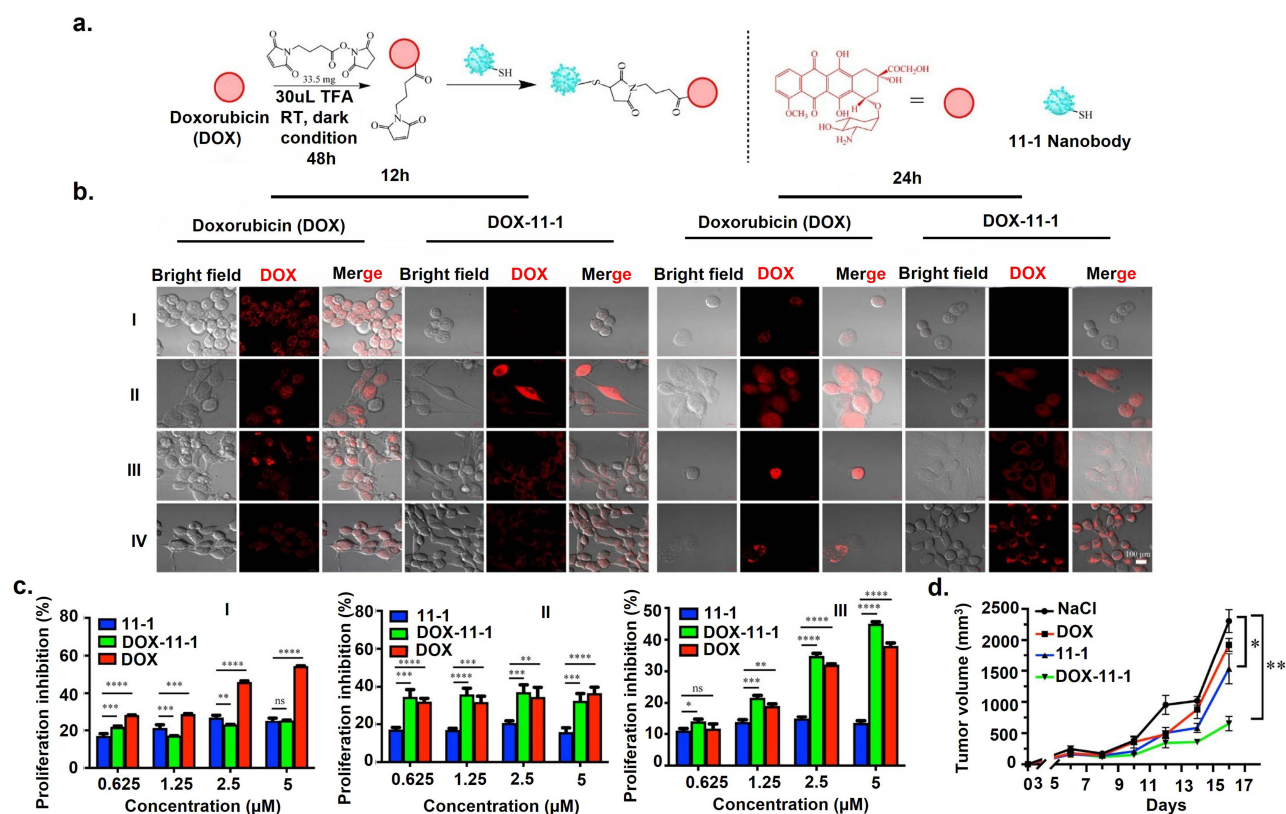


Figure 11 (a) The scheme of DOX-11-1 synthesis. (b) The distributions of free DOX and DOX-11-1 in different cell lines were observed by LSCM: (I) 293T, (II) U87, (III) HeLa, and (IV) 4T1 cells were treated with free DOX or DOX-11-1. Scale bar: 100 μm. (c) Antitumor effects of Nb 11-1, free DOX, and DOX-11-1 on different cells. Proliferation inhibition rates for 293T (I), U87 (II), and 4T1 (III) cells at 24 h after incubation with Nb 11-1, free DOX, and DOX-11-1, as determined by MTT assay. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; ns, not significant. (d) The tumor volume was significantly inhibited in DOX-11-1 compared with other groups at 16 days. Data are reported as mean ± SEM. Number of replicates (n = 5). *p < 0.05, **p < 0.01. Figure has been adapted from *Frontiers*, 2022.²³⁵

glycoprotein that promotes breast cancer progression.²³⁵ In vitro assays showed that DOX-11-1 was localized in the membrane and cytoplasm of HeLa (a human cervical cancer cell line) and 4T1 (a breast cancer cell line) cells, which highly express CD147, but not in 293T (a human embryonic kidney cell line) cells with low CD147 expression (Figure 11b). DOX-11-1 significantly inhibited U87 (a human glioblastoma cell line) and 4T1 cell proliferation, with higher inhibition in 4T1 cells, which highly express CD147, than in U87 cells with moderate CD147 expression. Free doxorubicin exhibited comparatively higher toxicity in cells with low CD147 expression or normal cells. DOX-11-1 exhibited little cytotoxicity in CD147-low 293T cells, even at higher concentrations, indicating low toxicity to CD147-negative or normal cells (Figure 11c). Conjugation of VHH-11-1 with DOX resulted in synergistic inhibition of tumor growth in mice bearing the 4T1 cell line (Figure 11d). This suggests that Nb-conjugated DOX-11-1 selectively targets and kills cancer cells with high CD147 expression. This therapeutic window is strongly dependent on CD147 over-expression, underscoring the importance of antigen density in determining NDC selectivity.

Xu et al (2023) developed a novel NDC for treating TROP2 (trophoblast cell surface antigen 2)-positive pancreatic cancer. The NDC, named HuNb_{TROP2}-HSA-MMAE, consists of TROP2-specific Nb conjugated to HSA and monomethyl auristatin E (MMAE) via a lysosomally cleavable linker (Figure 12a).²⁰⁷ In vitro studies demonstrated the internalization and potent cytotoxicity of this NDC against TROP2-positive pancreatic cancer cells (Figure 12b and c). Moreover, in a mouse xenograft model, this NDC exhibited significant antitumor effects and effectively eradicated tumors at high doses (Figure 12d). These findings highlight the potential of NDCs as a promising personalized treatment option for pancreatic cancer. However, the use of a membrane-permeable payload, such as MMAE, underscores the need for comprehensive toxicity profiling and evaluation of antigen-negative or low-TROP2 models to better assess potential failure rates.

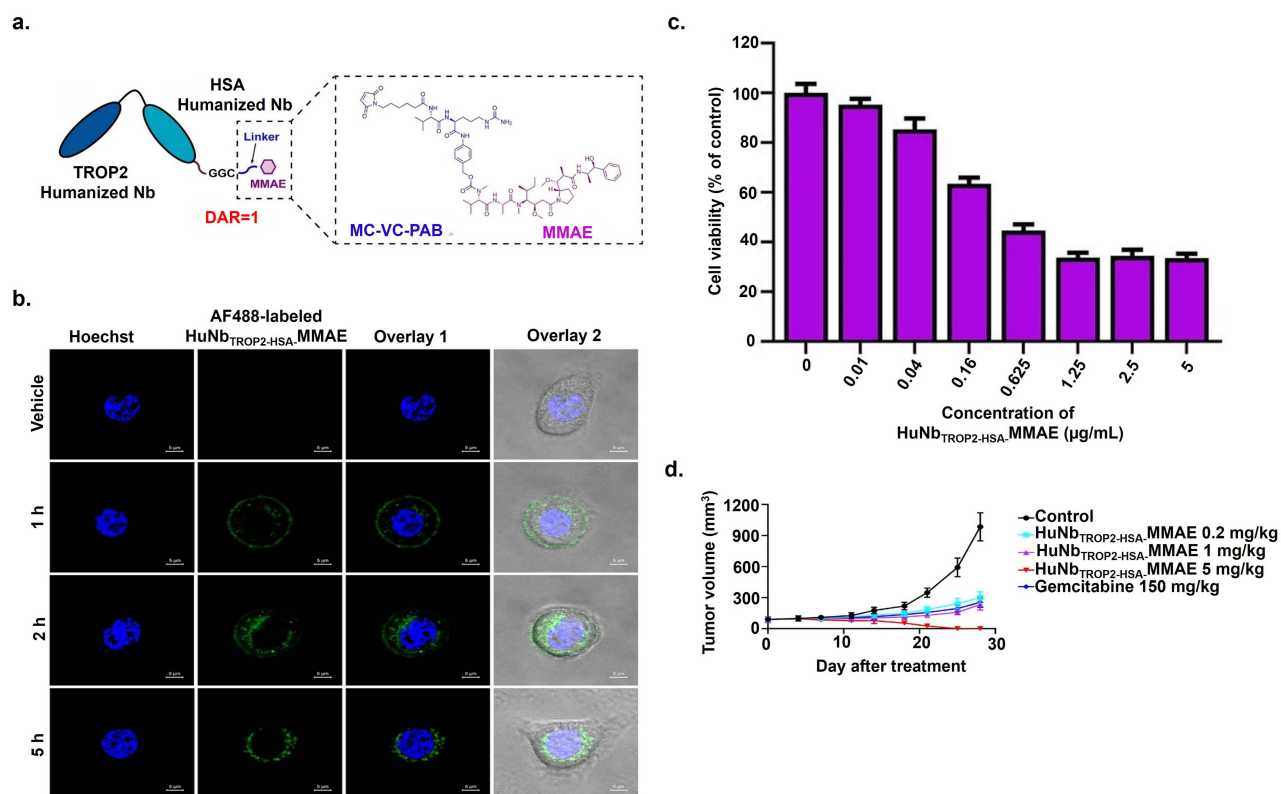


Figure 12 (a) Schematic structure of HuNb_{TROP2}-HSA-MMAE. (b) Fluorescence images of HuNb_{TROP2}-HSA-MMAE internalization into BxPC-3 (a human pancreatic cancer cell line) cells. Blue color indicates nuclei stained with Hoechst 33342, and green color shows HuNb_{TROP2}-HSA-MMAE labeled with Alexa Fluor™ 488. (c) The cytotoxic effect of HuNb_{TROP2}-HSA-MMAE treatment for 2 days on BxPC-3 cells was evaluated by MTT assay. (d) In vivo antitumor activity of HuNb_{TROP2}-HSA-MMAE was evaluated by measuring tumor volume over 4 weeks with various doses of the treatment. Figure reproduced with permission from *Journal of Nanobiotechnology*, 2023.²⁰⁷

Liu et al (2020) developed a novel Nb-ferritin platform for targeted drug delivery to EGFR-positive cancer cells.²²⁸ They loaded a photodynamic reagent, manganese phthalocyanine (MnPc), into a ferritin cavity, which serves as a protective environment for the drug (Figure 13a). Ferritin is a stable and biocompatible protein with a spherical structure that self-assembles reversibly at neutral pH.^{252–256} It can deliver drugs via transferrin receptor-1-mediated endocytosis^{257,258} with surface or genetic modifications such as Nb conjugation, enhancing its tumor-targeting abilities.²⁵⁹ The resulting MnPc@Nb-Ftn conjugate demonstrated efficient internalization into EGFR-positive A431 cancer cells, with no effect on EGFR-negative cells (Figure 13b).²²⁸ Upon laser irradiation at 730 nm, the MnPc@Nb-Ftn conjugate selectively induced the production of reactive oxygen species (ROS) in A431 cells, leading to cell death (Figure 13c). Nb-ferritin showed enhanced cytotoxicity in A431 cells (Figure 13d). This approach provides an effective targeted drug delivery system for cancer therapy. However, as with other photodynamic treatments, therapeutic efficacy is not determined solely by targeting efficiency or cellular internalization but is also constrained by physical factors, such as light penetration depth and tumor accessibility.^{260–262} As a result, although payload delivery is efficient, its clinical application is likely to be restricted to superficial or optically accessible tumors. This underscores the necessity of aligning the payload activation mechanism with the tumor's anatomical characteristics and the treatment context.

In another study, Deken et al (2020) demonstrated that HER2 was targeted by the Nb-photosensitizer conjugates 1D5-PS and 1D5-18A12-PS, in which 1D5 and 1D5-18A12 Nbs were conjugated to IRDye700DX, a photosensitizer (PS).²²⁵ These conjugates effectively induced significant tumor regression in trastuzumab-resistant HER2+ breast cancer after a single treatment session, highlighting the role of Nb specificity in achieving improved spatial selectivity in photodynamic therapy, particularly in tumors with limited responsiveness to conventional treatments. Likewise, Mashayekhi et al (2020) developed Nb-targeted photodynamic therapy (PDT) in vitro using an Nb-VEGFR2-IRDye700DX conjugate to target

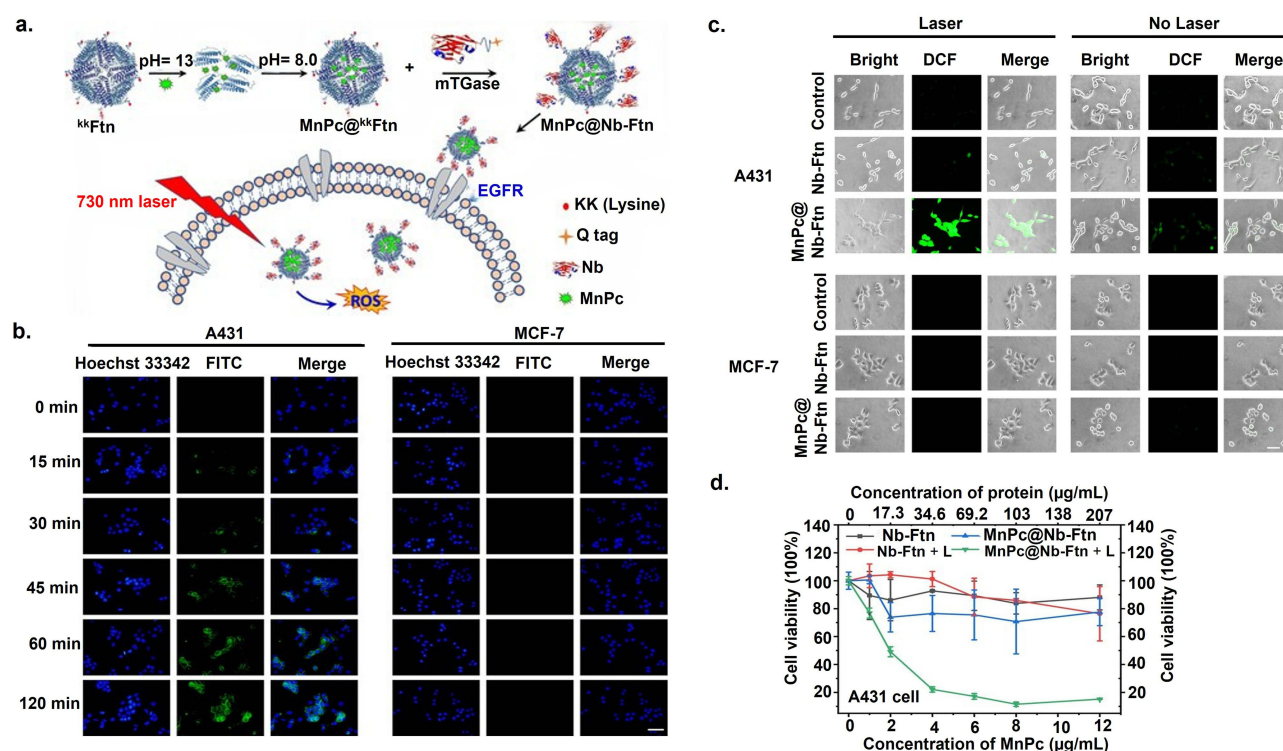


Figure 13 (a) Schematic illustration of the preparation and targeted delivery of photosensitizers by Nb-Ftn conjugate and the photoinduced ROS generation in EGFR-positive cancer cells. (b) Enhanced cellular uptake of Nb-Ftn conjugate by EGFR-positive A431 cells and with no detectable signal of the conjugate in EGFR-negative MCF-7 cells observed by fluorescence microscopy, indicating specific targeting of A431 cells. FITC-labeled Nb-Ftn (green) and Hoechst 33342-stained nuclei (blue) were used. Scale bar: 40 μm. (c) Fluorescence images of A431 and MCF-7 cells showing ROS generation using an assay kit containing a non-fluorescent probe DCFH-DA. Upon 730 nm laser irradiation, MnPc@Nb-Ftn selectively induced ROS in EGFR-positive A431 cells, where DCFH-DA was converted to fluorescent DCF, resulting in markedly higher green fluorescence and cell death compared to EGFR-negative MCF-7 cells. Scale bar: 40 μm. (d) MTT cell viability assay showing the percentage of A431 cell viability at different concentrations of MnPc@Nb-Ftn with or without laser irradiation, where “L” denotes laser irradiation. Figure reproduced with permission from *Chemistry – A European Journal*, 2020.²²⁸

VEGFR2 on tumor vasculature.²³⁴ When combined with an EGFR-targeted Nb, this approach showed improved efficacy in co-cultures of endothelial and cancer cells.

Xenaki et al (2021) developed HER2-targeted NDCs by fusing 11A4 Nb with an albumin-binding domain (ABD) to enhance solid tumor targeting and extend circulation half-life.¹⁸⁹ They fused HER2-targeted Nb 11A4 and irrelevant Nb R2 with ABD at their C-terminus, enabling effective binding and internalization into HER2-expressing cells without affecting their distribution in 3D spheroids. Fusion with ABD increased the serum half-life by 14.8-fold and prolonged the accumulation of 11A4-ABD in HER2-expressing BT-474 tumor xenografts, as confirmed by imaging, while preserving intratumoral distribution and reducing kidney retention. Furthermore, they showed that an NDC (11A4-ABD-AF) consisting of a HER2-targeted, ABD-fused Nb conjugated with a microtubule inhibitor, auristatin F (AF), caused significant tumor remission in HER2-positive NCI-N87 xenograft mice after a single dose. Overall, this Nb construct represents a comprehensive preclinical evaluation within current NDC platforms, highlighting the potential of Nbs as efficient targeting ligands for cancer therapy by enhancing circulation time and tumor accumulation.

Intracellular Tumor Targeting Using Nbs Conjugated with Platinum Anticancer Drugs

Platinum-based drugs, including cisplatin, carboplatin, and oxaliplatin, are the primary chemotherapeutic agents for various solid cancers.²⁶³ These drugs primarily exert their antitumor effects by interacting with DNA via both covalent and non-covalent means. However, non-specific drug binding and drug resistance can lead to dose-dependent side effects, such as nephrotoxicity and neurotoxicity, limiting the clinical application of these drugs. Therefore, there is a critical need for targeted administration of platinum drugs to ensure safer and more efficient treatments. Attaching platinum drugs to specific tumor-targeting moieties has the potential to enhance drug accumulation within tumor cells, improving overall drug effectiveness.

Huang et al (2019) utilized Nbs to design a versatile drug delivery system with diagnostic capabilities. They developed a biparatopic NDC, termed NGC, comprising an anti-EGFR Nb, a gadolinium-binding domain for MRI, and a C3-tag for drug conjugation.²²⁹ A maleimide-functionalized Pt(IV) cisplatin prodrug (Mal-Pt) was conjugated to the C3-tag of NGC via a click reaction to form Pt-NGC for targeted drug delivery to EGFR-positive A431 cancer cells (Figure 14a). They also developed Pt-NGCA, a variant of Pt-NGC with anti-albumin Nbs at the C-terminus, for prolonged in vivo circulation. FITC-labeled NGCs were analyzed using LSCM, showing binding to EGFR on A431 cells but not on EGFR-negative A375 cells (Figure 14b). Comparative biodistribution analysis using ICP-MS analysis of Balb/c nu/nu mice bearing A431 and A375 cancer cells showed that Pt-conjugated NGC/NGCA exhibited higher blood retention levels than freely administered cisplatin (Figure 14c). ICP-MS analysis in mice with A431 tumors showed that the EGFR-targeting of NGC enhances platinum accumulation at the tumor site. Furthermore, Pt-NGCA increases tumor specificity and reduces bioaccumulation in other organs, mitigating cytotoxic side effects compared to Pt-NGC (Figure 14d). In vivo studies in Balb/c nude mice showed that cisplatin inhibited the growth of both A375 and A431 tumors, but Pt-NGC and Pt-NGCA specifically targeted A431 tumors (Figure 14e). Fundamentally, this study provides a comprehensive preclinical evaluation and supports the feasibility of innovative Nb-based theranostic approaches by integrating target specificity, biodistribution, circulation engineering, pharmacokinetics, and imaging. In another study, Li et al (2021) described an EGFR-targeted Nb engineered with a C3-tag involving the Cys-Cys-Cys sequence for oxaliplatin prodrug Pt(IV) conjugation and a Q-tag glutamine motif for PEGylation, resulting in Pt@NbP to enhance tumor targeting and prolong oxaliplatin circulation (Figure 15a).⁵⁴ The results showed enhanced cellular uptake of Pt@NbP into EGFR-positive A431 cells due to EGFR receptor targeting by the anti-EGFR Nb (Figure 15b). Therefore, in vivo efficacy of the oxaliplatin-Nb conjugate also significantly increased (Figure 15c). While PEGylation and albumin binding significantly improved circulation time and tumor accumulation, such molecular enlargement may also influence renal clearance thresholds, tumor penetration, and immunogenicity, aspects that were not thoroughly investigated in these studies.

Therefore, platinum-based NDCs have improved selectivity and reduced off-target toxicity in preclinical studies; however, clinical translation is constrained by variability in intracellular processing and limited pharmacokinetic and safety data.

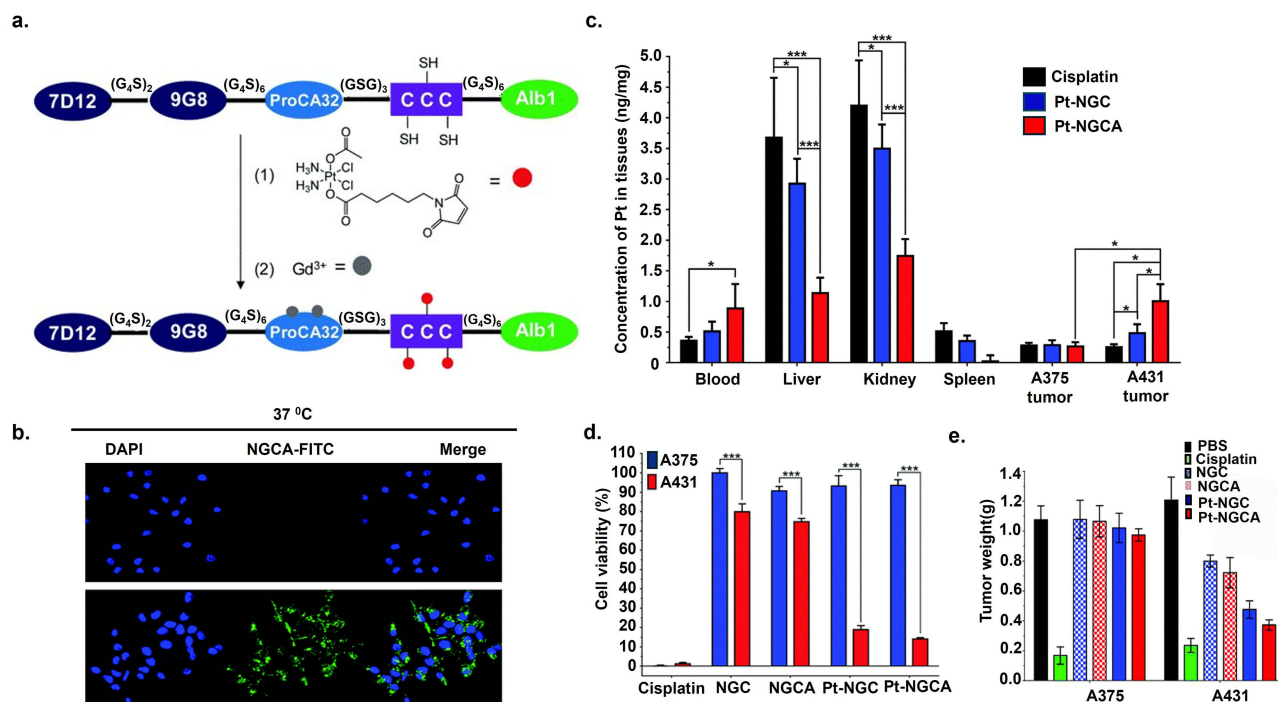


Figure 14 (a) The schematic presentation of conjugation of the Mal-Pt prodrug and the loading of Gd^{3+} into NGCA. $(G_4S)_n$ are linker sequences between the protein domains. (b) Cellular binding and internalization of FITC-labeled NGCA to A375 and A431 cells were measured by LSCM after 2 h of incubation at $37^\circ C$. The nuclei were stained with DAPI (blue). (c) The biodistribution of platinum measured by ICP-MS in Balb/c nude mice simultaneously bearing A375 and A431 tumors. Data are expressed as mean $\pm$ SD ($n = 3$). * $p < 0.05$, *** $p < 0.001$. (d) Comparison of in vitro cytotoxicity of Nb-Pt-drug constructs with the Nb-construct and free drug in A375 and A431 cell lines. Data are expressed as mean $\pm$ SD ($n = 3$). *** $p < 0.001$. (e) The tumor weights of A375 and A431 tumor-bearing mice treated with different agents by i.v. injection. Figure reproduced with permission from Royal Society of Chemistry, *Chem. Commun.* 2019.²²⁹

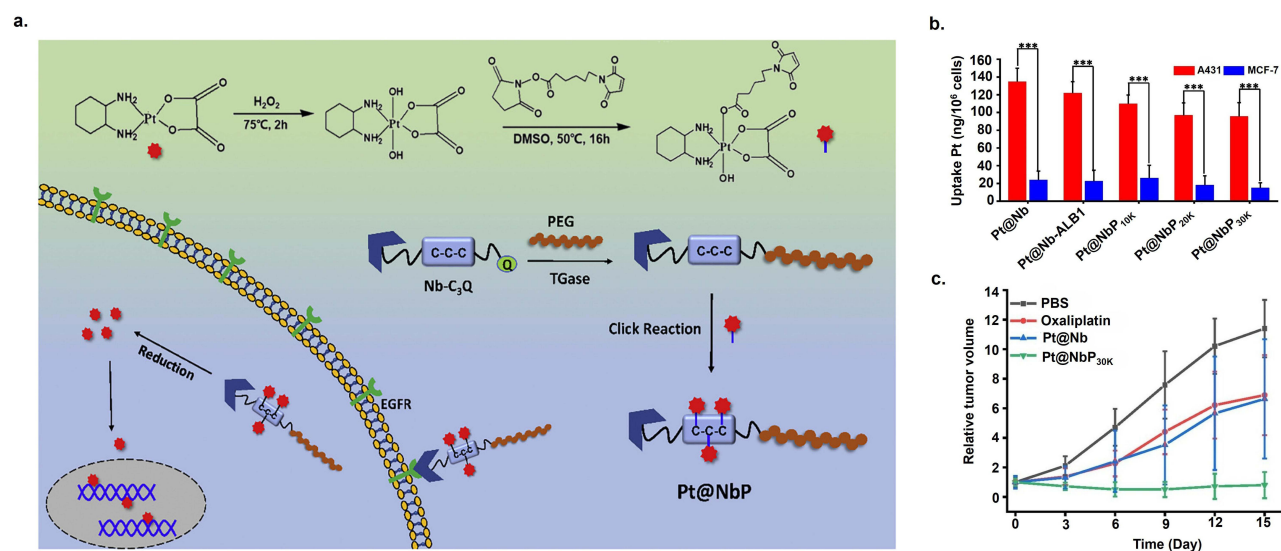


Figure 15 (a) Schematic representation of conjugation of oxaliplatin with PEGylated-Nb for enhancing tumor targeting and prolonging circulation. (b) Accumulation of Pt@Nb, Pt@Nb consisting of an anti-albumin Nb, and various PEGylated Pt@Nb conjugates in A431 and MCF-7 cells. *** $p < 0.001$. (c) Relative tumor volumes of mice treated with PBS, oxaliplatin, Pt@Nb, or PEGylated Pt@NbP_{30K}. Figure reproduced with permission from Elsevier, *Journal of Inorganic Biochemistry*, 2021.⁵⁴

Unlocking Precision in Cancer Therapy: The Synergy of Nbs and Nanoparticles in Drug Delivery

As discussed earlier in this review, owing to their small size, Nbs exhibit a reduced capacity for conjugation to multiple chemodrug molecules, potentially resulting in a decrease in the efficacy of NDCs as anticancer treatments. Moreover, new strategies should be developed for drug cleavage after reaching cellular targets; this is an important concern in Nb-mediated drug delivery systems, specifically in NDCs. In this context, NDv has the potential to overcome the challenges associated with conventional chemotherapy and to demonstrate superior drug loading efficiency compared to traditional ADCs or NDCs. The encapsulation efficiency of nanoscale drug delivery vehicles such as liposomes can improve the delivery of chemotherapeutic drugs, reduce adverse side effects, and enhance treatment effectiveness.²⁶⁴ NDvs enhance targeted drug accumulation by conjugating Nbs to the nanoscale drug delivery vehicle surface, offering the potential for treatment of cancer.^{158,169,219,230,238} This approach leverages the small size, stability, and controlled orientation of Nbs to improve specificity. Tumor cells, which often overexpress cancer antigens such as HER-2,²⁶⁵ EGFR,²⁶⁶ B7-H6,^{267,268} folic acid,²⁶⁹ MUC-1,²⁷⁰ VEGF,²⁷¹ and TGF- β ²⁷² are suitable targets for interaction with Nb-functionalized NDv. It has a higher drug-loading capacity than NDCs, primarily because of the presence of free volume within nanoscale drug delivery vehicles that can accommodate a diverse range of drug molecules, including both small and large compounds, as well as hydrophilic and hydrophobic drugs.⁴⁶ The release of drugs from the NDv can be controlled by incorporating responsiveness to external or internal stimuli using biocompatible materials that can undergo protonation, conformational changes, or hydrolytic cleavage, depending on NDv composition.⁴⁷ The development of stimulus-responsive NDv is influenced by tumor microenvironments such as enzymes, pH, or reducing agents.⁴⁷ These NDvs can also be designed to be controllable by external stimuli such as light, ultrasound, heat, electric, or magnetic fields. The efficacy of NDv applications relies on the selective affinity of the attached Nbs for cancer antigens and their ability to reach and penetrate tumors via the EPR effect (Figure 16).²⁷³ NDvs must extravasate into tumors and remain localized. Positive interstitial pressure in tumors may hinder large NDvs, directing diffusion through normal tissue. Smaller NDvs penetrate deeper through fenestrated tumor vessels, enhancing therapeutic potential.

NDv for Platinum Anticancer Drug Delivery

Nb-Modified Liposomes: Nanoscale Marvels for Targeted Delivery of Platinum Anticancer Drugs

Liposomes, ranging from nano- to micrometer scale and consisting of a spherical vesicle with an aqueous core enveloped by at least one lipid bilayer, are highly effective and versatile chemodrug carriers for targeted cancer therapy.^{274,275} Sayed-Tabatabaei et al (2022) improved metastatic breast cancer treatment using targeted liposomes containing nitroxoline (NIT) and cisplatin (CIS), leveraging camelid-derived Nb 11A4 for selective HER2 targeting (Figure 17a).²²¹ Nitroxoline, an anticancer antibiotic, inhibits cell migration by targeting cathepsin B,²⁷⁶ whereas cisplatin is a potent chemotherapeutic agent that cross-links DNA to prevent cancer cell division (Figure 17b).²⁷⁷ Nb-targeted liposomes showed increased cellular uptake and toxicity, specifically in TUBO (HER2+) cells, but not in MDA-MB-213 (HER2-) cells (Figure 17c and d). Targeted liposomes loaded with CIS-NIT showed enhanced tumor suppression compared to the control group, non-targeted liposomes, and free drugs (Figure 17e).

DNA-Nanocarrier Conjugated with Nb as a Drug Delivery System for Pt-Based Drugs

Customized DNA sequences can form nanoscale folded structures through domain hybridization. They can carry drugs and be programmed to trigger cellular responses within the biological microenvironment.^{278,279} These biocompatible polyhedral nanostructures with high drug-loading capacity enable targeted delivery and controlled release of therapeutics, gene therapy elements, imaging probes, and proteins. It has been proposed that the platinum-based aromatic DNA intercalator 56MESS can be efficiently loaded and delivered using this versatile DNA nanoplatform. Wu et al (2019) developed a DNA nanocarrier (Nb-TET56MESS) using a DNA double-bundle tetrahedron (TET) conjugated with anti-EGFR Nb to deliver the potent water-soluble platinum-based DNA intercalator, 56MESS (Figure 18a).¹⁶⁹ In vitro analysis revealed that TET carrying both chemodrug and Nb exhibited low cell viability in the EGFR+ A431 cell line compared to those treated with Nb or chemodrug alone, establishing NDv efficiency (Figure 18b). Nb-TET-56MESS DNA nanocarriers showed selective uptake, with strong internalization in EGFR-overexpressing A431 cells but no

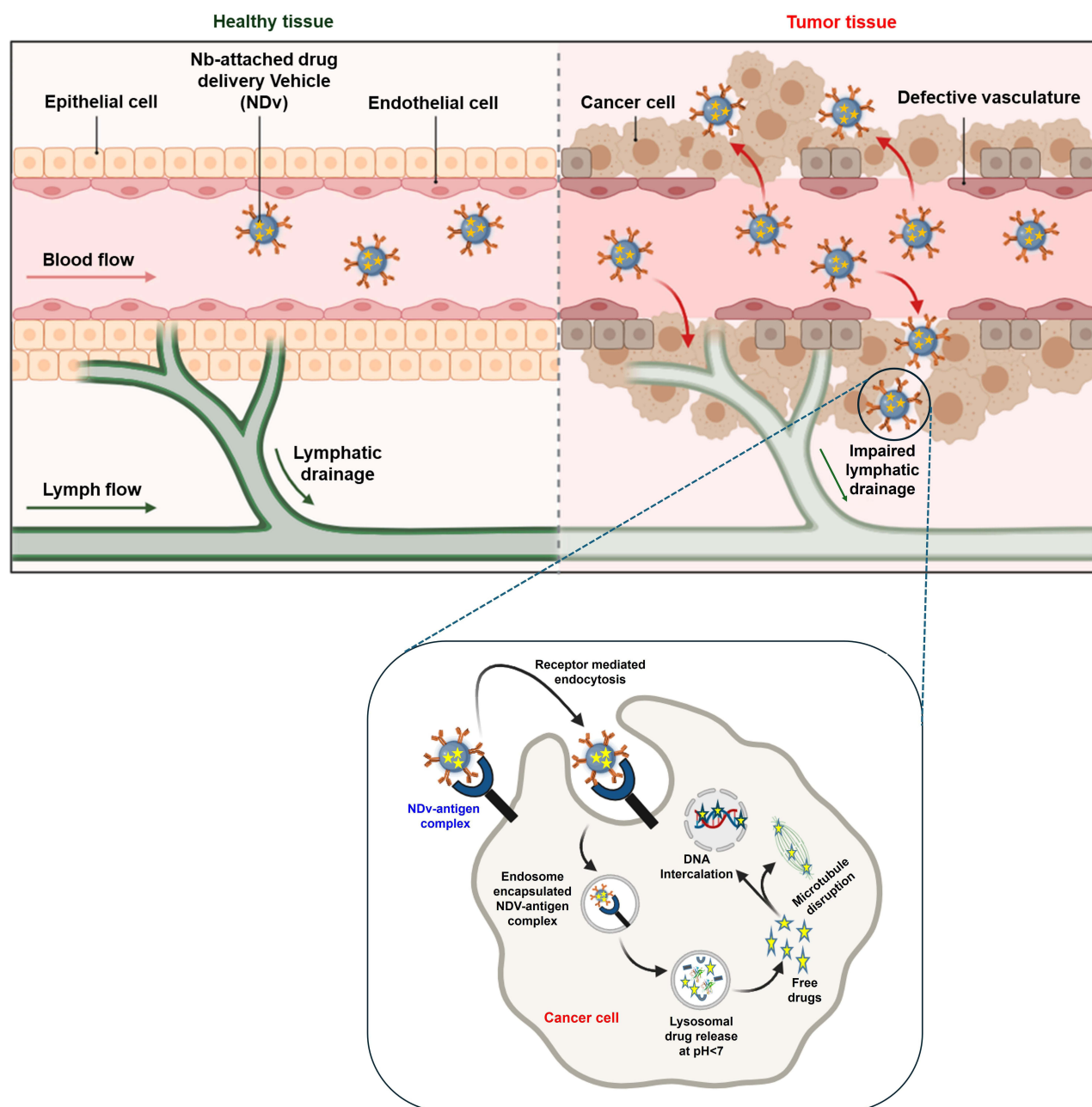


Figure 16 Therapeutic drugs are delivered to tumor cells using nanoparticles conjugated with Nbs, forming NDV. These NDVs are injected into the bloodstream and cross the poorly formed, leaky blood vessels of the tumor owing to the EPR effect. After escaping the blood vessels, the NDV must diffuse through the dense extracellular matrix to reach and attach to the tumor cells via Nb targeting. Once attached to the receptor, they are internalized via receptor-mediated endocytosis and processed within the endosomes and lysosomes, releasing chemotherapeutic drugs into the cytoplasm. These drugs inhibit cancer cell growth by disrupting DNA or cellular functions and triggering apoptosis. NDV releases drugs at the tumor site in response to internal or external stimuli, depending on the materials used.

detectable fluorescence in low EGFR-expressing MCF-7 and A2780 cells, confirming Nb-mediated cell selectivity (Figure 18c). A431-tumor-bearing mice treated with the compounds revealed that Nb-TET-56MESS significantly inhibited tumor growth in EGFR+ A431 cells in vivo (Figure 18d). This highlights the potential of DNA nanocarrier system by conjugating an anti-EGFR Nb to DNA on TET with a chemotherapeutic drug, achieving targeted and highly effective tumor inhibition of EGFR+ cells in vitro and in vivo without any side effects. However, its pharmacokinetics, payload release kinetics, and comparative performance with other nanosystems remain to be established.

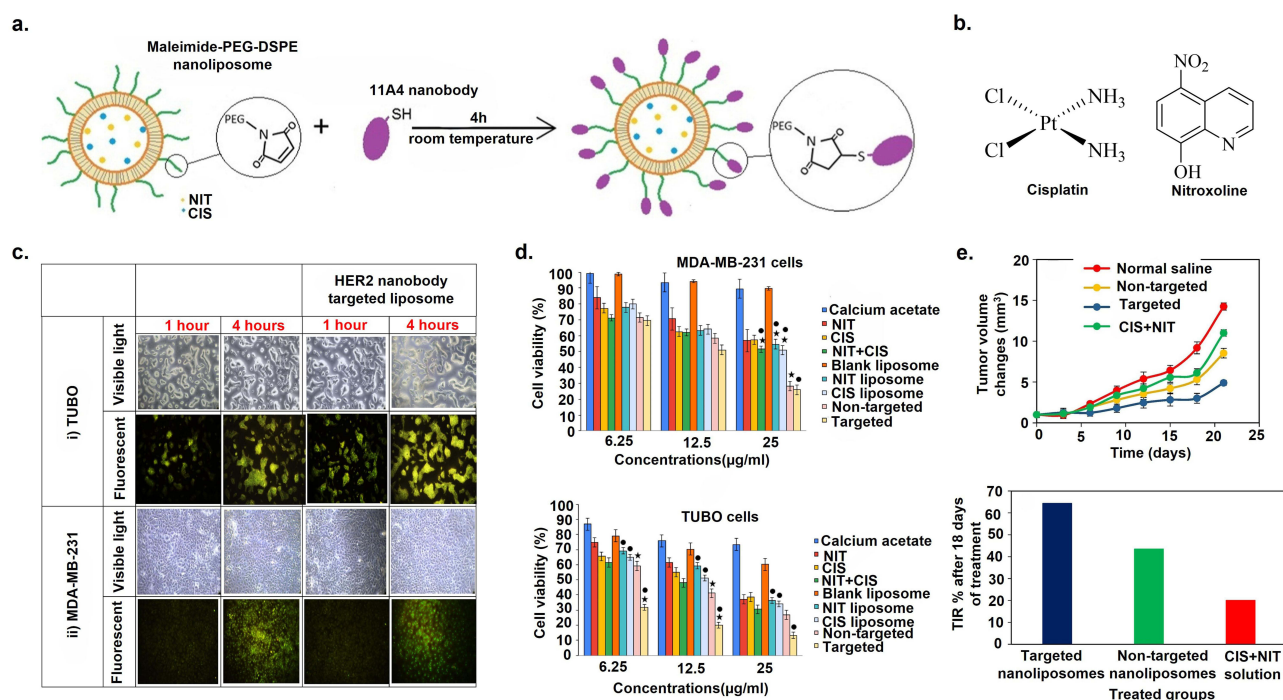


Figure 17 (a) Schematic representation of liposomes camouflaged by 11A4-Nb for co-delivery of cisplatin and nitroxoline in breast cancer tumors. (b) Structures of cisplatin and nitroxoline. (c) Fluorescent emission percentage of (i) TUBO cells and (ii) MDA-MB-231 cells after 1 and 4 h of incubation at 37 °C with non-targeted liposomes and Nb-targeted liposomes. (d) In vitro cytotoxicity of studied treatments on MDA-MB-231 cells and TUBO cells after 24 h of drug exposure. Data are presented as mean $\pm$ SD (n = 3). For each concentration, groups marked with the same symbol ($\bullet$ or $\star$) show statistically significant differences among those groups ($p < 0.05$). Statistical comparisons were performed within the same concentration only. (e) Antitumor efficiency including tumor volume changes and tumor inhibition ratio percentage (TIR%) of targeted and non-targeted liposomes loaded with cisplatin and nitroxoline in comparison to the solution of the two drugs in TUBO cell tumor-induced BALB/c mice. Figure reproduced with permission from Elsevier, *Journal of Inorganic Biochemistry*, 2022.²²¹

Dendrimer-Based Nanoparticles Conjugated with Nb Along with Platinum-Anticancer Drugs

Dendrimers are spherical polymeric nanoparticles composed of successive layers of branched monomers with radial symmetry. They are promising for targeted cancer therapy owing to their precise synthesis, excellent solubility, biocompatibility, multivalent surface, and stability.^{280,281} Recently, Wu et al (2020) developed a strategy using PEGylated dendritic nanoparticles conjugated with anti-EGFR Nb (7D12) to deliver a tetravalent Pt(IV) prodrug.⁵³ This approach demonstrated superior anticancer effect owing to the site-specific conjugation and clustering of the Pt(IV) prodrug (Figure 19a). Cell selectivity of the platinum drugs was demonstrated by the efficient internalization of FITC-labeled singlet NDC, PEGylated NDCs (NDC@PEG), and PEGylated clustered NDCs (cNDC@PEG) in EGFR-positive A431 cells, with no detectable fluorescence in EGFR-negative A2780 cells (Figure 19b). In vivo studies showed that cNDC@PEG had a longer half-life and higher tumor drug accumulation compared to NDC@PEG and free cisplatin due to its larger size, leading to increased blood retention and reduced renal clearance (Figure 19c). Nude mice with A431 (EGFR+) tumor xenografts showed significantly enhanced antitumor efficacy of cNDC@PEG compared to NDC@PEG and free cisplatin in vivo (Figure 19d). These results indicate the potential of Nb-conjugated dendrimer-based drug delivery systems for targeted and effective cancer treatment. While this study demonstrated enhanced in vitro and in vivo efficacy, the increased size of the PEGylated dendrimer-based NDVs may also limit deep tumor penetration and alter immune recognition. These factors remain important considerations for clinical translation and underscore the intrinsic design trade-offs in nanoparticle engineering.

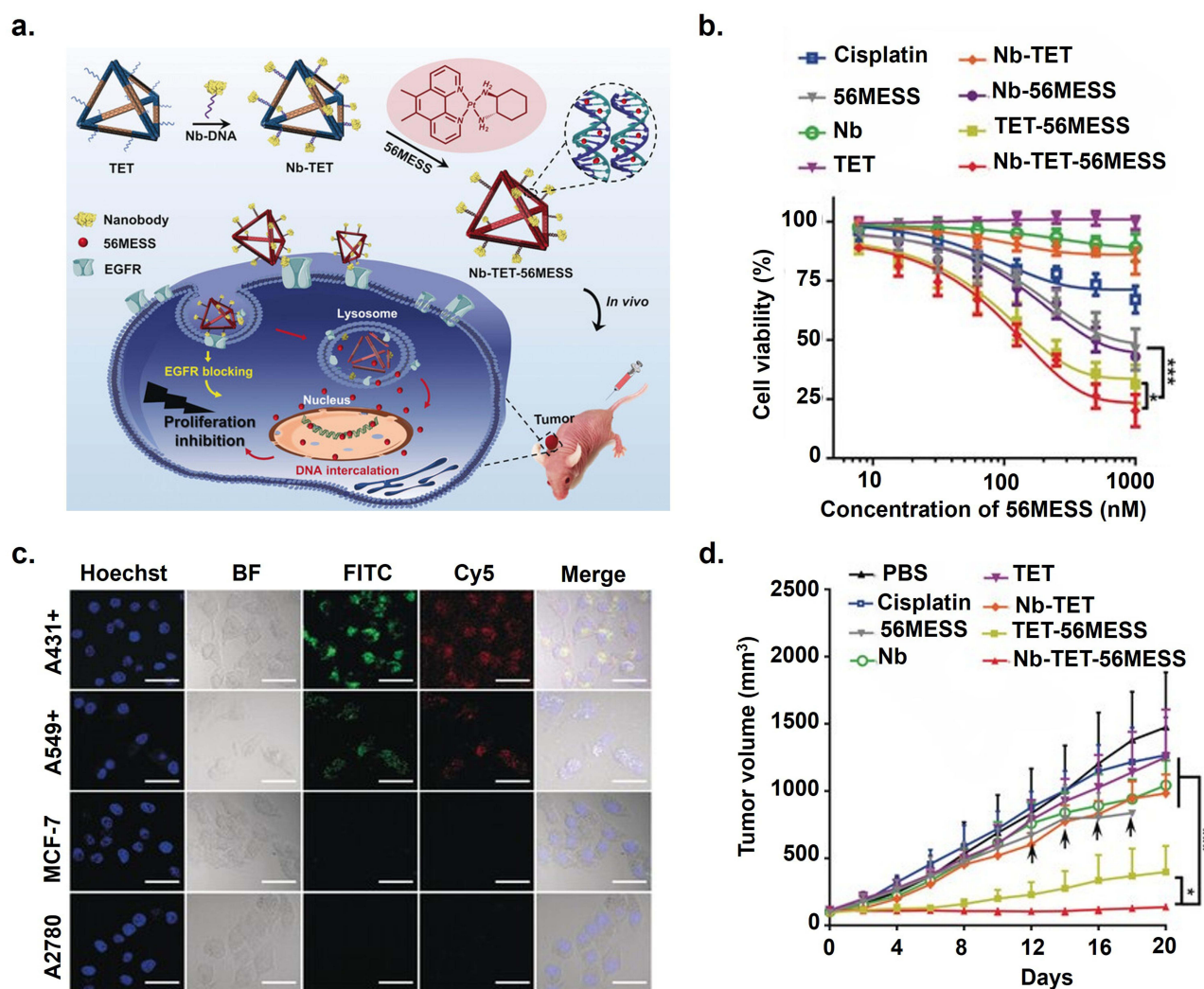


Figure 18 (a) Schematic illustration of an Nb-conjugated DNA nanoplatform for targeted platinum drug delivery. (b) Cell viability of A431 cells incubated with the indicated formulation for 48 h. The concentration of each drug was calculated based on 56MESS (56MESS: TET: Nb=120:1:12). *p < 0.05, ***p < 0.001. (c) LSCM images of Nb (FITC)-TET(Cy5)-56MESS in four cell lines with different EGFR expression levels. Scale bar: 50 μm. (d) Volumes of tumors from A431-tumor-bearing mice treated with PBS, cisplatin, 56MESS, TET, Nb-TET, TET-56MESS, or Nb-TET-56MESS. Black upward arrows represent deaths occurring during treatment with 56MESS. *p < 0.05, ***p < 0.001. Figure reproduced with permission from Angewandte, Communications, 2019.¹⁶⁹

Combination of Nanoparticle-Nb Conjugates with Other Drugs for Target-Specific Delivery Using Upconversion Nanoparticles (UCNPs)

Upconversion nanoparticles (UCNPs) convert low-energy near-infrared photons into high-energy emissions. They can resist photobleaching and offer sharp emission spectra and low background noise, making them ideal for precise long-term bioimaging and drug delivery.^{164,265,282,283} Therefore, Wu et al (2018) developed a targeted drug delivery system, the Nb_(PEG)DOX/HSA-UCNPs complex, by loading doxorubicin into HSA-coated UCNPs, which were linked to PEGylated Nbs with site-specific tags (Figure 20a).¹⁵⁸ In confocal microscopy study, the Nb_(PEG)DOX/HSA-UCNPs complex showed high doxorubicin accumulation in EGFR-positive A431 cells, but no detectable accumulation in EGFR-negative MCF-7 cells (Figure 20b). The Nb-conjugated drug-loaded UCNPs selectively reduced cell viability of EGFR+ A431 cells compared to those treated only with doxorubicin, while EGFR- MCF-7 cells remained largely unaffected, confirming targeted efficacy with reduced side effects (Figure 20c). Although upconversion nanoplatforms excel in imaging and tracking, their complexity and inorganic composition may raise concerns regarding long-term persistence, potential toxicity, and regulatory hurdles compared to purely organic carriers.

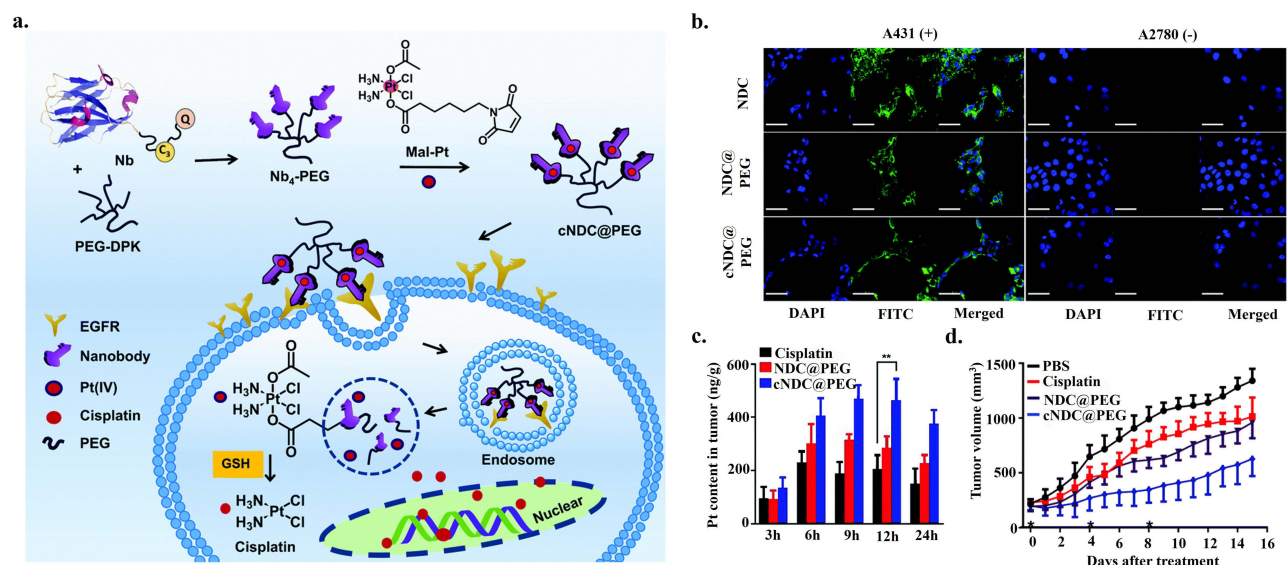
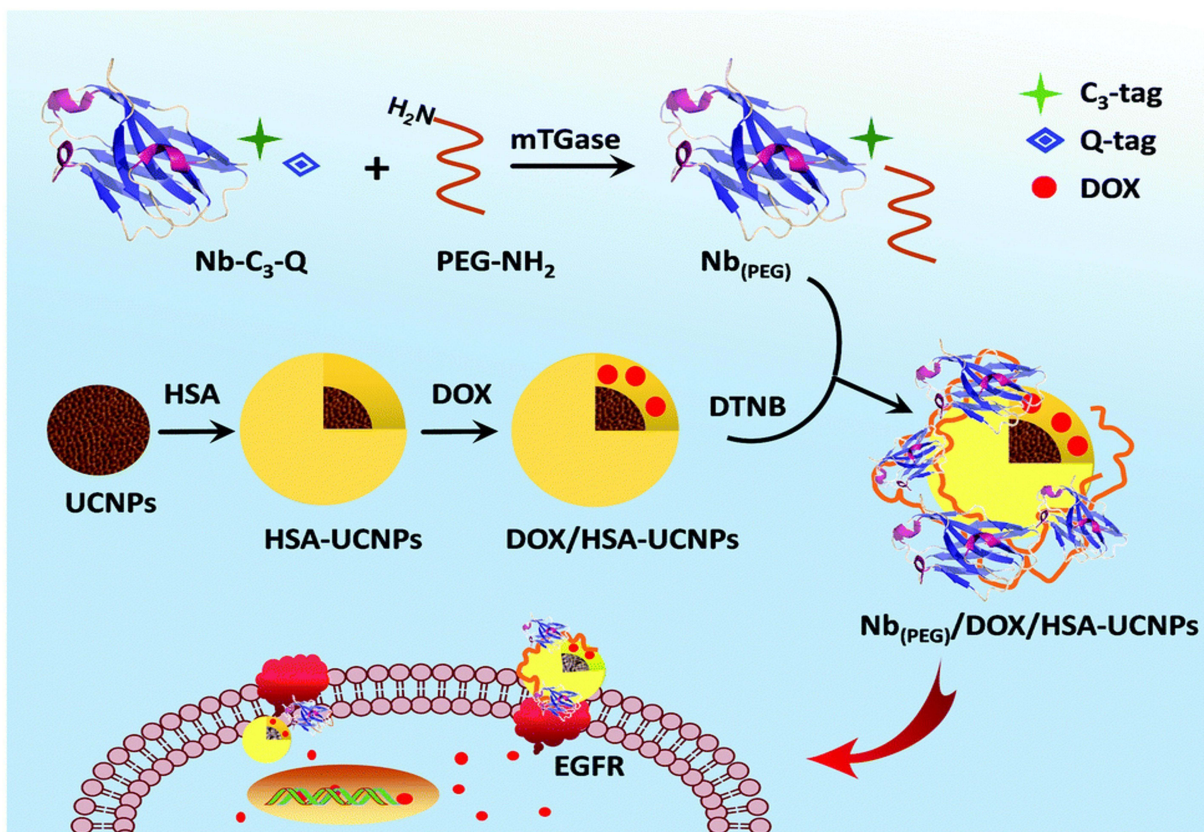


Figure 19 (a) Schematic illustration of the construction of cNDC@PEG and targeting EGFR-positive tumor cells. (b) LSCM images of FITC-NDC, FITC-NDC@PEG, and FITC-cNDC@PEG in EGFR+ A431 and EGFR- A2780 cells. Scale bar: 50 μm. (c) Platinum content in the tumor after administered with cisplatin, NDC@PEG, and cNDC@PEG. **p < 0.01. (d) Tumor volumes of mice treated with PBS, cisplatin, NDC@PEG, and cNDC@PEG. Drugs were intravenously injected with a platinum dose of 2 mg kg⁻¹ on the first day of treatment and 1 mg kg⁻¹ on days 4 and 8. * on the x-axis indicate the days of drug administration. Figure reproduced with permission from Royal Society of Chemistry, *Chem. Commun.*, 2020.⁵³

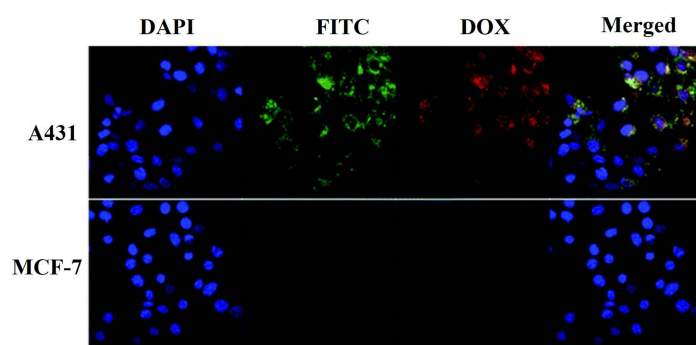
Using Nb-Conjugated Gold Nanoparticles Through Photothermal Therapy

Photothermal therapy uses nanoparticles that absorb near-infrared (NIR) light and convert the absorbed photons into thermal energy, causing tumor cell destruction.²⁸⁴ Branched gold nanoparticles, with their irregular shape and high surface-to-volume ratio, efficiently absorb NIR radiation and produce sufficient heat to destroy tumor cells, suitable for cancer therapy.^{285,286} In 2011, Van de broek et al used branched AuNPs functionalized with anti-HER2 Nbs to actively target and kill HER2+ breast and ovarian cancer cells through photothermal therapy (Figure 21a).²²⁰ Fluorescence-activated cell sorting (FACS) revealed that branched gold nanoparticles conjugated with anti-HER2 Nbs (anti-HER2 NP) specifically bound to HER2+ ovarian cancer SKOV3 cells, producing a significant fluorescence peak shift, whereas a minimal shift was observed with HER2- Chinese hamster ovary CHO cells in vitro (Figure 21b). The fluorescence peak shift was slightly less pronounced for anti-HER2 NP compared to free anti-HER2 Nbs due to the limited number of Nbs on the nanoparticles and potential quenching effects from their larger size. However, the shift remained significant and comparable, confirming that the Nbs retained their binding affinity after conjugation. When exposed to laser irradiation, cancer cells with anti-HER2 Nb-conjugated nanoparticles underwent significant photothermal destruction, indicating the potential of this approach for cancer treatment (Figure 21c). Branched gold nanoparticles conjugated with anti-PSA Nbs and free anti-PSA Nbs (negative controls), which specifically target prostate-specific antigen (PSA), showed low or negligible binding to both SKOV3 and CHO cells, confirming target specificity. Dark-field microscopy images confirmed that anti-HER2 NP specifically binds to HER2+ SKOV3 cells and showed strong orange scattering, whereas anti-PSA NP and free anti-HER2 Nbs showed minimal scattering (Figure 21d). HER2- CHO cells exhibited only slight, non-specific binding with both anti-HER2 and anti-PSA nanoparticles, consistent with the FACS results. Overall, this study demonstrates that nanobody-conjugated branched gold nanoparticles enable specific targeting and effective photothermal ablation of HER2-positive cancer cells, highlighting their potential as a selective and externally controllable therapeutic platform for targeted cancer therapy. Overall, this study demonstrates that Nb-conjugated branched AuNPs enable the specific targeting and effective photothermal ablation of HER2-positive cancer cells, highlighting their potential as a selective and externally controllable therapeutic platform for targeted cancer therapy.

a.



b.



c.

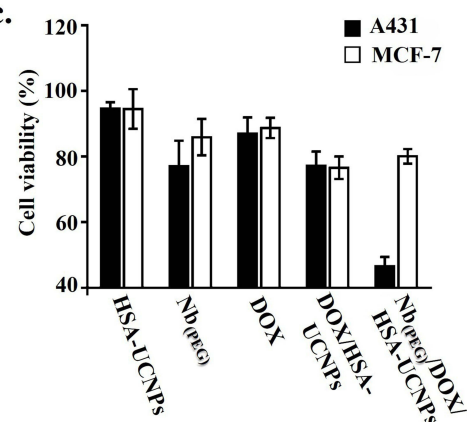


Figure 20 (a) Schematic illustration of the formation of **Nb(PEG)/DOX/HSA-UCNPs** for targeting tumor cells with overexpression of EGFR. (b) Cellular binding and internalization of **Nb(PEG)/DOX/HSA-UCNPs** to A431 cells and MCF-7 cells. (c) MTT assay showing cell viability of A431 and MCF-7 cells after treatment with HSA-UCNPs, **Nb(PEG)**, DOX, DOX/HSA-UCNPs, or **Nb(PEG)/DOX/HSA-UCNPs**. Figure reproduced with permission from Royal Society of Chemistry, *Journal of Materials Chemistry B*, 2018.¹⁵⁸

Using Nbs Conjugated with Liposomes for Targeted Drug Delivery

The simultaneous use of multiple antibodies targeting different tumor epitopes enhances drug binding and cytotoxicity, increasing the effectiveness of liposomal cancer therapies. Farasat et al (2019) developed a strategy for managing HER2-overexpressing breast cancers using Nb-bound liposomes to target cancer cells.²¹⁹ They produced and purified four anti-HER2 recombinant VHhs conjugated with PEGylated liposomes containing doxorubicin (Figure 22a). Nb-targeted liposomes are effectively bound to the HER2+ breast cancer cell lines SKBR3 and BT474 (Figure 22b). Oligoclonal VHH mixture-conjugated liposomes showed enhanced binding efficiency compared to their monoclonal counterparts.

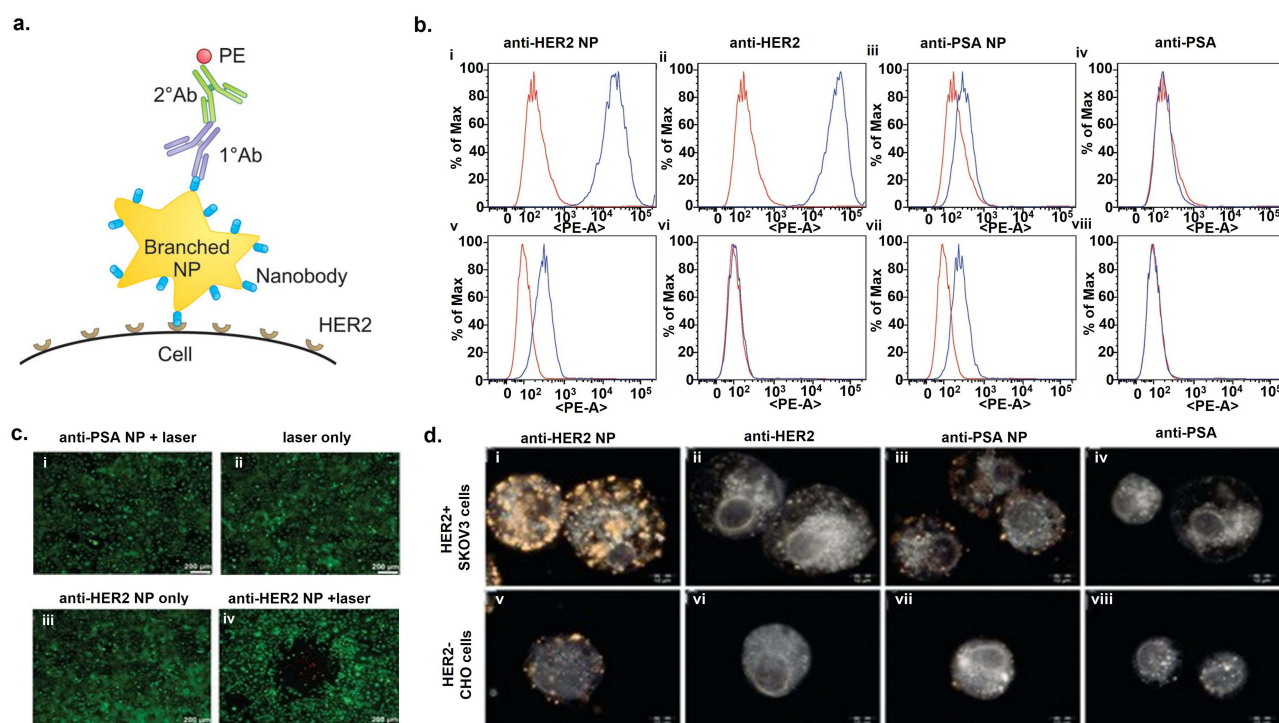


Figure 21 (a) Schematic representation of the fluorescent assay used for the FACS experiment. (b) FACS analysis of HER2+ SKOV3 cells incubated with (i) anti-HER2 NP, (ii) anti-HER2 Nb, (iii) anti-PSA NP, (iv) anti-PSA Nbs and HER2- CHO cells incubated with (v) anti-HER2 NP, (vi) anti-HER2 Nb, (vii) anti-PSA NP, (viii) anti-PSA Nbs. (c) Fluorescence microscopy images of (i) SKOV3 cells incubated with anti-PSA NP after laser irradiation, (ii) SKOV3 cells after laser irradiation without nanoparticles, (iii) SKOV3 cells with anti-HER2 NP without laser irradiation, (iv) SKOV3 cells incubated with anti-HER2 NP after laser irradiation. (d) Darkfield images of SKOV3 cells incubated with (i) anti-HER2 NP, (ii) anti-HER2 Nb, (iii) anti-PSA NP, (iv) anti-PSA Nbs and CHO cells incubated with (v) anti-HER2 NP, (vi) anti-HER2 Nb, (vii) anti-PSA NP, (viii) anti-PSA Nbs. Figure reproduced with permission from American Chemical Society, *ACS Nano*, 2011.²²⁰

Furthermore, Nb-targeted oligoclonal liposomes exhibited increased toxicity against HER2+ cells compared to non-targeted liposomes (Figure 22c). This approach represents a promising advancement in cancer therapy by combining the advantages of Nbs and liposomal drug delivery systems for targeted treatment of HER2+ breast cancer. Similarly, Nikkhai et al (2018) developed and characterized tetra-specific methotrexate-loaded VHH-PEG-liposomes with high binding affinity and cytotoxicity against HER2-overexpressing breast cancer cells, demonstrating an efficient strategy for making targeted cancer nanomedicines.²²² Although these oligoclonal approaches improve binding, increased ligand complexity could affect manufacturing reproducibility and immune recognition, factors important for clinical translation.^{287,288}

In another study, Noh et al (2025) developed a CD155 receptor targeting A5 Nb-conjugated liposomal nanocarriers loaded with doxorubicin (A5-LNP-DOX) for highly selective drug delivery to CD155-expressing lung cancer cells (Figure 23a).²³⁶ A5-LNP-DOX showed significantly higher uptake in CD155-positive H441 lung cancer cells but negligible uptake in CD155-negative human bronchial epithelial BEAS-2B cells, confirming selective Nb-targeted delivery of doxorubicin. Furthermore, confocal microscopy images also showed cytosolic doxorubicin-associated stronger green fluorescence in CD155-positive A549 cells treated with A5-LNP-DOX than with LNP-DOX, demonstrating effective A5 Nb-mediated active targeting (Figure 23b). A5-LNP-DOX showed stronger dose-dependent cytotoxicity than non-targeted LNP-DOX in CD155-positive lung cancer cells, demonstrating effective targeted drug delivery (Figure 23c). A5-LNP-DOX demonstrated superior CD155-targeted antitumor activity compared with non-targeted LNP-DOX, effectively suppressing tumor growth and proliferation while inducing apoptosis in both A549 orthotopic lung tumors (Figure 23d) and patient-derived lung cancer organoid xenografts (Figure 23e). Therefore, challenges associated with oligoclonal Nb-conjugated liposomes may be addressed by monospecific Nb-liposome platforms, such as CD155-targeted A5-LNP-DOX, which offer a more streamlined design and may better support clinical development.

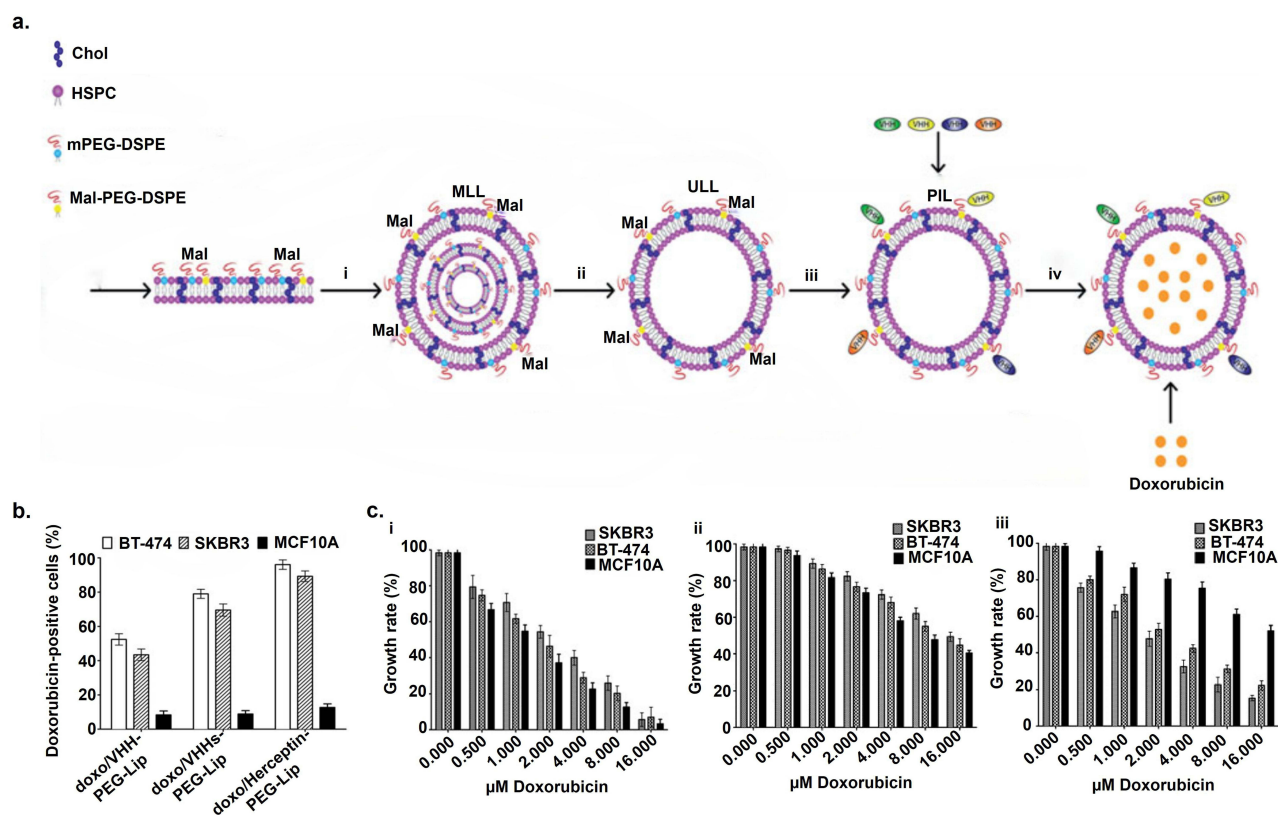


Figure 22 (a) Preparation of VHH-targeted liposomes. (i) Multilamellar liposomes (MLL) composed of cholesterol (Chol), saturated phospholipids like hydrogenated soy phosphatidylcholine (HSPC), a PEGylated lipid with a methoxy-PEG chain (mPEG-DSPE), and a PEGylated lipid with a maleimide group (Mal-PEG-DSPE) were prepared by a lipid film method. (ii) Then MLL was extruded to form unilamellar nano-scale liposomes (ULL). (iii) The VHHs were thiolated with Traut's reagent and conjugated on ULL. (iv) The resulting VHHs-PEG-Lip was loaded with doxorubicin. (b) The graph shows the percentage of doxorubicin-positive cells when BT-474, SKBR3, and MCF10A cells are treated with monoclonal doxo/VHH-PEG-Lip, oligoclonal doxo/VHHs-PEG-Lip, and doxo/Herceptin-PEG-Lip. (c) In vitro cytotoxicity assay of free doxorubicin (i), doxo/PEG-Lip (ii), and doxo/VHHs-PEG-Lip (iii) on SKBR3, BT-474, and MCF10A cell lines at 37 °C in a CO₂ incubator for 48 h. Figure reproduced with permission from Taylor & Francis, *Journal of Liposome Research*, 2019.²¹⁹

Nb-Dendrimer Conjugates

Yuen et al (2025) conjugated an anti-HER2 Nb to monomethyl auristatin E (MMAE)-loaded PEGylated polylysine dendrimer (Nb-G₃^{MMAE}) using unnatural amino acids and click chemistry (Figure 24a).²²⁴ Nb-G₃^{MMAE} selectively bound to HER2-overexpressing MDA-MB-231/HER2 cells with significantly higher affinity than non-targeted dendrimers, while showing minimal binding to HER2-negative MDA-MB-231 cells (Figure 24b). Nb-G₃^{MMAE} exhibited more than 100-fold greater cytotoxicity in MDA-MB-231/HER2 cells compared to non-targeted G₃^{MMAE}, while the two showed similarly low toxicity in HER2-negative MDA-MB-231 cells (Figure 24c). It also showed similar half-life and biodistribution to G₃^{MMAE} but predominant accumulation in the liver and subsequent renal excretion. These findings indicate that Nb conjugation did not significantly influence the systemic circulation or clearance pathways of the dendrimer-drug conjugate, although this effect may vary depending on dendrimer generation, PEGylation density, Nb orientation, and conjugation homogeneity.^{289–291} Nb-G₃^{MMAE} showed strong fluorescence inside HER2-overexpressing SKOV-3 tumor cells throughout the core and periphery of the tumor, whereas G₃^{MMAE} showed minimal signal, highlighting enhanced tumor cell internalization in vivo via Nb targeting (Figure 24d). Furthermore, Nb-G₃^{MMAE} strongly suppressed tumor growth in SKOV-3 xenograft mice compared to non-targeted G₃^{MMAE} and an ADC Kadcyla (a trastuzumab-emtansine conjugate).

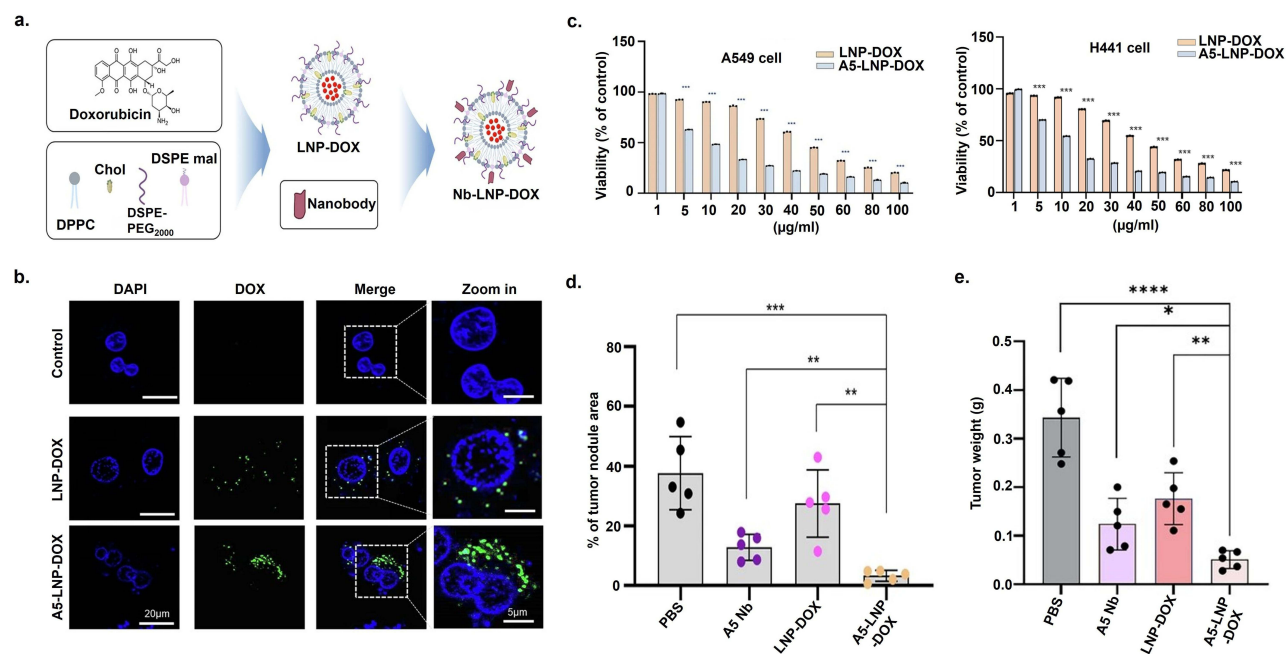


Figure 23 (a) Flowchart of the synthesis of A5-LNP-DOX. (b) Confocal imaging was performed to analyze the intracellular regions of liposomes. A549 cells were exposed to LNP-DOX and A5-LNP-DOX (DOX: green) for 4 h. The scale bars indicated 20 μm and 5 μm (magnification). (c) In vitro cytotoxicity of LNP-DOX and A5-LNP-DOX was evaluated in A549 and H441 cells by treating them with varying concentrations (1–100 $\mu\text{g/ml}$) of LNPs for 8 h, followed by medium replacement and a further 24 h of incubation. Data represent mean $\pm$ SD. ($n = 3$). *** $p < 0.001$. (d) The bar graph showed % of tumor regions in orthotopic A549-luc lung tissues from PBS-, A5 Nb-, LNP-DOX-, and A5-LNP-DOX-treated groups. $n = 5$. Each colored dot represents the measurement from an individual mouse. ** $p < 0.01$ and *** $p < 0.001$. (e) Weights of human lung cancer organoid xenograft mouse tumors treated with PBS, A5 Nb, LNP-DOX, and A5-LNP-DOX. Data were expressed as mean $\pm$ SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Figure reproduced with permission from Nature publishing group (Springer Nature), *Signal Transduction and Targeted Therapy*, 2018.²³⁶

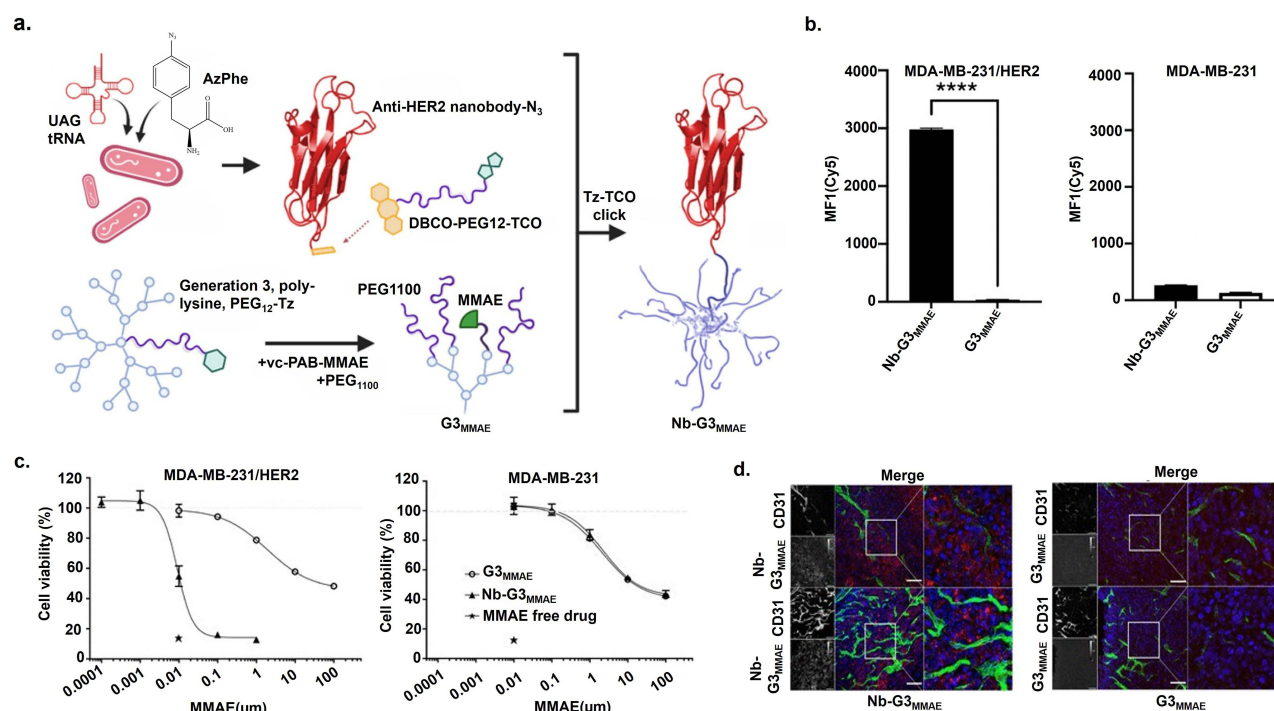


Figure 24 (a) Schematic representation of preparation of Nb-G3_{MMAE} where an anti-HER2 Nb site-specifically conjugated to a PEGylated dendrimer loaded with MMAE and Cy5 via Tz-TCO click chemistry for targeted drug delivery. (b) Cell mean fluorescence intensity (MFI) at 24 h was measured by flow cytometry after Nb-G3_{MMAE} and G3_{MMAE} were incubated with HER2+ MDA-MB-231 and HER2- MDA-MB-231 cells. *** $p < 0.0001$, $n = 3$. (c) AlamarBlue cell viability assay of MDA-MB-231/HER2 and MDA-MB-231 cells incubated with Nb-G3_{MMAE} and G3_{MMAE} for 48 h at 37 $^{\circ}\text{C}$. * $p < 0.05$. (d) Confocal images of 5–8 μm SKOV-3 tumor sections collected 24 h after treatment with Nb-G3_{MMAE} or G3_{MMAE} and then fixed and immunostained with anti-CD31-FITC (green) and Hoechst 33342 (blue), showing dendrimer Cy5 fluorescence (red). Scale bar: 100 μm . Figure reproduced with permission from American Chemical Society, *ACS Nano*, 2025.²²⁴

Nb-Modified Polymeric Micelles

Polymeric micelles (PMs) are self-assembling complexes of amphiphilic molecules that act as biocompatible drug carriers. They can enhance stability, solubility, and targeted accumulation of chemotherapeutic agents in areas with damaged vasculature.²⁹² To address the limitations of passive targeting and to enhance antitumor efficacy, researchers are combining the intrinsic pharmacological activity of actively targeted ligand-modified nanocarriers with the cytostatic effects of incorporated chemotherapeutic agents. Using Nbs as ligands, Nb-modified PMs were developed to enhance targeted cancer treatment.²³⁰ These micelles inhibit tumor growth even in the absence of drugs and are significantly more effective when loaded with chemotherapeutic agents.

Talelli et al (2012) found that EGa1 Nb-modified polymeric micelles with covalently entrapped doxorubicin, EGa1-DOX-PM, improved tumor therapy effectiveness both in vitro and in vivo²³⁰ (Figure 25a). Confocal microscopy analysis showed that EGa1 Nb-modified micelles significantly improved cellular uptake in a squamous cell carcinoma cell line compared to unmodified micelles (Figure 25b). Nb-modified DOX-PM was significantly more effective at killing cancer cells, exhibiting a lower EC₅₀ value compared to untargeted DOX-PM (Figure 25c). EGa1 Nb-modified micelles with doxorubicin conjugated to a macromolecular carrier (EGa1-DOX-MA-PM) significantly inhibited tumor growth by enhancing drug retention and accumulation at the tumor site (Figure 25d).

Similarly, researchers have developed thermosensitive micelles from block copolymers and attached EGa1 Nbs for targeted drug delivery.²³¹ This approach has shown improved cellular uptake of drugs in EGFR-positive tumors compared to untargeted micelles. Costa et al (2018) developed the DOX-pAcF-ELP_{BC}-EgA1 drug delivery system in which doxorubicin is precisely attached to elastin-like polypeptides (ELP) decorated with EgA1 Nb for enhanced targeting of cancer cells (Figure 26a).²³¹ Upon heating above its critical micellization temperature, this diblock ELP constructs self-assemble into stable micelles with a doxorubicin-loaded hydrophobic core and a hydrophilic block displaying EgA1 Nbs on the surface (Figure 26b). The p-acetylphenylalanine (pAcF) linker connecting doxorubicin with diblock ELP constructs features a pH-sensitive oxime bond^{293,294} that facilitates the release of doxorubicin in

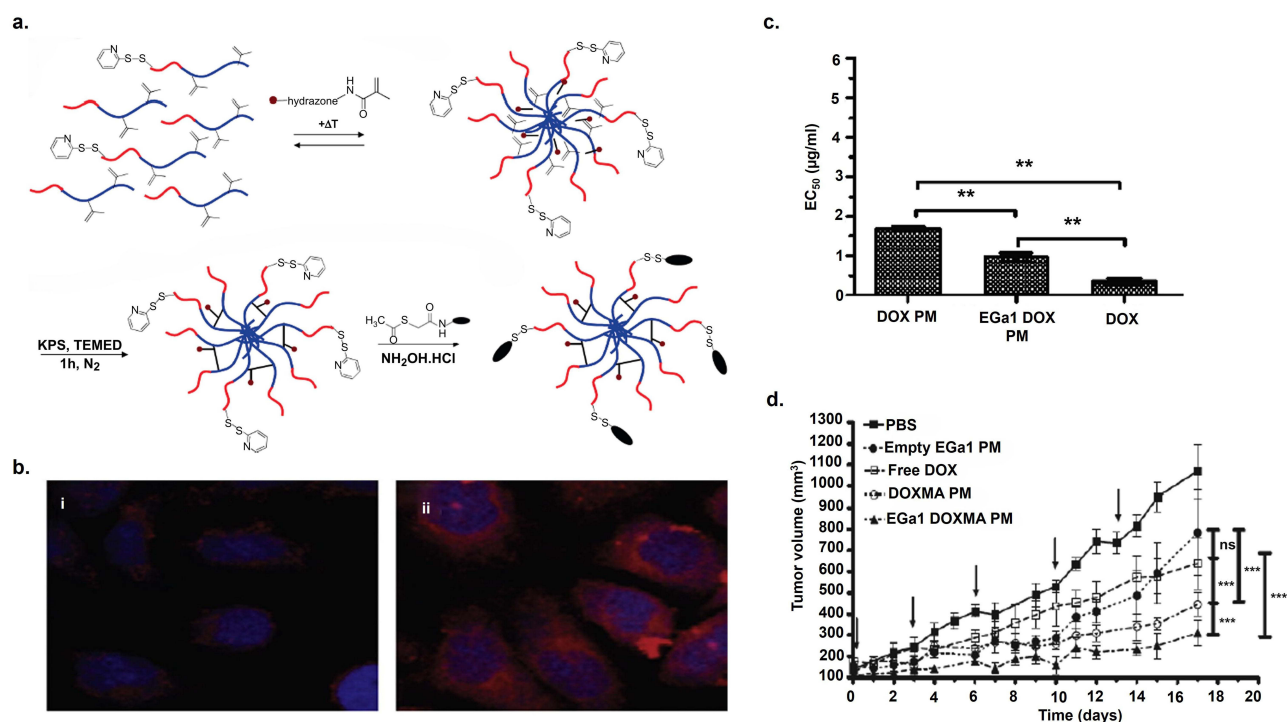


Figure 25 (a) Synthetic scheme for the preparation of Nb-modified PM. (b) Uptake of untargeted (i) and Nb-modified PM (ii) containing rhodamine (as a model drug; in red) by 14C cells upon 4 h of incubation at 37 °C. (c) In vitro cytotoxicity was evaluated using the WST-I assay. 14C cells were pulse-incubated with the indicated formulations for 4 h, followed by medium replacement and allowed to grow for another 68 h. **p < 0.01. (d) In vivo, tumor volumes were measured in mice treated with PBS, drug-free EGa1-modified PM, free doxorubicin, DOX-containing PM, or EGa1-modified DOX-containing PM. ***p < 0.001 and downward arrows indicate the time points of intravenous administration of the respective treatments. Figure reproduced with permission from Elsevier, *Biomaterials*, 2012.²³⁰

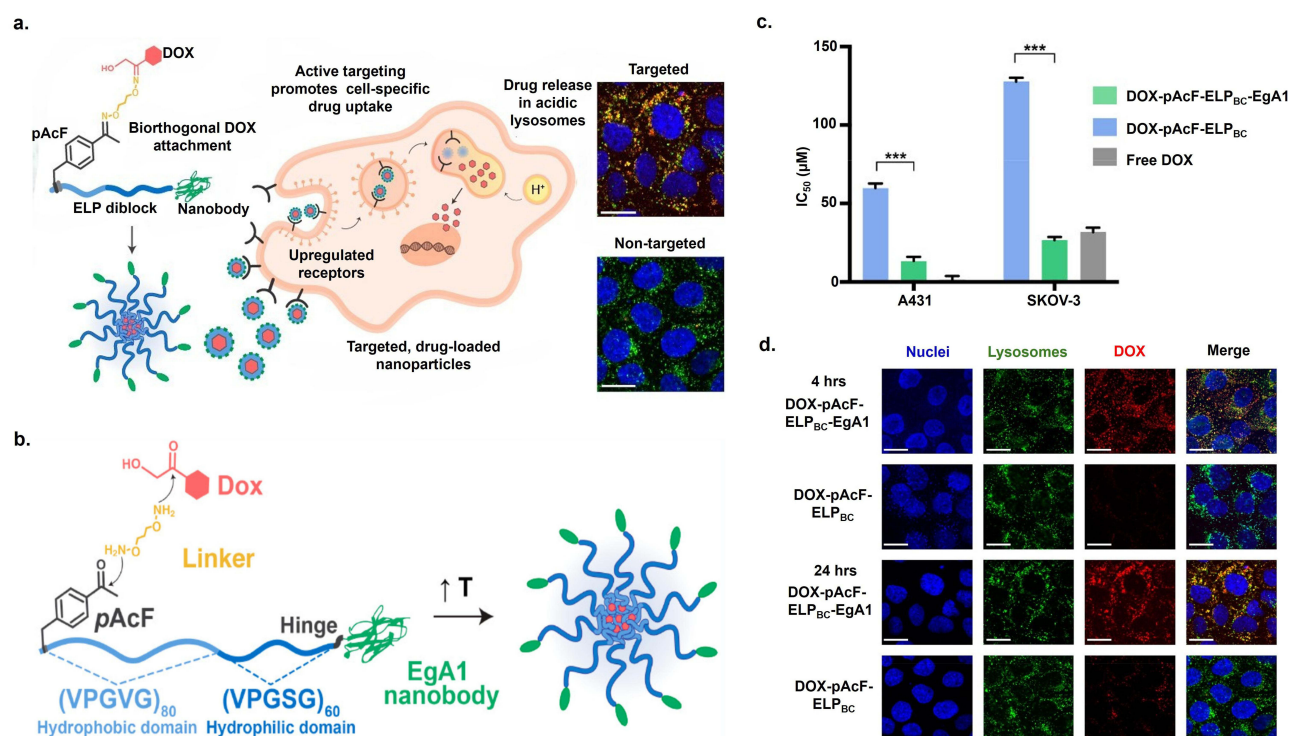
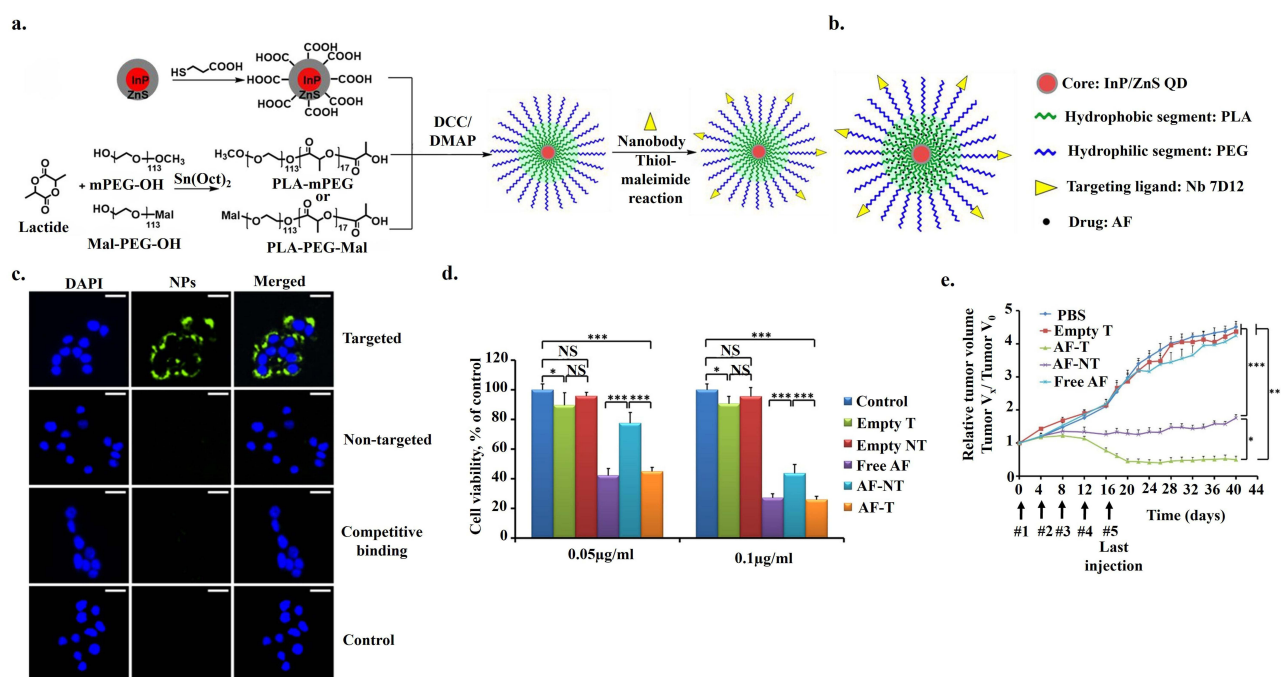


Figure 26 (a) Schematic representation of the active targeting of cancer cells by an Nb-decorated polypeptide micelle with bioorthogonally conjugated doxorubicin. The accompanying confocal images illustrate cell-specific uptake; white scale bars represent 20 μm. (b) Schematic showing the design and assembly of DOX-pAcF-ELP_{BC}-EgA1 nanoparticles. Dox (red) is conjugated to the pAcF residue (dark gray) at the N-terminus of the amphiphilic ELP_{BC} chain (blue) by a telechelic hydroxylamine linker (yellow). The hydrophilic ELP block is fused to the EgA1 Nb (green) by a flexible hinge (black). The upward arrow next to T indicates that nanoparticle self-assembly is triggered by increasing the temperature above the critical micellization temperature. (c) Cell viability assay of A431 and SKOV-3 cells confirm that the targeted DOX-pAcF-ELP_{BC}-EgA1 is more highly cytotoxic than the non-targeted control, DOX-pAcF-ELP_{BC}. ***p < 0.001. (d) Confocal images of A431 cells incubated with DOX-pAcF-ELP_{BC}-EgA1 or DOX-pAcF-ELP_{BC} for 4 and 24 h shows that doxorubicin from both constructs colocalizes with lysosomes in A431 cells. Hoechst staining (blue) marks nuclei, CytoPainter dye (green) labels acidic endolysosomal compartments, and red fluorescence indicates localization of doxorubicin. The merged images reveal the colocalization (yellow) of doxorubicin with lysosomes. The white scale bars in all microscopy panels represent 20 μm. Figure reproduced with permission from American Chemical Society, *Nano Letters*, 2018.²³¹

the acidic lysosomal compartments of cells after cellular uptake via receptor-mediated endocytosis. In the cytotoxicity study, targeted DOX-pAcF-ELP_{BC}-EgA1 particles had significantly lower IC₅₀ values than non-targeted DOX-pAcF-ELP_{BC} polymeric micelles in both A431 (high EGFR expression) and SKOV-3 (intermediate EGFR expression) cell lines, indicating enhanced Nb-targeted drug delivery efficacy (Figure 26c). A subcellular trafficking study revealed significantly higher and time-dependent colocalization of DOX-pAcF-ELP_{BC}-EgA1 with lysosomes compared to non-targeted DOX-pAcF-ELP_{BC}, demonstrating more efficient cellular uptake and drug release in EGFR-positive A431 cell lines (Figure 26d). This study reported a highly appealing NDv system for targeted cancer therapy.

In 2017, Wang et al developed a unique NIR fluorescent theranostic micelle for EGFR-overexpressing cancers, particularly triple-negative breast cancer (TNBC), using InP/ZnS quantum dots (QDs) functionalized with the amphiphilic block copolymer PLA-PEG. This micelle was conjugated with anti-EGFR Nbs, 7D12 Nbs, and the anticancer drug aminoflavone (AF) (Figure 27a and b).²³³ The study showed that AF-loaded Nb-targeted micelles (QD-PLA-PEG-Nb or AF-T) has 67-fold higher cellular uptake (Figure 27c), and greater cytotoxicity (Figure 27d) in EGFR-overexpressing MDA-MB-468 cells compared to non-targeted micelles (QD-PLA-PEG or AF-NT). AF-T accumulated to a greater extent in tumors and led to better tumor regression than AF-NT micelles in a TNBC mouse model (Figure 27e). Furthermore, no systemic toxicity was observed with the treatments, suggesting the QD-based and Nb-conjugated micelles as an effective theranostic nanoplatform for EGFR-overexpressing cancer such as TNBC.



Overall, these studies established Nb-modified polymeric micelles as a promising strategy to improve targeted drug delivery. However, therapeutic benefit is not intrinsic to Nb decoration alone and instead depends on coordinated optimization of ligand presentation, micelle architecture, and release mechanisms.^{295–297} At the same time, micellar carriers face inherent design challenges, including dynamic assembly/disassembly behavior in vivo, sensitivity to dilution, and stability at physiological temperatures, all of which can influence drug release and biodistribution.^{298–300} Therefore, rigorous pharmacokinetic evaluations and further preclinical and clinical studies are required to define their safety, robustness, and translational relevance.

Emerging Directions in Nb-Based Drug Delivery

The studies discussed in this review predominantly represent early-stage preclinical investigations and collectively demonstrate the versatility of Nb-based drug delivery strategies, including NDCs and NDVs. Both NDCs and NDVs exploit Nb specificity, receptor-mediated internalization, and modular conjugation strategies to enable tumor-targeted therapy; however, each approach is associated with distinct trade-offs. NDCs demonstrate promising preclinical efficacy driven by high target selectivity and adaptable conjugation chemistries; however, comparative analyses have revealed sensitivity to target heterogeneity, linker design, and circulation behavior. Dual-targeting or bispecific formats enhance selectivity but depend strongly on the co-expression of target antigens and may underperform in heterogeneous tumors. Strategies aimed at extending systemic exposure, such as PEGylation or incorporation of albumin-binding domains, improve tumor accumulation but may adversely affect renal clearance, tumor penetration, or immunogenicity.^{301,302} While advances in site-specific conjugation and cleavable linker chemistries improve construct homogeneity and release control, some activation strategies rely on external stimuli or chemical conditions that may complicate clinical translation. In parallel, NDVs, including liposomes, DNA nanostructures, dendrimers, inorganic nanoparticles, photothermal systems, and polymeric micelles, offer additional functionality through enhanced payload loading, and in some cases,

stimulus-responsive release. Although several NDv systems have demonstrated encouraging *in vivo* efficacy, most supporting data still arise from early-stage preclinical models. Factors such as carrier size, tumor penetration, circulation and clearance behavior, ligand density, structural stability, and release kinetics can influence the performance of Nb-targeted drug-loaded nanocarriers; however, these parameters have not been fully investigated. Moreover, long-term safety and *in vivo* stability, including accumulation in clearance organs such as the liver and kidneys, remain under-explored, limiting cross-study comparability and translational potential. Across both NDC and NDv platforms, building on this preclinical foundation, well-designed translational and clinical studies are essential to more rigorously define the therapeutic potential of Nb-based targeting strategies.

Beyond the established NDC and NDv platforms, an emerging and conceptually important direction is the development of simplified, carrier-free, or minimal-carrier Nb-based delivery systems aimed at improving translational feasibility and therapeutic efficiency. This paradigm aligns closely with the intrinsic properties of Nbs, including their small size, modular architecture, high stability, and compatibility with site-specific functionalization. There is a growing trend in nanomedicine toward carrier-free or minimal-carrier self-assembling systems that maximize the active payload while preserving targeting precision, pharmacokinetic control, and therapeutic potency, thereby addressing several translational limitations associated with larger, multicomponent nanocarriers. In that context, self-assembled nanoparticles derived entirely from natural drug molecules exhibited high drug loading, synergistic therapeutic activity, and enhanced tumor-targeted phototherapy without the need for traditional nanocarriers, illustrating a broader push for molecularly efficient delivery platforms that minimize non-active mass while maximizing functional payload and safety.^{303–306} In another example, a carrier-free affibody–drug conjugate, a nano-sized protein ligand (~6–7 kDa), can spontaneously self-assemble into nanomicelles containing high drug payloads. This system enhances tumor targeting and exhibits potent antitumor activity in HER2-positive models, illustrating how small protein ligands can act as their own delivery vehicles without requiring an inert carrier.³⁰⁷ This shift reflects a broader move toward molecularly efficient delivery systems that rely on the targeting ligand itself rather than a high carrier mass.

Following these examples, carrier-free or minimal-carrier strategies could involve direct Nb–payload conjugation, compact multivalent assemblies, or self-assembling Nb–drug conjugates, potentially offering advantages in manufacturing reproducibility, tumor penetration, reduced off-target accumulation, and regulatory simplicity, while maintaining targeting specificity. Importantly, mechanistic insights from preclinical NDC and NDv studies, particularly regarding target biology, internalization kinetics, linker and release chemistry, conjugation homogeneity, and pharmacokinetic behavior, provide a strong foundation for the rational design of these next-generation systems. While current evidence supports their mechanistic promise, most data remain preclinical, underscoring the need for rigorously designed clinical studies to establish durable clinical benefits in targeted cancer therapy.

Conclusion

In conclusion, research on Nb-based drug delivery systems for cancer therapy highlights the remarkable potential of Nbs in revolutionizing current treatment paradigms. Nb offers a plethora of advantages, including high aqueous solubility, stability, low immunogenicity, deep tumor-penetration capability, and exceptional specificity and affinity toward cancer biomarkers. Leveraging these properties, various strategies have been developed, such as NDCs and NDvs, aiming to deliver cytotoxic agents selectively to cancer cells while minimizing damage to normal cells. The efficacy and specificity of Nb-based drug delivery systems have been demonstrated through numerous studies, showing their ability to inhibit tumor growth, target specific cancer biomarkers such as EGFR, HER2, and VEGFR2, and enhance drug uptake and cytotoxic effects in cancer cells, both *in vitro* and *in vivo* in animal models. Moreover, Nbs exhibit versatility in their applications, being utilized in various drug delivery systems, including liposomes, DNA-based carriers, dendrimer-based nanoparticles, PMs, UCNPs, and gold nanoparticles, among others. These approaches may offer enhanced specificity compared to conventional chemotherapies and could potentially reduce off-target effects, as suggested by preclinical studies. Looking toward the future, Nb-based drug delivery systems hold immense promise for overcoming the limitations of traditional therapies in cancer treatment. Continued research efforts are exploring strategies to address challenges such as rapid blood clearance and renal uptake, with preclinical studies in animal models showing that approaches such as PEGylation or incorporation of albumin-binding domains can improve tumor accumulation,

highlighting opportunities for further advancements in this field. Furthermore, Nb-based, carrier-free self-assembling drug delivery systems can be explored to further enhance the development and therapeutic efficiency of Nb-targeted drug delivery platforms. Ultimately, the versatility and potential of Nbs underscore their significance as central tools in targeted drug delivery systems, offering hope for more effective and personalized treatment approaches for cancer.

Abbreviations

ADC, antibody-drug conjugate; mAb, monoclonal antibody; Nb, nanobody; sdAb, single-domain antibody; HCAb, heavy-chain-only antibody; NDC, Nb-drug conjugate; NDv, Nb-attached drug delivery vehicle; HSA, human serum albumin; DAR, drug-to-antibody ratio; EPR, enhanced permeability and retention effect; CPP, cell-penetrating peptide; IgG, immunoglobulin G; Fv, variable fragment; Fab, antigen-binding fragment; scFv, single-chain variable fragment; Fc, fragment crystallizable region; ECM, extracellular matrix; TME, tumor microenvironment, CAF, cancer-associated fibroblasts; FR, framework region; CDR, complementarity determining region; VHH, variable heavy domain of the heavy chain; PEG, polyethylene glycol; pAcF, *p*-acetylphenylalanine; CAIX, carbonic anhydrase IX; FcRn, fragment crystallizable; LV, lentiviral vector; T3SS, type III secretion system; LSCM, Laser scanning confocal microscopy; NP, nanoparticle; EV, extracellular vesicle; GPI, glycosylphosphatidylinositol; DOX, doxorubicin; ROS, reactive oxygen species; PS, photosensitizer; PDT, photodynamic therapy; ABD, albumin-binding domain; NIT, nitroxoline; CIS, cisplatin; UCNP, upconversion nanoparticle; NIR, near-infrared; FACS, fluorescence-activated cell sorting; PSA, prostate-specific antigen; Chol, cholesterol; HSPC, hydrogenated soy phosphatidylcholine; MLL, multilamellar liposomes; ULL, unilamellar nano-scale liposomes; MFI, mean fluorescence intensity; PM, polymeric micelle; ELP, elastin-like polypeptide; AF, aminoflavone; HER2, human epidermal growth factor receptor 2; VEGFR2, vascular endothelial growth factor receptor 2; EGFR, epidermal growth factor receptor; TAT, transactivator of transcription; C_H, constant domains of heavy chain; C_L, constant domain of light chain; V_H, variable domain of heavy chain; V_L, variable domain of light chain; TNF α , tumor necrosis factor- α ; RSV F, respiratory syncytial virus fusion protein; CXCR2, C-X-C motif chemokine receptor 2; MMAE, monomethyl auristatin E; SMCC, succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; PSMA9, prostate-specific membrane antigen 9; TROP2, trophoblast cell surface antigen 2; MHC-II, major histocompatibility complex class II; VCAM-1, vascular cell adhesion molecule-1; Lip, liposome; MnPc, manganese phthalocyanine; QD, quantum dot; MUC1, mucin-1 receptor; MCS, multicellular spheroids; ADM, adriamycin; DM1, mertansine; AF647, alexa fluor 647; Ftn, ferritin; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; MRI, magnetic resonance imaging; TGF- β , transforming growth factor-beta receptor; VEGF, vascular endothelial growth factor receptor; TIR%, tumor inhibition ratio percentage; LNP, liposomal nanocarriers; TNBC, triple-negative breast cancer.

Data Sharing Statement

All data in the review are presented in the manuscript. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics Approval and Consent to Participate

This manuscript is a review article that does not require prior approval.

Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest.

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