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REVIEW

Strategies for Non-Covalent Attachment of Antibodies to PEGylated Nanoparticles for Targeted Drug Delivery

Kai-Wen Ho[1,2], Yen-Ling Liu[2,3], Tzu-Yi Liao[2,3], En-Shuo Liu[2,3], Tian-Lu Cheng[1–3]

[1]Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan; [2]Drug Development and Value Creation Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan; [3]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan

Correspondence: Tian-Lu Cheng, Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, No. 100 Shih-Chuan 1st Road, Kaohsiung, 80708, Taiwan, Tel +886 7 3121101 2697, Fax +886 7 3227508, Email tlcheng@kmu.edu.tw

Abstract: Polyethylene glycol (PEG)-modified nanoparticles (NPs) often struggle with reduced effectiveness against metastasis and liquid tumors due to limited tumor cell uptake and therapeutic efficacy. To address this, actively targeted liposomes with enhanced tumor selectivity and internalization are being developed to improve uptake and treatment outcomes. Using bi-functional proteins to functionalize PEGylated NPs and enhance targeted drug delivery through non-covalent attachment methods has emerged as a promising approach. Among these, the one-step and two-step targeting strategies stand out for their simplicity, efficiency, and versatility. The one-step strategy integrates streptavidin-tagged antibodies or bispecific antibodies (bsAbs: PEG/DIG × marker) directly into PEGylated NPs. This method uses the natural interactions between antibodies and PEG for stable, specific binding, allowing the modification of biotin/Fc-binding molecules like protein A, G, or anti-Fc peptide. Simply mixing bsAbs with PEGylated NPs improves tumor targeting and internalization. The two-step strategy involves first accumulating bsAbs (PEG/biotin × tumor marker) on the tumor cell surface, triggering an initial attack via antibody-dependent and complement-dependent cytotoxicity. These bsAbs then capture PEGylated NPs, initiating a second wave of internalization and cytotoxicity. Both strategies aim to enhance the targeting capabilities of PEGylated NPs by enabling specific recognition and binding to disease-specific markers or receptors. This review provides potential pathways for accelerating clinical translation in the development of targeted nanomedicine.

Keywords: PEGylated nanoparticle, monoclonal antibody, antibody-containing nanodrugs, functionalization, targeted cancer therapy

Introduction

Polyethylene glycol (PEG)-based nanoparticles (NPs) are diverse and promising drug delivery vehicles. PEG is water-soluble, non-toxic, and used as an excipient in medication formulations to improve pharmacokinetics, biodistribution, solubility, and stability, reducing side effects.[1–3] PEG polymers coupled to a lipid anchor inhibit the reticuloendothelial system (RES). The PEGylated NPs escape from the RES system and are stable within PEGylated liposomes, extending their half-life and enhancing therapeutic efficacy.[4–8] The use of nanotechnology to develop these systems has been well-established over the past decade, both in pharmaceutical research and the clinical setting. For instance, Doxil and Caelyx, which are PEGylated liposomal doxorubicin (PLD)[9–11] have been utilized in treating AIDS-related Kaposi's sarcoma and solid cancers.[10] The pharmacokinetic profile of Doxil at 50 mg/m² in humans shows a 300-fold increase in the area under the curve (AUC) compared to the free drug.[10,12] Metastatic pancreatic cancer patients can receive Onivyde, a PEGylated liposomal irinotecan.[13,14] After administering Onivyde 60–180 mg/m^2 to cancer patients, the overall AUC increased by 46.2 times compared to nonliposomal irinotecan.[15,16] Tumor accumulation of PEGylated NPs can boost treatment efficiency due to the enhanced permeability and retention (EPR) effect.[17–19] Although non-targeted PEGylated NPs exhibit improved pharmacokinetics by evading the RES, EPR effects-lacking metastases and liquid tumors present difficulties. PEGylated NPs may internalize less in metastatic or hematopoietic cancers. PEGylated NPs had one-third of

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the intracellular absorption of non-PEGylated NPs.[20] PEGylated NPs are less effective against metastasis or liquid tumors because the EPR effect is weaker, reducing tumor cell uptake and therapeutic efficacy. Thus, actively targeted liposomes with tumor selectivity and internalization augmentation are needed to promote tumor uptake and therapeutic efficacy.

The significance of active targeted PEGylated NP internalization lies in its pivotal role in optimizing drug delivery to solve hematologic malignancies, metastasis, and drug resistance.[21–25] Hematologic malignancies like leukemia lack the EPR effect. Chen et al developed a bispecific antibody (bsAb: mPEG × CD20) that simultaneously binds PEGylated NPs and CD20.[25] This yielded multivalent αCD20-armed liposomes, enhancing internalization and anticancer efficacy against CD20-expressing lymphoma cells. The one-step formulation of mPEG × CD20 modified PLD, resulting in αCD20/PLD specifically targeting Raji cells, demonstrating 56-fold increased internalization. In metastasis, the distribution of MM-302, an anti-human epidermal growth factor receptor 2 (HER2) Ab-targeted PEGylated liposomal doxorubicin, was also associated with better activity.[26] Cu-MM-302 PET/CT data revealed its ability to access difficult metastatic locations, such as brain and lung metastasis.[24] Cheng et al[23] developed a bsAb (mPEG × HER2) to confer the PLD with tumor tropism to enhance PLD internalization. The αHER2/PLD specifically accumulated doxorubicin in tumor-bearing mice and produced significantly greater antitumor activity against doxorubicin-resistant MDA-MB-361 ($P < 0.05$) tumors compared to untargeted PLD. Various strategies can be employed to transform conventional NPs into targeted NPs, ensuring their precise delivery to specific cells or tissues and internalization enhancement. These methods include bsAbs, peptide conjugation, and ligand-receptor interactions. Further advancements in active targeting of NP techniques may revolutionize drug development, possibly ushering in the third generation of therapeutic interventions.

The chemical covalent conjugation of NPs is often used to incorporate Abs into cancer treatment. Chemical conjugation between Abs and NPs can occur via Ab amino acids like lysine and cysteine.[27–29] The limitation is that reactive and abundant amine groups cause random conjugation and heterogeneity in Abs and NP mixtures. Many chemical conjugation techniques use site-specific conjugation to solve heterogeneity, such as thiol chemistry,[30] 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry,[31] and Click chemistry.[32] However, the modification of these strategies is complicated. To address these drawbacks, one-step formulation strategies have been proposed that involve the utilization of streptavidin-tagged Ab, Ab, or bsAb (PEG/DIG × marker), which possess the ability to attach to PEGylated NPs that will modify the biotin/Fc binding domain (such as protein A, G or anti-Fc peptide)/mPEG/digoxigenin (DIG) molecules separately, and these strategies can bind to tumor markers simultaneously. The simple one-step mixing of bsAb with these PEGylated NPs can confer tumor tropism and internalization enhancement. Moreover, in the two-step targeting strategy, pre-targeting is conducted using a bispecific adaptor that can accumulate on the tumor cell surface and capture the PEGylated NPs to induce a second attack by enhancing internalization and cytotoxicity against targeted tumor cells. The two-step targeting strategy maintains the original formulation and characteristics of PEGylated NPs with minimal modification. This review details these innovative approaches, the one-step formulation and the two-step targeting strategy (Figure 1), which improve PEGylated NP tumor targeting and cellular internalization. After tumor cells internalize NPs, they can release chemotherapeutic drugs, nucleic acids, or imaging agents, improving efficacy and reducing side effects. The one-step formulation and two-step targeting strategy of creating targeted NPs has great potential for use in precision medicine and personalized cancer therapy.

Content

One-Step Formulation Strategy for Active Targeting

Biotin-Based × Streptavidin-Tagged Formulation

Numerous Ab-NP systems have been developed using the biotin-streptavidin ligation technique.[33,34] Biotinylated NPs were formed by activating the carboxylic group of biotin and covalently attaching it to the amine group of PEG-amine.[35] Streptavidin interacts with biomaterials like Abs via thiol conjugation. The biotin-streptavidin ligation technique is employed to attach monoclonal Abs (mAbs) or other ligand types onto the surface of liposomes. This technique involves a one-step formulation process, creating targeted liposomes that can selectively bind to tumor receptors (such as HER2, EGFR, CD20, and others) and enhance drug internalization (Figure 2). For instance, by using streptavidin-tagged anti-TfR monoclonal Abs

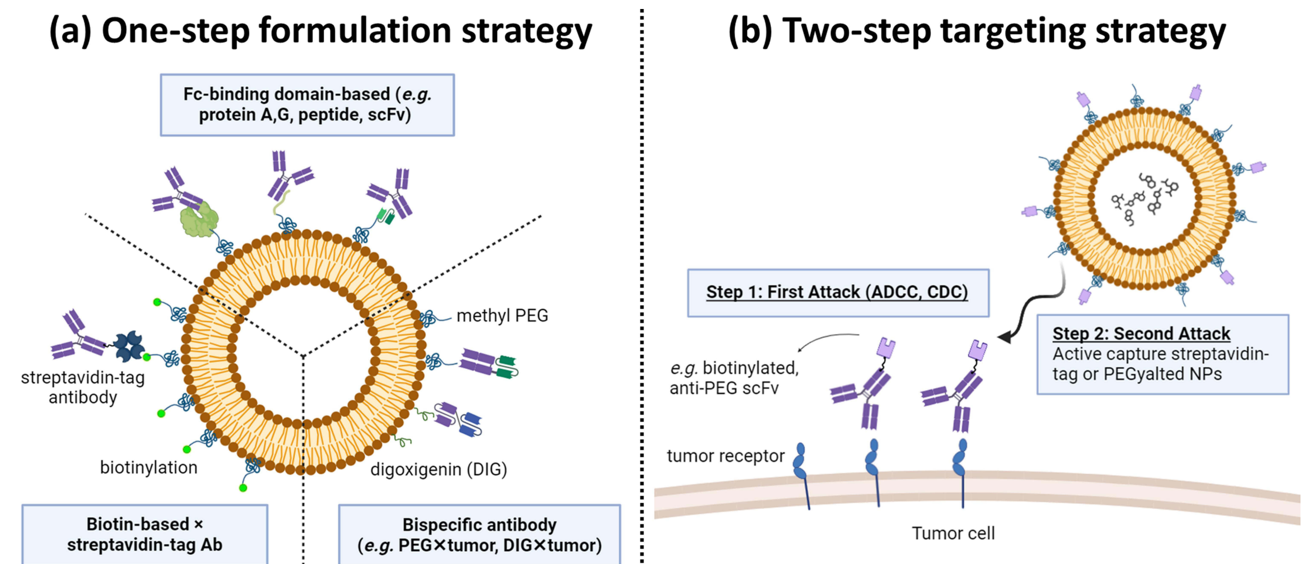

Figure 1 Schematic representation of one-step and two-step targeting nanoparticles (NPs) for enhanced drug delivery. (**a**) One-Step Formulation Strategy: The strategies use streptavidin-tagged antibody (Ab), Ab, or bispecific Ab (bsAb: PEG/DIG × marker) that can be mixed in one step with NPs which are modified with biotin/Fc binding domain (eg, protein A, G, or anti-Fc peptide)/polyethylene glycol (PEG)/digoxigenin (DIG) molecules separately. These strategies can confer the NPs with tumor tropism and enhance internalization and therapeutic efficacy. (**b**) Two-Step Targeting Strategy: The bsAb (PEG/biotin × tumor marker) accumulates on the tumor cell surface to activate the first attack of Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) through Abs. Then, bsAbs can capture PEGylated/streptavidin-tagged NPs to trigger a second attack by increasing internalization and cytotoxicity.

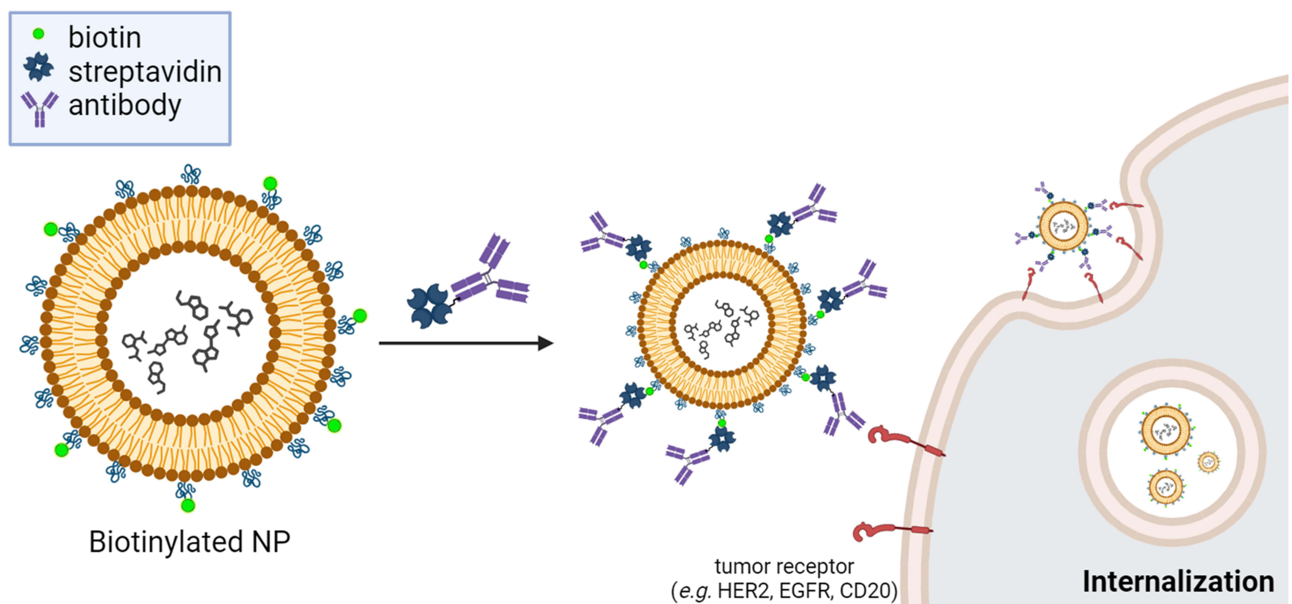

Figure 2 Schematic of one-step mixing of biotin-based NPs with streptavidin-tagged Abs for targeted drug delivery and internalization enhancement. Biotinylated NPs were formed by activating the carboxylic group of biotin and covalently attaching it to DSPE–PEG_{2000} using EDC/NHS chemistry. The biotinylated NPs one-step mix with streptavidin-tagged Abs to form targeted NPs. The antibody specifically binds to its target antigen such as transferrin receptor (TfR), CD33, CD7, etc. The targeted NPs allow for the specific delivery of therapeutic payloads to the desired tumor site, enhancing drug efficacy and minimizing off-target effects.

conjugating through the biotin group located at the distal end of the PEG spacer of PEGylated immuno-lipopolyplexes (PILP) for gene delivery. The targeted PILP transfection efficiency (67.5 ± 3.8%) increased by nearly 3-fold compared to non-targeted PILP (25 ± 4%).[36] Attachment of anti-TfR monoclonal Abs to PEGylated NPs increases the evaluated in vitro and in vivo gene transfection efficiency, stability, and cytotoxicity without increasing toxicity.[37,38] Also, Chen et al[39] developed biotinylated anti-CD33 and biotinylated anti-CD7 Abs that were coupled to chemo-drug cytarabine encapsulated streptavidin-tagged

liposomes (SALs) by using the biotin-streptavidin technique. The internalization efficacy was 93%, 74%, and 81% of targeted SALs compared to 63%, 27%, and 18% of non-targeted SALs in the three cell lines, respectively. The biotin-streptavidin technique has also been applied to specific protein assay systems for immobilizing Abs.[40,41] Biotin-streptavidin ligation involves the specific binding of biotin to streptavidin, creating a stable and robust linkage widely used in various applications, such as biomolecule immobilization, targeted drug delivery, and diagnostic assays. In immobilizing Abs on NPs, the challenge is to prevent aggregation and ensure proper orientation, which is crucial for maintaining functionality and paratope accessibility.[42] Therefore, careful optimization is essential in biotin-streptavidin ligation (Table 1).

The biotin-streptavidin interaction is well-known as one of the most robust non-covalent interactions (affinity: K_d ~10^{-15} M) found in nature. High affinity prevents the ligand from detaching from NPs, resulting in high stability and enabling the efficient operation of Abs.[59] However, biotin-streptavidin ligation introduces foreign entities (biotinylated ligands) to which the immune system may respond. Streptavidin, a bacterial protein, may trigger an immune response leading to clearance by phagocytic cells in humans.[60,61] This may result in the formation of anti-streptavidin Abs, impacting the pharmacokinetics and efficacy of the delivery system. The immune response generated in the context of biotin-streptavidin ligation might lack specificity and control, potentially leading to unintended immune reactions. Streptavidin's tetrameric structure provides numerous binding sites,[62] resulting in different biotin attachment orientations. This heterogeneity may impair biotin-streptavidin interactions and tests or therapeutic applications that rely on this binding affinity. To provide consistent and dependable results in biological and medicinal applications, conjugation

Table 1 One-Step Formulation Strategy for Active Targeting

Adaptor-based formulation	Engager	Nanoparticles (NPs)	Payload	Ref
Biotin-based × streptavidin-tagged	Streptavidin-tagged TfR (8D3) Ab	Biotinylated lipopolyplexes	DNA	[34]
	Streptavidin-tagged TfR (8D3) Ab	Biotinylated NP	Phosphatidic acid	[35]
	Biotinylated TfR (OX26) Ab	Streptavidin-tagged NP	FITC	[36]
	Biotinylated CD33 Ab	Streptavidin-tagged NP	Cytarabine	[37]
	Biotinylated CD7 Ab	Streptavidin-tagged NP	Cytarabine	[37]
	Biotinylated HSA Ab	Streptavidin-tagged NP	N/A	[38]
	Streptavidin-tagged CD8 Ab	Biotinylated NP	HRP	[39]
Fc-binding domain-based NPs × Ab	HER2 Ab	Protein A-conjugated quantum dots (QDs)		[43]
	HER2 Ab	Immunostimulating complexes	Doxorubicin or paclitaxel	[44]
	HER2 Ab	Protein A-conjugated QD		[45]
	CD147 Ab	Protein A-conjugated NP	Doxorubicin	[46]
	CD31 Ab	Protein A-conjugated NP	Doxorubicin	[46]
	CD3 Ab and EGFR Ab	FcBP conjugated NP	Fluorescent	[47]
	PD-1 Ab and PD-L1 Ab	αFc Ab-conjugated NP	N/A	[48]
	EGFR Ab	αFc scFv-conjugated NP	RNAi	[49]
	HER2 Ab	FcBP conjugated NP	N/A	[50]
	EGFR Ab	FcBP conjugated NP	Nucleic acids	[51]
Bispecific antibody	mPEG × EGFR (Fab × scFv)	PEGylated NP	Doxorubicin	[52]
	mPEG × HER2 (Fab × scFv)	PEGylated NP	Doxorubicin	[21,53,54]
	mPEG × CD20 (Fab × scFv)	PEGylated NP	Doxorubicin	[23]
	mPEG × CD19 (scFv × scFv)	PEGylated NP	Doxorubicin	[55]
	mPEG × CD22 (scFv × scFv)	PEGylated NP	Doxorubicin	[55]
	mPEG × CD38 (scFv × scFv)	PEGylated NP	Doxorubicin	[55]
	mPEG × EGFR (scFv × scFv)	PEGylated NP	Doxorubicin	[56,57]
	Digoxigenin (DIG) × CD33 (scFv × Fab)	DIGylated NP	SIRNA	[58]

methods must be carefully considered and optimized.[63] This optimization process aims to achieve consistent and controlled binding interactions between biotinylated molecules and streptavidin, preventing the formation of varied or mixed conjugates. Proper conditions during ligation are essential for optimizing the effectiveness to maintain the integrity and specificity of Ab-NP conjugates.[64]

Fc-Binding Domain-Based NPs × Ab Formulation

In this section, three methods to improve the antigen-binding activity of liposome-tagging Abs for capturing Abs are discussed: (1) protein A and protein G, which are bacterial proteins known for their high affinity to the Fc region of Abs, (2) anti-Fc scFv, a single-chain variable fragment engineered to specifically bind to the Fc region of Abs, and (3) anti-Fc peptide, short peptide sequences designed to mimic the binding site of Abs' Fc region. Each method offers unique advantages and applications to confer the PEGylated NPs with tumor tropism and internalization enhancement (Figure 3 and Table 1).

To improve the antigen-binding activity of liposome-tagging Abs, IgG Fc-binding proteins, like protein A or protein G, were conjugated to the surface of NPs using numerous chemical methods such as carbodiimide chemistry,[65] maleimide chemistry[43,65,66] or Click chemistry.[43] This strategy can be used to couple Abs to NPs in an oriented manner, and the Fc binding affinity also allows the Ab orientation to face the antigen binding site outward.[44] This non-covalent approach has emerged as a prevalent strategy for the facile attachment of diverse targeting or therapeutic Abs to NPs.[45,46] As an example, protein A was conjugated to quantum dots (QDs) that were subsequently employed for the immobilization of anti-HER2 Abs.[45] Delivery efficiency was assessed in breast cancer cells with both HER2 overexpression and HER2 non-overexpression evaluated by confocal microscopy. The findings showed that HER2-targeted QDs enhanced cellular uptake by endocytosis.[47] This non-covalent coupling system could serve as an effective carrier for drug delivery targeting breast cancer cells with HER2 overexpression. Also, Hama et al demonstrated rapid Ab modification on high-affinity protein A-displaying liposomes (PAR28-PEG-lipo), allowing specific uptake by CD147- and CD31-positive cells. Liposomes modified with anti-CD147 Abs significantly increased cellular uptake of doxorubicin-capsuled PAR28-PEG-lipo and enhanced delivery compared to controls.[67] Apart from proteins A and G, growing interest in site-specific Ab coupling has spurred research on affinity peptides derived from protein.[68,69] However, the unexplored potential of protein A or protein G derivatives in the creation of conjugates

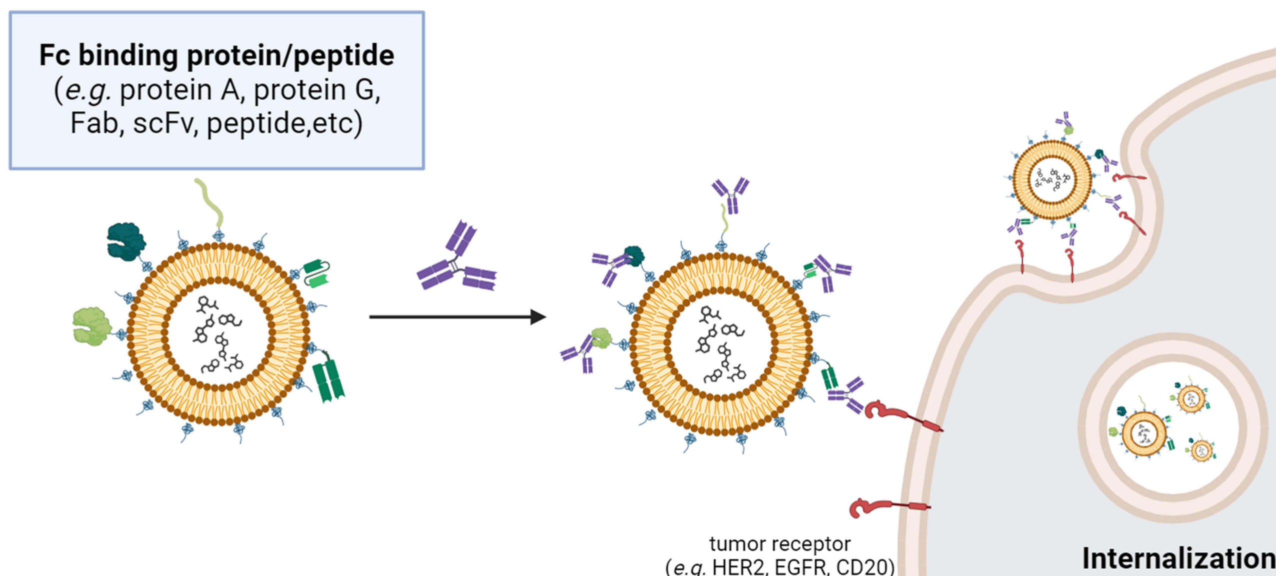

Figure 3 Schematic representation of one-step mixing of Fc-binding domain-based NPs with Abs for targeted drug delivery. The Fc-binding domain-based NPs are engineered with Fc-binding domains (eg, protein A, protein G, Fab, peptide) displayed on their surface. The Fc-binding domain, such as protein A/G, was covalently attached to NPs through N-hydroxysuccinimide ester groups at the PEG end (bis-NHS esters). On the other hand, the Ab fragments and Fc-binding peptides were synthesized by conjugating with maleimide-PEG-DSPE through amide bond formation. These domains exhibit a high affinity for the Fc region of Abs, facilitating specific binding (such as HER2, EGFR, CD147, CD31, etc.) and subsequent functionalization of the NPs with therapeutic payloads. Upon mixing, the Abs readily interact with the Fc-binding domains on the NPs, forming targeted NPs. This one-step mixing strategy offers a simple and efficient approach for the development of targeted NPs and holds significant promise for various biomedical applications, including targeted drug delivery and precision therapeutics.

between anticancer Abs and NPs remains an active area of investigation. In a different investigation, Gil-Garcia et al constructed a tripartite fusion protein, incorporating ZapB (for assembly), green fluorescent protein (GFP, for cell imaging), and the Z-domain (an engineered analog of the B-domain of *Staphylococcus aureus* protein A with high IgG affinity).[70] The resulting ZapB-GFP-Z NPs were then selectively bound to the Fc regions of anti-EGFR and anti-CD3 Abs in a controllable ratio, forming a bsAb mimic capable of redirecting T lymphocytes to cancer cells.

Similar to biotin-streptavidin ligation, protein A or G ligation might provide limited control over immune activation, potentially causing non-specific immune reactions. Abs and smaller Ab fragments, such as Fab' and scFv, are hypothesized to be less immunogenic.[48,71] Jiang et al devised a versatile platform for Ab immobilization. They conjugated oxidized anti-IgG (Fc specific) Abs (αFc) onto amino-functionalized polystyrene NPs (αFc-NP). Subsequently, anti-programmed death-1 (anti-PD-1) and anti-PD-L1 monoclonal Abs (mAbs) were immobilized onto αFc-NPs through Fc-specific noncovalent interactions.[49] These resulting immunomodulating nano-adaptors demonstrated the ability to co-engage T cells and tumor cells using two types of mAbs, showcasing their potential for efficient Ab-based cancer immunotherapy. Moreover, Kampel et al employed an Anchored Secondary scFv Enabling Targeting (ASSET) strategy[50] that enables anchoring of anti-IgG Fc single chain Fv (scFv) to the LNP surface.[51] The ASSET strategy allowed for the noncovalent and uniform attachment of anti-EGFR monoclonal Abs and facilitated targeted delivery using Cy5-labeled LNPs; they explored tumor localization and observed targeted LNPs in direct association with the cell surface of head and neck cancer.[69] Specialized peptides have been utilized to immobilize Abs for the functionalization of NPs. In the synthesis of a peptide conjugate NP using maleimide chemistry, the NP surface is initially activated with maleimide groups, facilitating the subsequent conjugation reaction. For instance, Shim et al covalently attached an Fc-binding peptide (FcBP, amino acid: DCAWHLGELVWCT), selected from a cyclic peptide library and refined through monovalent phage display, to the lactic-co-glycolic acid-polyethylenimine (LGA-PEI) NP. The liposome can non-covalently target HER2-specific Ab, enabling targeted delivery of the Ab-conjugated LGA-PEI NPs to HER-expressing cells.[26] HER2/FcBP-NPs showed 5.3-fold higher binding affinity to HER2 than isotype IgG Ab-modified NP and demonstrated significantly higher cellular uptake in HER2-positive cells compared to other formulations. Similar results were also reported by Lü et al, who covalently attached the anti-EGFR Ab cetuximab, enabling targeted delivery of therapeutic nucleic acids loaded in the Ab-conjugated LGA-PEI NPs to EGFR-expressing pancreatic cancer cells. Anti-EGFR Ab/FcBP-LGA-PEI/miR-198 mimic NPs showed enhanced internalization in Mia-MSLN cells compared to non-targeted NPs ($P < 0.01$).[72] In the Fc-binding domain-based NP strategy, an Ab can be non-covalently attached to the surface of FcBP-expressing NPs with precise orientation.[26]

Protein A (or protein G) is a bacterial protein that may be recognized by the immune system, potentially triggering an immune response. This can lead to the formation of anti-protein A Abs. Similar to streptavidin, the immunogenicity of protein A may impact the stability and effectiveness of the delivery system. The presence of anti-protein A Abs could affect the system's performance. Protein A and protein G display differing affinities for various Ab isotypes,[73,74] resulting in variations in the binding specificity of Abs to NPs. The alignment of Abs attached to NPs impacts their function and effectiveness in therapy. In 1984, Uhlén et al discovered five locations where Protein A binds to IgG. Various binding locations and Ab conformations create difficulty in standardizing the orientation of NP Abs.[75,76] Variability in protein A and protein G binding can introduce challenges in achieving stable and reproducible Ab-NP conjugates. Rigorous optimization and quality control measures are essential to address issues related to heterogeneity. The potential dissociation protein A (or protein G) and Abs can have a significant impact on the stability and targeting efficacy of NP formulation. The binding affinity (10^{-7} to 10^{-8} M)[77] between protein A and Abs on NPs plays a crucial role in determining the specificity and stability of the conjugation. A higher binding affinity ensures strong and specific interactions, promoting a stable and durable association between protein A and Abs on the NP surface.[78] The FcBPs have varying K_d values (10^{-9} M to 10^{-5} M),[79–83] enabling selection for unique application needs. Drug administration uses affinity ligands because of their high selectivity, stability, low toxicity, affordability, and configurable affinity capacity. However, FcBPs may attract different Ab subclasses and species, resulting in inconsistent binding efficacy and stability of targeted NPs. FcBP sequences must be optimized and selected based on target Ab attributes for optimal binding affinity and performance.

Bispecific Ab Formulation

BsAbs are engineered proteins designed to simultaneously target two different antigens. According to this concept, one end of the bsAb recognizes a specific hapten such as PEG digoxigenin (DIG), while the other end targets a tumor marker such as HER2, EGFR, CD20, etc. This innovative bsAb is engineered to simultaneously target multiple antigens, offering unique therapeutic advantages in various applications, including cancer treatment, immunotherapy, and other medical interventions.[52,53] It is essential to balance bsAb functionalization strategies with the stealth properties of PEGylated NPs when aiming to enhance in vivo tumor accumulation or increase NP uptake by tumor cells (Figure 4). The humanized bsAb platform can confer PEGylated NPs with tumor tropism in a simple one-step formulation. Several studies achieved the successful fabrication of methoxy PEGylated NPs through the straightforward mixing of PEGylated NPs with different formats of bsAbs such as Fab × scFv format[23,25,54–57] or scFv × scFv format.[58,84,85] These bsAbs comprised a humanized anti-mPEG (clone: h15-2b)[86] Fab fragment along with an anti-tumor antigen (EGFR, HER2, CD19, CD20, CD22, or CD38) scFv. Take the bsAb strategy reported in Kao et al for example,[54] cell imaging was conducted by combining bsAbs with PEG-imaging probes (Lipo/Rho, $Qdot_{565nm}$, or FeOdots) and then assessing probe localization in cancer cells using confocal microscopy and magnetic resonance (MR) imaging. Confocal microscopy of αEGFR-Lipo /Rho added to SW480 ($EGFR^+$) and SW620 ($EGFR^-$) cancer cells revealed a red fluorescence signal exclusively on SW480 cells. In addition, the αHER2/PLD effectively increased doxorubicin accumulation and cytotoxicity in both HER2-amplified MCF-7/HER2 breast cancer cells and DOX-resistant MDA-MB-361 cells. PEGylated NPs lack efficacy for hematologic malignancies due to the absence of the EPR effect. To address this, a one-step formulation of mPEG × CD20 modified PLD was used, resulting in αCD20/PLD specifically targeting leukemic cells, which demonstrated a 56-fold increased internalization and 15.2-fold higher cytotoxicity compared to controls. mPEG × CD20 modification presents a convenient strategy to enhance the efficacy of PEG-NPs against hematologic malignancies.[25] Similarly, Moles et al reported that bsAb-assisted enhancements in leukemia cell-targeting and cytotoxic potency correlated with receptor expression minimally affected normal peripheral blood mononuclear cells and hematopoietic progenitors in vitro and in vivo, and contributed to extended overall survival in patient-derived xenograft models of high-risk childhood leukemia, with reduced drug accumulation in the heart and kidneys (Table 1).

In addition to PEG binding, small chemical haptens such as digoxigenin (DIG)[87] incorporated into the lipid formulation, allow anti-DIG bsAbs to be recognized and confer the DIG-containing NPs with tumor tropism. Irmgard et al developed two bsAb formats for decorating siRNA-containing NPs with DIGs. DIG-binding scFv are fused at the

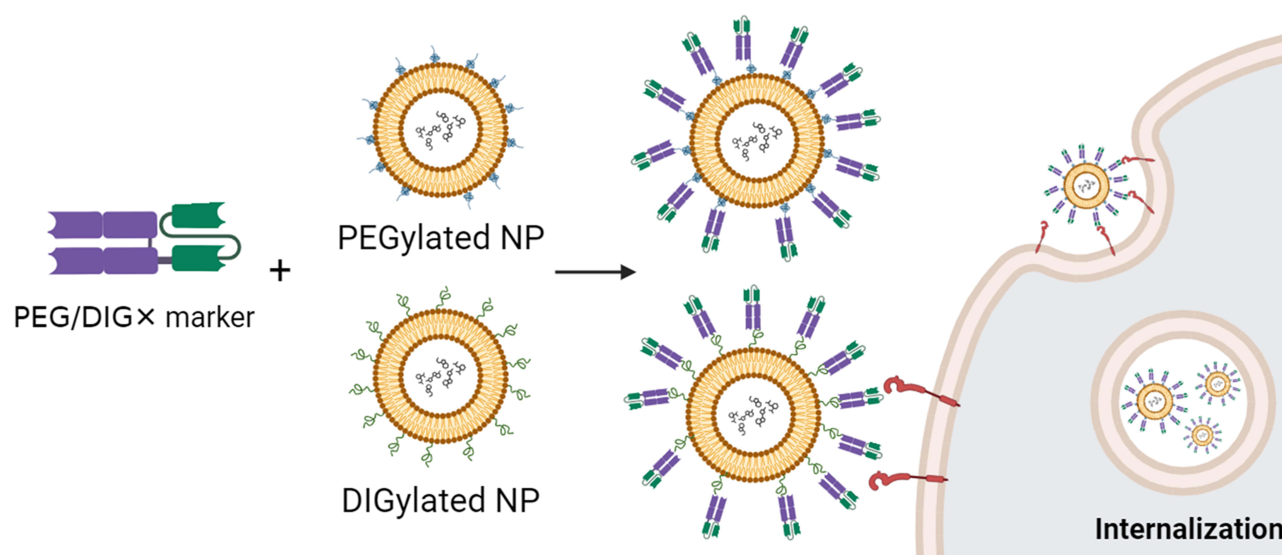

Figure 4 Schematic representation of one-step mixing of PEGylated or DIGylated (digoxigenin-modified) NPs with a bsAb for the formation of targeted NPs. DIGylated NPs are synthesized by chemically coupling NHS- or maleimide-DIG molecules to PEG. The bsAb is engineered to simultaneously recognize PEG or DIG and a specific tumor marker. Upon mixing, the bsAbs readily interact with the PEG/DIG moiety of NPs and confer NPs with tumor tropism. This one-step method streamlines NP functionalization and delivers therapeutic payloads to the tumor site efficiently and specifically, potentially improving treatment efficacy.

C-terminal to the anti-CD33 IgG or Fab.[88,89] Using such αCD33/NPs, they achieved a knockdown efficiency of up to 90% knockdown compared to the control NPs (less than 15%). The DIG-decorating NPs become internalized along with the anti-CD33 bsAb and accumulate in the endosomal compartments as observed by confocal microscopy.

BsAbs offer precise targeting capabilities by simultaneously binding to two different antigens. This design enhances specificity, reducing the off-target immune responses. This can be advantageous in minimizing collateral damage to healthy tissues. Also, bsAbs are constructed from humanized Ab fragments, which display low immunogenicity in patients.[90] The orientation of Abs when combined with NPs is a critical aspect that can significantly influence the performance and efficacy of the resulting conjugates. The anti-mPEG segment of the bsAbs is designed to specifically bind to the methoxy ends of mPEG molecules on the surface of PEGylated NPs. This results in a uniform orientation of the anti-cancer scFv portion of the bsAbs, facilitating precise targeting of PEGylated NPs to cancer cells. The one-step method involves simply mixing the bsAbs with PEGylated NPs, demonstrating a straightforward approach to confer tumor-targeting capability to PEGylated NPs. Under the one-step formulation strategy, how bsAbs stably remain on the NPs becomes a major issue. Cheng et al[23] evaluated the stability of mPEG × HER2 on PLD. The results showed that αHER2/PLD remained HER2-bound after storage at 4°C for 7 days or incubation in 10% human serum for 72 h at 37°C. The results showed that the one-step formulation of bsAbs was retained on the NPs, and the HER2-targeting ability of targeted NPs might not be influenced. On the other hand, Howard et al reported that bsAb-functionalized PEG particles showed similar accumulation ($p > 0.05$) in EGFR-expressing tumours in vivo compared to their non-targeted counterparts in mouse xenografts. Furthermore, when the concentration of bsAbs exceeded 170 bsAbs per particle, the targeted PEG particles exhibited notably increased accumulation in the spleen compared to non-targeted particles or those with a lower bsAb density. This suggests that elevating the functionalization of bsAbs beyond a specific threshold compromises the stealth characteristic of PEGs, resulting in unintended off-target accumulation in vivo.

Pharmacokinetics of Non-Covalent Antibody-Conjugated PEGylated Nanoparticles

The pharmacokinetics of non-covalent antibody-conjugated PEGylated nanoparticles are crucial because they influence the biodistribution, stability, and therapeutic efficacy. The high-affinity interaction between biotin and streptavidin (affinity: K_d ~10^{-15} M) can be compromised in vivo due to competitive displacement by endogenous biotin. This displacement can significantly alter the biodistribution of nanoparticles, leading to increased uptake by the liver, kidney and spleen, primarily due to rapid clearance by the reticuloendothelial system (RES).[91,92] Studies have demonstrated that such a mechanism can result in a substantial proportion of nanoparticles being sequestered in these organs, thereby reducing their availability for target engagement. Although biotin-streptavidin interactions are initially stable, endogenous biotin can displace streptavidin, resulting in the dissociation of the complex and a decreased half-life of the nanoparticles.[93,94] The dissociated components are rapidly cleared by the liver and kidneys, further shortening the circulation time of these nanoparticles.

Nanoparticles conjugated to the anti-Fc domain exhibit a stable interaction with antibodies under physiological conditions. However, the dynamic environment of the bloodstream can gradually lead to the dissociation antibody from the Fc-binding domain.[78] Dissociated antibodies or nanoparticles, particularly those small enough, may be filtered by the kidneys, leading to renal clearance.[95] This process reduces the half-life of the nanoparticles and may impact their therapeutic effectiveness.

Bispecific antibodies are engineered to enhance targeting specificity by binding both to the nanoparticle and the target antigen. However, the non-covalent nature of this interaction introduces the risk of dissociation in vivo, which can reduce targeting efficiency and increase the rate of clearance. This, in turn, affects the biodistribution of the nanoparticles and potentially diminishes therapeutic efficacy. Research highlights that the balance between stability and dissociation is critical for maintaining the desired biodistribution and clearance profiles. Although these nanoparticles are designed to remain in circulation long enough to effectively target specific antigens, the non-covalent binding of bispecific antibodies to nanoparticles may lead to a reduced half-life if dissociation occurs, resulting in faster clearance from the bloodstream.

Comparison of One-Step Formulations for Targeted Liposomal Drug Delivery

In the case of one-step formulation, the direct attachment of engagers to liposomes may expose the entire construct to the immune system, potentially leading to an immune response against the liposomal drug delivery system.[96] The

immunogenicity profiles of three one-step formulation targeted NP strategies differ based on their molecular components and origins. Biotin-based and streptavidin-tagged targeted NPs generally have higher immunogenicity due to biotin-streptavidin, which are foreign substances that the immune system may react to. Targeted NPs based on Fc-binding domains have the potential to trigger immunological responses, particularly when sourced from non-human sources like protein A/G and peptides. Therefore, it is preferable to use human-derived or modified domains to reduce immunogenicity. BsAb targeted NPs offer enhanced targeting through dual specificity and may not induce immune responses, particularly when derived from humanized or full Abs are preferred to reduce immunogenicity. Overall, while bsAbs may offer lower immunogenicity, careful consideration and optimization are required for all strategies to ensure the development of safe and effective targeted drug delivery systems.

The biotin/streptavidin or bsAbs are directly attached to the liposome surface, potentially leading to varied orientations and spatial heterogeneity. This direct coupling may result in a mix of orientations, affecting the accessibility of binding domains and potentially influencing targeting efficiency. The orientation of targeting ligands or engagers on liposomes can significantly influence the binding affinity and targeting efficiency of NPs. In biotin-based and streptavidin-tagged targeted NPs, the orientation of biotin or streptavidin molecules is generally heterogeneous, as Streptavidin's tetrameric structure provides numerous binding sites, which can limit the application of the NPs. In Fc-binding domain-based targeted NPs, the orientation of Fc-binding domains may vary depending on the coupling strategy, potentially affecting the binding affinity and stability of the NPs. For bsAb-targeted NPs, the anti-mPEG component of bsAb binds to the methoxy ends of the mPEG molecules on the surface of PEGylated NPs. This enables the anti-cancer scFv portion of the bsAbs to be homogeneously oriented and allows for the targeted delivery of PEGylated NPs to cancer cells. Overall, the precise orientation of engagers coupled to liposomes is essential for optimizing the binding affinity, stability, and targeting efficiency of targeted NPs across all strategies, highlighting the importance of careful design and characterization in NP development.

The modification complexity of Abs and NPs utilizing a one-step formulation differs significantly from the two-step targeted technique. In the one-step formulation, both the Ab and NPs are modified simultaneously, which simplifies the process. However, a one-step formulation might make it more difficult to fine-tune particular changes that increase complexity. In biotin-based and streptavidin-tagged targeted NPs, the modification process is generally straightforward, involving the attachment of biotin or streptavidin molecules to liposomes followed by the binding of their respective partners, which require more complex engineering to achieve optimal targeting efficiency. For Fc-binding domain-based targeted NPs, the coupling process may require more intricate engineering to ensure proper orientation and stability of the Fc-binding domains on liposomes, potentially increasing the complexity of the modification process. In bsAb-targeted NPs, the modification may offer simpler modification processes. BsAbs can simplify and expedite the conversion of non-targeted PEGylated NPs into targeted NPs without any chemical modification compared to biotin-based or Fc-binding domain-based strategies (Table 2).

Two-Step Targeting Strategy for NP Active Targeting

Pre-Targeting Bispecific Ab + PEGylated NPs

Pre-targeting bsAbs can be accumulated on the surface of target cells to capture the subsequently administered NPs. These pre-targeting strategies can solve problems such as conformational changes, compromise of the stealth feature and hindrance of tumor uptake of NPs[97,98] (Figure 5). Su et al generated a humanized bsAb (PEG engager) by merging a humanized anti-mPEG Fab with a human anti-EGFR scFv which can induce endocytosis of PEGylated NPs into $EGFR^+$ triple-negative breast cancers (TNBCs). This strategy could remarkably enhance the antitumor efficacy of PEGylated therapeutic agents in vitro and in vivo.[99] However, loss of the Fc fragment led to loss-of-function of ADCC and CDC of Abs that might decrease the therapeutic efficacy. A bsAb made by fusion of an anti-mPEG scFv to the C-terminus of an anti-HER2 Ab named HER2 ×mPEG BsAb (IgG-scFv format) was introduced by Chen.[100] Results indicated that bsAbs enhance accumulation and retention by 2.2-fold in the $HER2^{high}$ MCF7/HER2 tumor compared to the HER^{low} MCF7/neo1 tumors. Previously, we also proved that the IgG-scFv format of bsAbs can solve the non-specific targeting cytotoxicity and reduced EPR effect seen in liquid tumors.[101] By using clinical anti-CD20 mAb (Ofatumumab)[102] fused with humanized anti-mPEG scFv, the bsAb enhanced internalization of NPs and internalized up to 56% into CD20-expressing Raji cells compared to the control groups which show no detectable signal of

Table 2 Comparison of One-Step Formulation for Targeted Liposomal Drugs Delivery

Strategy		Immunogenicity	Orientation	Modification complexity	Ref.
Biotin-based × streptavidin-tag	Direct binding of biotinylated Ab (TfR,CD33, CD7, etc.) to streptavidin-tagged NPs	Biotin-streptavidin, foreign compounds, makes streptavidin-tag targeted NPs more immunogenic.	Biotin and streptavidin molecules have varied orientations because streptavidin's tetrameric shape affords many binding sites.	Streptavidin molecules to NPs and their partners' binding require more complicated engineering to optimize targeting efficiency.	[42,59–62]
Fc-binding domain-based NPs × Ab	Interaction of Fc-binding domain-based NPs with Abs (HER2, CD147, CD31, etc).	Fc-binding domains, especially protein A/G and peptides from non-human sources, might cause immune reactions.	Fc-binding domain orientation may change based on the coupling method	To ensure liposome Fc-binding domain orientation and stability, coupling may require more complex engineering.	[73,74,76]
Bispecific antibody (PEG/DIG × marker)	The bsAb (PEG/DIG × markers) can bind to PEGylated or DIGylated NPs.	Humanized bsAbs can reduce immune responses.	Anti-cancer scFv portion of the bsAbs to be homogeneously oriented	BsAbs may quickly and easily convert non-targeted PEGylated NPs into targeted ones without chemical modification.	[87]

internalization within 24 h. Huckaby et al generated a "Fab-IgG" bsAb against ICAM-1, which is constitutively expressed on the luminal surface of the gastrointestinal (GI) epithelium and PEG on the surface of "mucus-penetrating" particles (MPP). The results indicated that the uptake of both non-targeted and actively targeted NPs was substantially decreased by half or more ($p < 0.0001$) when compared to pre-targeted NPs.[103] Parker et al used OrthoMab,[104] a bsAb platform, to construct two bispecific pretargeting molecules (tandem Fab and Fab-IgG1) that bind both breast cancer cell-overexpressed HER2 receptors and PEG on PEGylated liposomal doxorubicin (PLD) and polystyrene beads.[105] In mice treated with a pretargeting dosage of bsAb, the ratio of doxorubicin concentration in the tumor to serum increased by ~3-fold compared to mice treated with PLD alone or bsAb (Table 3).

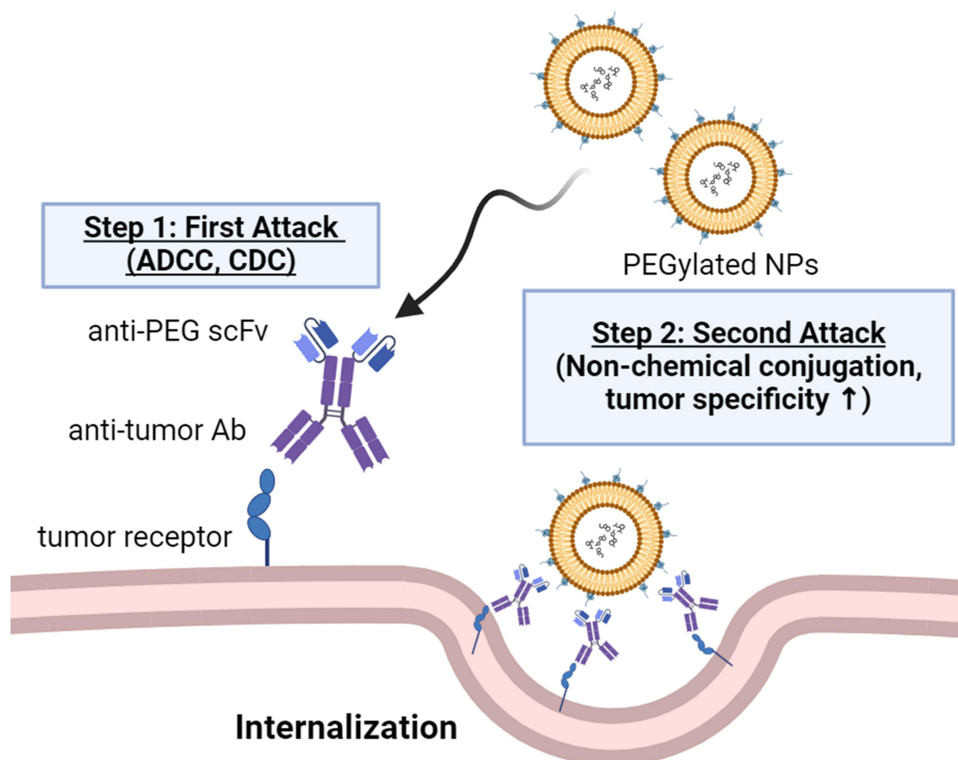

Figure 5 Schematic representation of the two-step strategy for bsAb (PEG × tumor marker) combined with PEGylated NPs. The bsAbs accumulate on the tumor cell surface to activate the first attack of Ab-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Then, bsAbs can capture PEGylated NPs to trigger a second attack by increasing internalization and cytotoxicity. Without chemical modification, the two-step strategy maintains naïve NPs with tumor specificity and enhances drug accumulation in tumor cells.

Table 3 Two-Step Targeting Strategy for Nanoparticles Active Targeting

Two-step targeting strategy	Engager	Nanoparticle (NP)	Payload	Ref
Pre-targeting bsAb + PEGylated NPs	mPEG × EGFR (Fab × scFv)	PEGylated NP	Doxorubicin	[91]
	mPEG × HER2 (scFv × IgG)	PEGylated NP	Doxorubicin	[92]
	mPEG × CD20 (scFv × IgG)	PEGylated NP	Doxorubicin	[94]
	PEG × ICAM-1 (IgG × Fab)	PEGylated NP	N/A	[95]
Pre-targeting bispecific fusion protein	Biotinylated EGFR Ab	Streptavidin-tagged NP	Doxorubicin	[106]
+ PEGylated NPs	Streptavidin-tagged CD20 fusion protein	Biotinylated NP	N/A	[107]

Pre-targeting bsAbs first bind to the tumor antigen and then capture the NPs without affecting conformational changes to enhance the accumulation and internalization in the tumor. By using fully human or humanized Abs to construct the bsAbs the immune response of the bsAbs can be minimized during treatment. Also, the pre-targeting strategy does not affect the physicochemical properties of NPs, including size, shape, surface charge, and composition, which play crucial roles in determining their immunogenicity. BsAbs utilize 15–2b or 6.3 scFv to specifically attach to the methyl group or backbone of PEG.[108,109] These scFv alter NP interactions and properties by specifically targeting locations on PEG molecules. This adaptability enhances the stability, distribution, and effectiveness in targeting for medical treatment and diagnostic purposes, thus advancing nanomedicine. By engineering the variable domains of the bsAbs, researchers can tailor the binding specificity to different tumor antigens or epitopes, allowing for precise targeting of cancer cells. A significant hurdle arises when considering higher doses of pre-targeting bsAbs, as this approach might potentially reduce the binding and accumulation of NPs in tumor cells. The challenge stems from the spatial and temporal heterogeneity in tumor receptor expression.[110] Hence, developing a "personalized" pre-targeting strategy could be optimized and allow an ideal concentration of bsAbs against the heterogeneous nature of cancer cells, especially in cases of relapse or highly metastatic tumors.[106] It is known that attaching targeting ligands directly to NPs may compromise the stealth characteristics of the NPs, leading to faster clearance and diminished uptake in tumors.[97] The two-step targeting strategy of bsAbs is not expected to impact the pharmacokinetics (PK) and stability of NPs during treatment. However, Yang et al indicated that extra circulating bsAbs binding to NPs afterward can enhance NP clearance before NPs extravasate into tumors.[106,107] To optimize both tumor distribution and retention while addressing systemic clearance, diverse strategies have been employed, such as modulation of treatment timing,[53,54] size,[111] valency,[112] and composition[113–115] of pre-targeting bsAbs. The chemical conjugation of Abs onto NPs may impact the translation of drugs to the clinic including the amount of time required, the ligand aggregation phenomenon, the bioactivity of targeting ligand reduction, and cost.[116] Also, Su et al mentioned that variations between different batches of targeted NPs can impede the process of applying them to clinical use and making them available for commercial purposes.[99]

Pre-Targeting Biotinylated Ab + PEGylated NPs

Pre-targeting with biotinylated Abs can capture streptavidin based on PEGylated NPs to improve tumor targeting and drug accumulation at the tumor area. In the biotin-streptavidin ligation technique, the tumor-targeting proteins such as Abs and derivatives conjugate either biotin or streptavidin, and then attach the NPs to the complementary biotin or streptavidin component (Figure 6). Lehtinen et al employed EGFR Abs conjugated with biotin, which were affixed onto the exterior of PEGylated NPs with streptavidin. They explored the process of cellular uptake influenced by receptors and assessed the effectiveness of EGFR-targeted liposomes in causing cell death in human ovarian adenocarcinoma cells.[117] In summary, the direct targeting of liposomes to EGFR proved to be both specific and effective in ovarian cancer cells when tested in vitro. Hang et al used biotinylated anti-CEA Abs to bind cancer cells and collect streptavidin-based NPs for molecular ultrasound imaging and chemotherapy in ovarian cancer treatment. Red fluorescence from the confocal laser scanning microscope showed that DiI-labeled NPs were more attracted to SKOV3 cells than the drug-loaded direct-

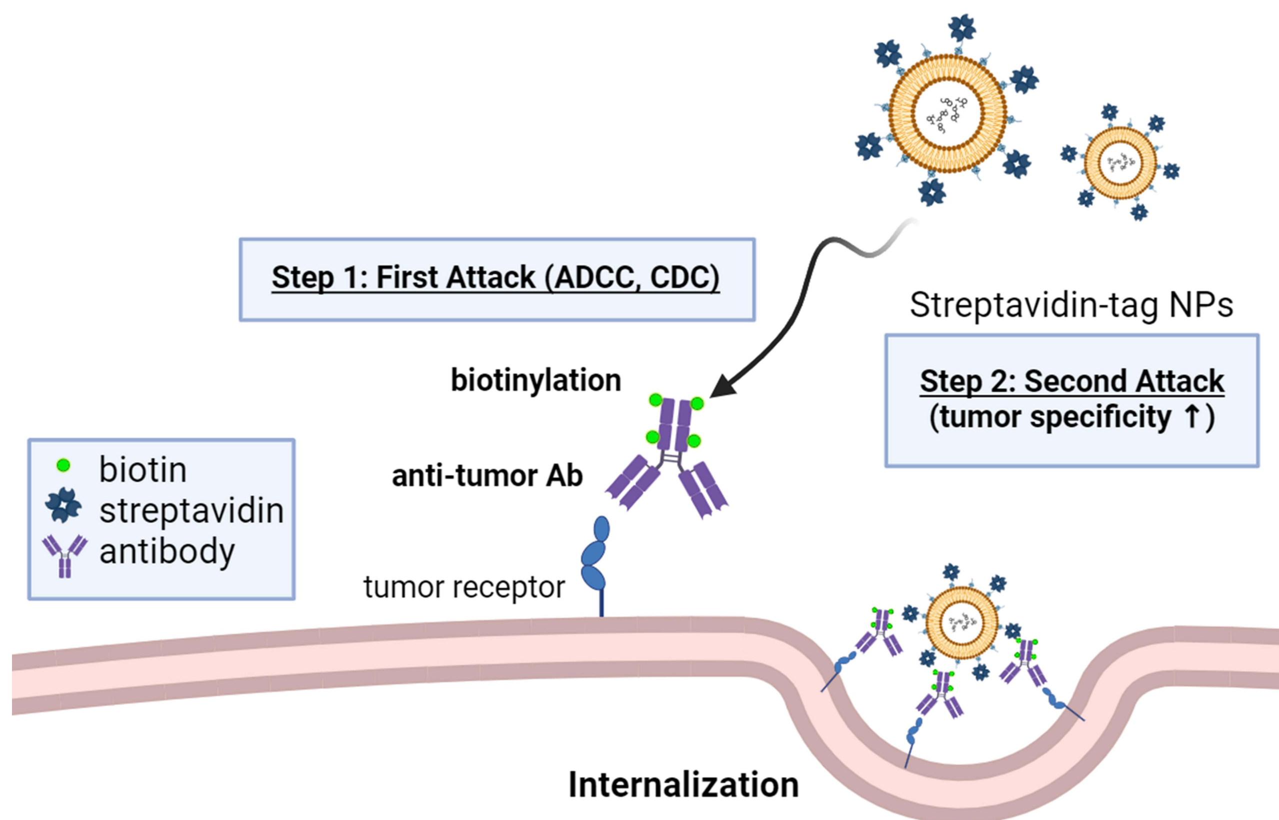

Figure 6 Schematic representation of the two-step strategy for biotinylated Abs combined with streptavidin-tagged NPs. The N-hydroxysuccinimide biotin cross-links to a primary amine of Ab to form biotinylated Abs. The biotinylated Abs accumulate on the tumor cell surface to activate the first attack of ADCC and CDC. Then, the biotin on the Abs can interact with the streptavidin which is modified on the NPs to trigger a second attack by increasing internalization and cytotoxicity. This two-step strategy offers a versatile approach for the development of streptavidin-tagged NPs, allowing for enhanced specificity and efficacy of drug delivery applications.

targeting group and the control group. Yang et al also examined a pre-targeting approach to enhance the delivery of NPs to various target cells. These cells were pre-treated with diverse bispecific streptavidin-scFv fusion proteins (*eg*, CD20, tumor-associated glycoprotein) and then the absorption of biotinylated NPs was assessed.[118] Pre-targeting of Raji cells with streptavidin-αCD20 scFv resulted in a ~15-fold greater uptake of fully biotinylated PS-PEG-biotin NPs compared to PEGylated NPs without biotin functionalization ($p < 0.01$) (Table 3).

Product-related impurities in bsAb or fusion proteins, such as aggregation and fragmentation are critical considerations in the development and manufacturing of biotinylated Abs. Aggregation refers to the clumping or clustering of protein molecules. In biotinylated Abs, it can occur when individual protein units form aggregates, compromising the product's quality, safety, and efficacy resulting in loss of targeting function and immune response.[119–122] Process-related impurities like imidazole can arise during the manufacturing and purification processes. Improper washing and elution steps during chromatography can lead to the carryover of imidazole into the final product. Also, the presence of imidazole above permissible levels may have safety implications. Those impurities are essential for ensuring the safety, efficacy, and regulatory approval of biotinylated Abs in therapeutic applications. Continuous monitoring, optimization of manufacturing processes, and adherence to regulatory standards are key elements in mitigating these challenges. The immunogenicity of biotinylated Abs is an important consideration in drug development, as an immune response can impact the safety and efficacy of the therapeutic. The risk of immunogenicity is influenced by several factors, including the degree of humanization,[123] choice of expression systems,[124,125] and post-translational modifications.[126] Monitoring immunogenicity during clinical trials through robust assays for anti-drug Abs is standard practice, allowing for early detection and evaluation of potential immune reactions. Addressing immunogenicity challenges in biotinylated Abs is crucial to ensure their safety, efficacy, and successful clinical translation. The stability of biotinylated Abs on NPs is crucial for their effective therapeutic application. The interaction between

biotinylated Abs and NPs can impact the stability of both entities, influencing their pharmacokinetics and overall therapeutic performance.[127] Proper stabilization is crucial to prevent protein unfolding, or degradation during storage and administration.[128,129] Factors such as surface modifications of NPs, choice of coupling techniques, and the physicochemical properties of the fusion protein play essential roles in maintaining stability.[130] Advanced analytical techniques, such as size-exclusion chromatography and dynamic light scattering, rigorously assess the stability of biotinylated Abs on NPs. Addressing stability challenges ensures the preservation of the therapeutic integrity, bioactivity, and safety of these complexes, promoting their successful application in targeted drug delivery and precision medicine.

Comparison of Two-Step Targeting Strategies for Targeted Liposomal Drug Delivery

On the other hand, pre-targeting bsAbs or fusion proteins capture liposomes involving a two-step process, where bsAbs or fusion proteins first bind selectively to target cells before the introduction of liposomes. This strategy may mitigate immunogenicity concerns, as the liposomes are introduced after the initial targeting step, potentially reducing the exposure of the entire construct to the immune system. The immunogenicity of bsAbs and biotinylated antibodies is influenced by their molecular characteristics and origins. Biotinylated antibodies are typically derived from natural Abs and undergo biotinylation, which involves the attachment of biotin molecules to specific sites on the Ab structure. The biotinylation process is generally well-tolerated, and biotin itself is a naturally occurring molecule in biological systems, leading to low immunogenicity. Humanized or fully human bsAbs are developed to minimize immunogenicity by reducing the potential for immune recognition of non-human sequences. However, due to the foreign protein component, streptavidin-tagged NPs, as opposed to PEGylated NPs, could cause immunogenicity by repeated administration over time.

The modification strategies for PEGylated NPs in comparison with streptavidin-tagged NPs differ in their complexity and the nature of the surface modifications. Clinical use of PEGylated NPs has been established for an extended period. In contrast, Streptavidin-tagged NPs require further incorporation of streptavidin through chemical conjugation increasing the complexity and potential for off-target effects. The additional chemical conjugation steps can complicate the nanoparticle synthesis process, potentially leading to reduced reproducibility and increased variability in nanoparticle properties, which can affect their stability, biocompatibility, and targeting efficiency. The choice of nanoparticle modification strategy should be carefully considered based on the desired properties, application requirements, and potential immunological considerations to optimize the performance and safety of the nanoparticles in therapeutic and diagnostic applications (Table 4).

Comparison of the One-Step Formulation and Two-Step Targeting Strategies

A comparison between one-step formulation and two-step targeting is pivotal in understanding their distinct advantages. Both approaches contribute to advancing targeted drug delivery, offering tailored solutions for diverse therapeutic applications. In the one-step formulation, the Ab and NPs are simultaneously formulated, potentially leading to faster production times and simpler procedures. However, this method may result in lower stability due to non-specific interactions between the Ab and NPs during formulation. Increasing the affinity of Abs might ensure a stable and specific binding, preventing premature dissociation of bsAbs from liposomes in physiological conditions. This stability is

Table 4 Comparison of Two-Step Targeting Strategies for Targeted Liposomal Drugs Delivery

Pre-targeting Strategy	Capture Strategy	Immunogenicity	Nanoparticle modification	Affinity of bsAb interact with NPs (K_d)	Targeted internalization	Ref.
PEG bsAbs	PEGylated NPs	Humanized antibodies can reduce the immunogenicity	Naïve PEGylated NPs is no need to modification	10^{-8}~10^{-9} M	Multiple-epitopes interaction can enhance internalization of NPs	[98,99,101–105,108,109]
Biotinylated Abs	Streptavidin-based NPs	Biotinylated Abs can cause immune response limited the sequential treatments	Need to modify streptavidin. Chemical conjugation damage to Ab and modification complexity	~10^{-15} M	Multiple- epitopes interaction can enhance internalization of NPs	[111–122]

Table 5 Comparison of Two Strategies for Targeted Liposomal Drugs Delivery

Strategy	Stability during treatment	Treatment Timing
One-step formulation	Lower stability due to non-specific interactions between the antibody and NPs during formulation.	Allowing immediate administration after completion
Two-step targeting	The antibody and NP components are adjusted separately to manage their interactions and possibly improve stability.	Longer preparation time before treatment administration

essential for maintaining the integrity of the targeted liposomes during circulation in the bloodstream, preventing nonspecific interactions, and reducing the risk of premature payload release.[131] Ultimately, the choice between a one-step or a two-step strategy depends on the specific requirements of the application and the desired balance between efficiency and stability.

The comparison between the one-step formulation and the two-step targeting strategy of Ab and NPs also extends to treatment timing. In the one-step formulation, immediate administration can be conducted upon completion. This streamlined approach may be advantageous for situations requiring rapid treatment initiation. However, the two-step targeting strategy involves separate optimization of Ab and NP modifications before conjugation, which may result in a longer preparation time before treatment administration. In the two-step targeting strategy, it is crucial to minimize the circulating levels of pre-targeting bsAbs during the administration of NPs. Elevated concentrations of bsAbs can result in the entrapment of NPs within the bloodstream, impeding their efficient extravasation to the intended targeted tissues. This emphasizes the need for a precise balance in the timing and concentration of bsAbs to support, rather than hinder, the effective delivery of NPs to the intended target sites.[105,132] Maintaining the persistence of bsAbs on the tumor cell surface at the time of NP administration is crucial, preventing premature internalization that could hinder subsequent NP binding. Achieving an optimal time interval between bsAb and NP administration, balancing systemic clearance and tumor surface retention, is vital for effective pre-targeting drug delivery.[133] Understanding the kinetics of bsAb clearance and their behavior at the tumor site is imperative for developing an efficient pre-targeting drug delivery system. Therefore, the choice between one-step and two-step strategies depends on the urgency of treatment initiation and the desired level of control over the conjugation process (Table 5).

Conclusion and Future Prospects

The comparison of the one-step formulation technique and the two-step targeting strategy highlights the intricate factors involved in creating efficient, targeted drug delivery systems. Combining biotin/streptavidin, Fc-binding domain, or bsAbs directly onto liposomes in a one-step process simplifies manufacturing but could present issues with immunogenicity and inconsistent Ab orientations, which might affect targeting effectiveness. Pre-targeting bsAb or fusion proteins that trap liposomes offer a two-step method that may decrease immunogenicity and provide more consistent Ab orientations, potentially improving targeting accuracy. The selection of a strategy depends on therapeutic objectives, taking into account aspects including immunogenicity, targeted specificity, and the complexity of the drug delivery system.

PEGylated products remain widely accessible in clinical practice. The continuous advancement of nanomedicine and biotechnology holds promise for enhancing these techniques and ultimately improving the precise delivery of therapeutic agents for various biological applications. Anti-PEG bsAbs give PEGylated NPs targeted capabilities, improving their clinical potential. The incorporation of anti-PEG bispecific antibodies offers a transformative approach to overcome this limitation by conferring PEGylated NPs with targeted capabilities. Anti-PEG bispecific antibodies enable the specific and dual targeting of PEGylated NPs to both PEG and a specific cell surface marker, enhancing the precision, efficiency, and selectivity of nanoparticle delivery. This targeted approach can significantly improve therapeutic efficacy, reduce systemic toxicity, and enhance patient compliance by minimizing off-target effects and the required drug dosage. Furthermore, the active targeting facilitated by anti-PEG bispecific antibodies can broaden the clinical applications of PEGylated NPs, particularly in the treatment of heterogeneous or resistant cell populations and in immunotherapy. Overall, the one-step formulation of anti-PEG bsAb and PEGylated NP not only accelerates the development of targeted

nanomedicine but also enhances its clinical feasibility, scalability, and translational potential, paving the way for the advancement of personalized and precision medicine approaches in various therapeutic areas.

In current clinical trials, bispecific antibody-targeted lipopolysaccharides (LPS)-packaged micelles are a leading strategy for targeted cancer therapy, as shown by the Phase I trial of EGFR-targeted, paclitaxel-loaded minicells.[134] While these micelles are advancing in clinical studies, PEGylated nanoparticles, despite extensive preclinical research, have yet to enter clinical trials, underscoring the challenges of bringing PEGylated formulations to clinical use. Currently, non-covalent conjugation methods, particularly in the context of PEGylated nanoparticles, are still primarily in the preclinical or early clinical phases, and thus not extensively represented in the available clinical trial databases or published studies. Additionally, much of the existing literature and ongoing trials tend to focus on more established covalent conjugation techniques, further contributing to the scarcity of clinical data on non-covalent methods. We recognize the importance of this area and understand that more detailed clinical reporting and further research are essential to fully elucidate the clinical translation potential of these nanoplatforms.

Abbreviations

Ab, antibody; ADCC, Ab-dependent cellular cytotoxicity; AUC, area under the curve; bsAb, bispecific antibody; CDC, complement-dependent cytotoxicity; DIG, digoxigenin; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; EPR, enhanced permeability and retention; FcBP, Fc-binding peptide; GI, gastrointestinal; HER2, Human epidermal growth factor receptor 2; LGA-PEI, lactic-co-glycolic acid-polyethylenimine; MR, magnetic resonance; NP, nanoparticles; PEG, polyethylene glycol; PILP, PEGylated immuno-lipopolyplexes; PK, pharmacokinetic; PLD, PEGylated liposomal doxorubicin; QD, quantum dot; RES, reticuloendothelial system; SAL, streptavidin-tagged liposome; scFv, single chain Fv; TfR, transferrin receptor; TNBC, triple-negative breast cancer.

Author Contributions

All authors made a significant contribution to the work reported, whether that is 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 report no competing interests in this work.

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