Review

*Discovery Medicine* 2023; 35(178): 697–714
https://doi.org/10.24976/Discov.Med.202335178.67

# Current Protein Conjugation Strategies and Pioneering Anti-Cancer Applications

Candice Maria Mckertish[1], Veysel Kayser[1,*]

[1]Sydney School of Pharmacy, Faculty of Medicine and Health, The University of Sydney, Sydney, NSW 2006, Australia
*Correspondence: veysel.kayser@sydney.edu.au (Veysel Kayser)
Published: 1 October 2023

**The escalating global impact of cancer demands targeted and efficient therapies. Many targeted cancer therapeutics have been fostered into the market in recent years. The leading class of targeted therapeutics is monoclonal antibodies (mAbs). The success of mAbs sparked significant interest and subsequently paved the way for the development of various recombinant protein-based products that were conjugated to other compounds to form antibody-drug conjugates (ADCs) for the treatment of cancer. This review explores diverse mAb conjugation strategies and pioneering approaches for cancer applications, to optimize drug delivery through site-directed conjugation, prodrug conjugates and antibody conjugated nanoparticles. It also highlights promising innovations and addresses challenges including stability issues of ADCs, drug resistance, payload retention and some of the shortcomings of ADCs such as limited bioavailability, short serum half-life, and reduced drug buildup caused by obstacles in the tumor environment primarily associated with heightened interstitial fluid pressure (IFP).**

**Keywords:** site-specific conjugation; ADC; antibody prodrug conjugates; antibody conjugated nanoparticles

## Introduction

Cancer is a growing disease burden worldwide and the rate of mortality amongst some cancer types such as lung cancer is on the rise when compared to other cancers. It is estimated that the overall global new cancer cases will reach up to 27.5 million by the year 2040, a substantial increase from 18.1 million in 2018 [1–3]. In the last three decades, extensive effort has been made to develop monoclonal antibody (mAb) based therapeutics that selectively target tumors with fewer side effects compared to conventional therapies such as radiation or chemotherapy, which is mostly associated with systemic distribution and hence linked to an array of side effects (e.g., nausea) [4,5]. mAbs can be designed to bind to a wide range of overexpressed target receptors on the surface of tumors, subsequently triggering the immune system and exerting its therapeutic effects through different mechanisms: complement-dependent cytotoxicity (CDC); antibody-dependent cell-mediated cytotoxicity (ADCC); antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cellular cytotoxicity (CDCC) [6–8].

The advancements in mAb based therapeutics have paved the way for the development of antibody-drug conjugates (ADCs), a treatment approach that demonstrated efficacy in the delivery of the payload to the tumor site to cause cell death and apoptosis at lower concentrations than the mAb solely while providing target specificity [9–11]. However, there are many factors that are responsible for the failure rates of ADCs in pre-clinical studies [12]. Linker instability is one of the contributing factors [12]. In recent years researchers have found that the widely enzymatically cleavable valine-citrulline (VC) linker, specifically the VC linker is mis-cleaved by an extracellular carboxylesterase 1c (Ces1c) enzyme, consequently the payload is released prematurely in the mouse xenograft model [13]. The primary concern of poor ADC stability in the circulation is adverse effects resulting in poor patient adherence and treatment delays. Recent findings suggest that the use of glutamic acid-glycine-citrulline (EGCit) linkers in design of an ADC can mitigate this concern as the linker has the ability to defy degradation mediated by the neutrophil protease [14].

Traditionally, the linker-payload moiety is conjugated to a mAb mainly via cysteine or lysine residues in an ADC, C- or N-terminals, or via site-directed conjugation [12]. Although there is plenty of surface lysine, free cysteine is usually formed by breaking one or more disulfide bonds on a mAb which can result in structural instability. Site-directed conjugation involves engineering specific sites on the antibody to attain a homogenous ADC. This approach can overcome the limitations associated with heterogenous ADC design in some of the former methods wherein the structural integrity, stability, therapeutic index, and pharmacokinetics might be compromised [10,15–18]. There are different site-directed conjugation strategies including THIOMAB™ drug conjugate (TDC) technology, incorporation of unnatural amino acids (UAAs), site-specific glycan conjugation, and peptide tags for site-specific conjugation [10,16–18].

Copyright: © 2023 The Author(s). Published by Discovery Medicine. This is an open access article under the CC BY 4.0 license.
Note: *Discovery Medicine* stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

The antibody conjugated nanoparticle (ACNP) is a relatively novel format where targeted drug delivery and enhanced efficacy of nanoparticles are achieved by the specificity of antibodies [19–22]. Nanoparticles (NPs) are commonly designed in various sizes, i.e., inorganic NPs are usually up to 100 nm in size to ensure tumor entry and liposomal NPs are usually 80–100 nm [23,24]. Smaller NPs warrant penetration but suffer from poor accumulation in the tumor site. On the other hand, larger NPs present good accumulation with poor tumor penetration characteristics [25]. Tumor microenvironments can differ due to factors such as variation in acidity, overexpressed enzymes, and other factors [22,24,26]. Thus, by tuning the size and modification of the surface via different functional groups, tumor penetration and retention of nanoparticles could be tailored to the tumor microenvironment [22,24–28].

There are various classes of nanoparticles including polymeric, inorganic and lipid nanoparticles that possess many characteristics such as prolonged circulation time, and transportation across membranes, that can overcome common limitations in drug transport and delivery [29,30]. Out of which gold nanoparticles (AuNPs) have been widely studied along with magnetic iron oxide NPs, composed of magnetite ($Fe_3O_4$) or maghemite ($Fe_2O_3$) which can be used as drug delivery vehicles and thermal based therapeutics [22,31]. However, some of the limitations including low solubility and toxicity concerns require further investigation [30,32].

So far, there have been numerous liposomal nanomaterials that have gained regulatory approval [33,34]. Out of which, Doxil®, Onivyde®, Marqibo kit®, Myocet®, Mepact®, and Vyxeos® DaunoXome®, Lipusu®, Abraxane®, Genexol-PM®, PICN®, Paclical®, Hensify®, DHP107®, NanoTherm®, Nanoxel®, Depocyt®, were approved for the treatment of various types of cancer [7,34–36]. PEGylated (PEG) liposomes have shown an improved circulation time and enhanced bioavailability compared to non-PEGylated liposomes. Along with the various advantages of PEGylated liposomes, off-target toxicities can also be minimized with the attachment of mAbs onto the surface of liposomes [20].

With the growing burden of drug resistance for the treatment of some cancers, the development and discovery of prodrug conjugates as a new modality emerged as a promising approach. The idea of prodrug conjugates was somewhat based on the mechanism of action of cleavable linkers requiring cleavage by a specific protease to release the payload [37]. To this end, novel prodrug conjugates such as peptide drug conjugates (PDCs) have gained interest recently. Much like an ADC, a PDC is comprised of a peptide that is attached to a linker and a cytotoxic payload [38,39]. The small size of the peptide is a highly advantageous characteristic for some applications. For example, for cancers that tend to metastasize in the brain, small size peptide constructs have the ability to penetrate the blood-brain barrier (BBB) and can be quickly cleared from the blood circulation [40,41]. Realizing the full potential of this approach is important considering the limited options currently available to treat this type of cancer, and inoperable or unresectable brain tumors due to their location and the risk of injury to the brain entailed during surgery [42]. Moreover, the prevalence of brain cancer is small with a ten-year survival rate of less than 32% [43]. This alarming figure affirms that research towards the treatment and development of novel therapies against brain cancers is of considerable importance.

This review delineates various mAb conjugation strategies and approaches that have the potential to enhance drug delivery and therapeutic efficacy, for various cancers particularly in the challenging context of brain cancers, where limited treatment options and low survival rates demand novel therapeutic approaches to potentially revolutionize the treatment landscape.

## Antibody Site-Directed Conjugation Strategies

Commonly used conjugation strategies for ADCs involved conjugation of a linker-drug complex to glycosylation groups, cysteine, or lysine residues on the mAb that produce a more heterogenous ADC [44]. Site-specific drug attachment leads to more homogeneous conjugates and allows control of the site of drug attachment along with several other advantages including improved therapeutic index and an extended serum half-life [45]. This process involves conjugating a known number of cytotoxic payloads to engineered sites on the mAb, that are stable conjugation sites without affecting its ability to bind to the antigen [10,16–18] and attaining a specific drug-antibody-ratio (DAR) or pharmacokinetics [46]. Leading the way is the application of the recently developed AJICAP® technology involving chemical conjugation to produce site-specific ADCs [47]. A recent study utilized the AJICAP® technology to develop Trastuzumab-AJICAP-MMAE, the study employed various analytical methods to determine the DAR of the purified and unpurified Trastuzumab-AJICAP-MMAE. The resulting DAR for the purified sample was 2.0 and the unpurified sample was 1.6. A similar result was generated across all analytical methods that were used in the study [48]. There have been several approaches to achieving site-specific conjugation. The current conjugation strategies are cysteine (Cys) engineering using THIOMAB™ technology, incorporation of UAAs, site-specific glycan conjugation, peptide tags for site-specific conjugation, and C- or N-terminal conjugation [49–52]. Fig. 1 illustrates commonly used site-specific conjugation techniques. All figures were created using Adobe illustrator software (version 26.0.2, Adobe Inc., San Jose, California, USA).

Overall, each site-specific conjugation approach has its strengths and weaknesses. Site-directed mutation using THIOMAB™ offers controlled, homogenous ADCs

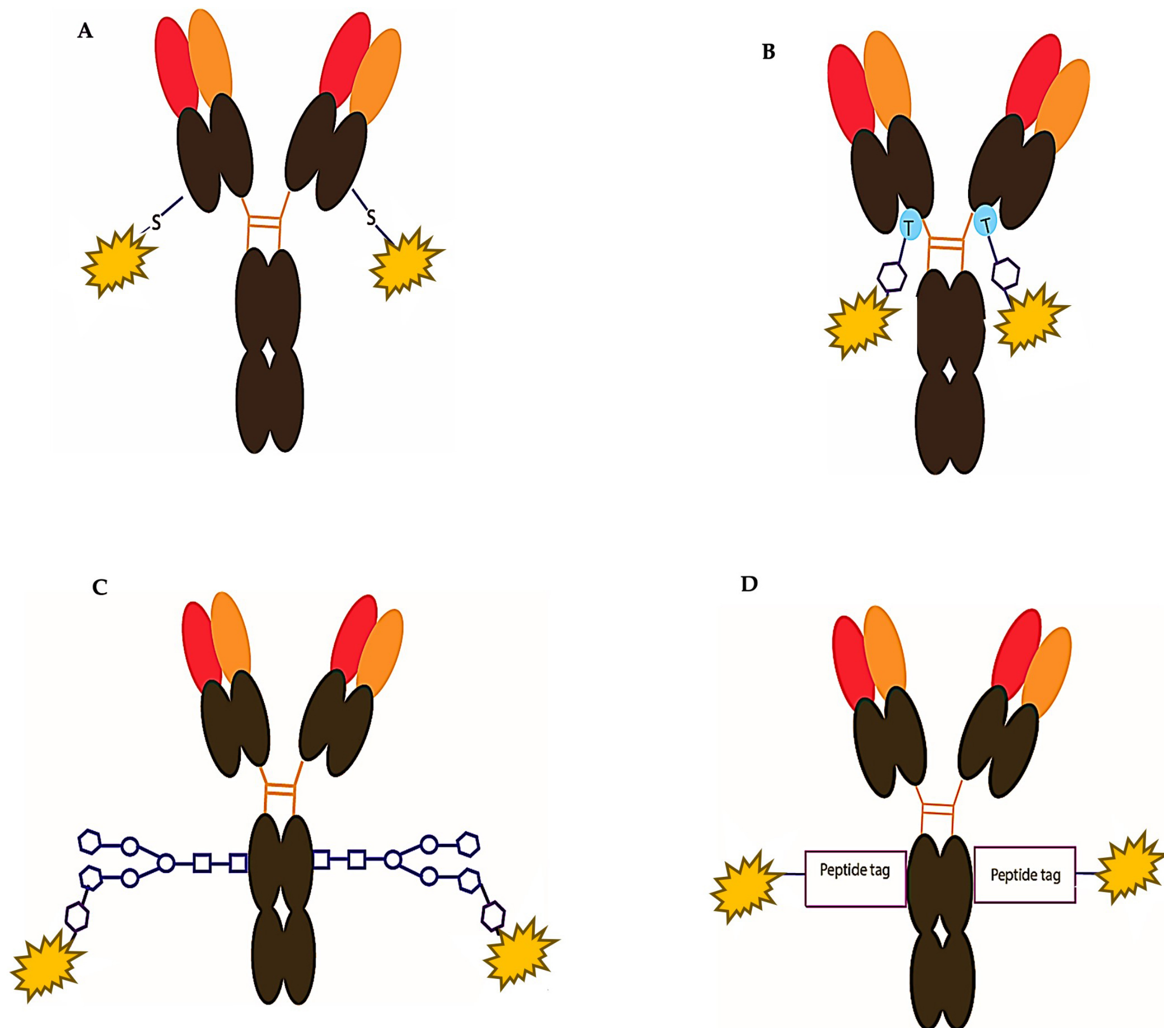


**Fig. 1. Major classes of site-specific conjugation techniques.** (A) Cysteine-mediated conjugation using THIOMAB™ technology, (B) antibody reengineering to incorporate unnatural amino acids, (C) site-specific glycan conjugation, and (D) coupling unique short peptide tags to drug-linkers.

with improved stability and selectivity, while UAAs offer promise in achieving enhanced *in vitro* and *in vivo* effects. Site-specific glycan conjugation preserves antigen binding and offers consistent glycan patterns, but the process is complex. Peptide tags provide a versatile method for enzyme modification but require specific tag introduction and costly enzymes. These site-specific conjugation approaches are explored in the following sections.

### *Site-Directed Cysteine Conjugation*

A commonly used method of Cys conjugation incorporates disulfide bond breakage and results in a heterogenous ADC [12,50]. *In vivo* studies, using this method of Cys conjugation demonstrated that a DAR of 4 was optimal, but when the DAR was decreased to 2, the performance of the ADC decreased while tolerability was improved [53]. Reducing the interchain disulfides involves using a reducing agent such as DTT that results in a heterogenous ADC that exposes between 2–8 sulfhydryl groups preceding linker-payload conjugation [12,50,54]. This method of conjugation using disulfide bond breakage was adopted in the development of the ADC, Brentuximab vedotin (Adcetris®: Seattle genetics) [12]. However, ADC heterogeneity makes it challenging to predict the stability, therapeutic window, structural integrity, and pharmacokinetics [55–57].

However, site-directed mutation can be used to address ADC heterogeneity and this method has many advantages. THIOMAB™ or TDC technology which is Cys engineering method involves the introduction of Cys residues on the heavy and light chains of the constant domain of the fragment antigen-binding (Fab) region on mAb to attain a homogenous ADC which allows control in the number and location of conjugation sites without hindering the binding site of the mAb as well as other advantages such as high potency, stability, structural integrity and selectivity (Fig. 1A) [49,58–61].

This method of conjugation was employed with the ADC Polatuzumab vedotin (Polivy®: Genentech/Roche) [62,63]. A study that focused on targeting Mucin 16 (MUC16), an antigen overexpressed in ovarian cancer, compared the *in vivo* properties of an ADC with a DAR ~3.1 and TDC with a DAR ~1.6, and interestingly demonstrated that the TDC had comparable antitumor activity with lower toxicity and an improved therapeutic index in comparison to the ADC with a higher drug loading [17]. According to these results, an improved therapeutic index of the TDC means better tolerability and thereby a higher dose administration.

Site-directed conjugation can also be used to address antigen binding. Antigen binding in some cases is compromised following conjugation. Glycoengineering approaches ensure the antigen site is unaffected by conjugation, attributed to the distance from the engineered site to the antigen binding site. However, the greatest concern with this approach is the generation of homogenous glycans. Apart from this, it is challenging to control the location of the modification on the mAb and the sum of modifications [64].

Enzymatic modifications involving the use of short peptide tags have advantageous selectivity and stability capabilities. Additionally, this method has the potential to attain a more predictable DAR [64]. Although there is no requirement for any engineering, specific enzymes and reagents necessary for this method of conjugation are expensive, moreover, the immunogenicity of peptide tag conjugation has not been widely studied [52].

### *Engineering the Antibody to Incorporate Unnatural Amino Acids (UAAs)*

Site-specific incorporation of UAAs involves specific alteration of the ketone groups without hindrance from other amino acids (Fig. 1B) [65]. It requires co-expression of tRNA/aminoacyl-tRNA synthetase (aaRS), an amber suppressor and the antibody in a mammalian cell culture such as Chinese hamster ovary (CHO) cells, to integrate engineered unnatural amino acids such as p-acetylphenylalanine (pAcF) or selenocysteine onto an antibody using an amber stop codon (UAG) [66,67]. The pAcF unnatural amino acid contains a ketone functional group that is absent in natural amino acids [68].

A study that site-specifically integrated pAcF on an anti-HER2 (human epidermal growth factor receptor 2) antibody comprised of a potent tubulin inhibitor auristatin via an oxime linker showed promising results, demonstrating potent *in vitro* cytotoxicity [68].

In another study, a different approach was employed using cell free protein synthesis to demonstrate that the incorporation of para-azidomethyl-l-phenylalanine (pAMF) promotes conjugation between the auristatin payload and the HER2 binding mAb [69]. As a result, the ADC showed promising *in vitro* results. Moreover, an anti-HER2 ADC, ARX788 that incorporated unnatural amino acids into its design [70,71] is currently undergoing Phase I clinical trials for solid tumors that express the HER2 antigen (NCT03255070) [72]. Compared to TDCs, UAAs tend to exert superior effects *in vivo* [49].

### *Site-Specific Glycan Conjugation*

Glycosylation is important for the aggregation propensity and structural stability of glycoproteins including monoclonal antibodies [8,56,73,74]. Site-specific glycan conjugation involves conjugating the drug-linker to existing glycans that present in the fragment crystallizable (Fc) region attached to N297 at the CH2 domain instead of coupling hydrophobic cytotoxins via linkers into amino acid residues such as Cys or lysine (Lys) (Fig. 1C) [49]. Alternatively, additional glycosylation sites can be introduced for this purpose [74]. This site-specific conjugation approach has several advantages; it does not hinder the antigen binding domains since conjugation is at the Fc region of the antibody and in addition, glycan remodeling is ideal since the glycosylation pattern around the antibody is fairly consistent [51]. There are several different monosaccharides present at the non-reducing terminus of the glycans: including fucose, galactose, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), and sialic acid (SA) [49,51,74,75]. The monosaccharides act as a chemical handle to conjugate cytotoxic payloads preceding oxime ligation of the payload.

There are two steps involved in conjugating the drug-linker to the glycans: the first step involves trimming the antibody's native glycan via $\beta$-galactosidase, which cleaves the terminal galactose moieties or via endoglycosidase, which cleaves after the innermost GlcNAc moiety. The second step involves the rebuilding of the glycan by installing an unnatural sugar moiety containing a handle for further bioconjugation [54]. For conjugation using the GlyCLICK® (Genovis AB, Sweden and Cambridge, MA, USA) procedure to produce conjugates with DARs of 2 and 4, the first step is performed at room temperature which involves cleavage of the native glycan after the innermost GlcNAc moiety endoglycosidase, the trimmed antibody is readily separated from the immobilized enzyme. Step 2 involves $\beta$-1,4-galactosyltransferase-mediated installation of the azide-functionalized unnatural sugar that occurs overnight at 30 °C [54,76]. In the last step, the alkyne-functionalized payload reacts with the azide handle at room temperature.

Furthermore, an anti-NaPi2b (sodium-dependent phosphate transport protein 2b) ADC (XMT-1592), developed by Mersana therapeutics, is currently undergoing Phase I/II clinical trials (NCT04396340), indicated for ovarian cancer or non-small cell lung cancer (NSCLC) that express the antigen sodium-dependent phosphate transport protein 2b (NaPi2b) [72]. The ADC employed the auristatin microtubule inhibitor, F-hydroxypropylamide

(F-HPA). It uses Synaffix GlycoConnect™ technology that is similar to GlyCLICK® technology, cleaving native glycans on the mAb, followed by attaching a sugar handle for the payload conjugation [54,77]. The anti-NaPi2b ADC yielded a DAR of 6 [78].

### *Peptide Tags for Site-Specific Conjugation*

Peptide tag site-specific conjugation introduces unique short peptide tags onto antibodies for enzyme modification either *in vivo* or *in vitro*. They allow specific amino acids in the peptide tags to be functionalized and coupled to the drug-linkers, i.e., coupling of cytotoxins to specific short peptide tags that contain four to six amino acid residues (Fig. 1D) [49,66,79].

There are multiple strategies to utilize peptide tags in site-specific conjugation. Bond forming enzymes such as microbial transglutaminase (mTG), sortase A (SrtA) and formylglycine generating enzyme (FGE) recognize and commonly catalyze the attachment of glutamine moieties to the mAb at the C or N terminal the conjugation of these unique peptide tags to the mAb [50,51,67,79,80]. These tags can be attached to small molecules or to amino acids at different sites on the antibody. Sortase A usually recognizes the five-residue motif, LPxTG. It exchanges a part of the recognition sequence with a separate peptide conjugated to a labelling moiety through cleavage of the amide bond between glycine and threonine of the LPxTG motif and thereby SrtA catalyzes the attachment of glycine functionalized drugs at the C-terminal of the mAb [50,51,80,81]. FGE enzyme works by oxidizing a cysteine residue to formylglycine. It recognizes the six-residue motif, LCxPxR which is an aldehyde-tag [50,80,81].

An ADC, TRPH-222, targeting the CD22 antigen with a DAR of 2, incorporated fully-galactosylated IgG on each heavy chain of the mAb. It employed a non-cleavable linker and a maytansinoid microtubule inhibitor, and it has completed Phase I clinical trials (NCT03682796) for the treatment of relapsed/refractory B-cell lymphoma [72].

Moreover, microbial transglutaminase (mTG) could catalyze bond formation using a wide range of amino substrates by forming isopeptide bonds between primary amines and glutamine [45,51,82,83]. In addition, Strop *et al*. [55] demonstrated the efficacy of mTG in recognizing the glutamine tag and catalyzing the reaction by engineering a glutamine tag (LLQG) on the mAb. They showed that mTG successfully conjugated twelve out of ninety sites in an anti-epidermal growth factor receptor (anti-EGFR) antibody. They used an amine containing drug valine-citrulline monomethyl auristatin D (VC-MMAD) to amplify the ability of the enzyme to recognize and transfer the drug to glutamine tags LLQGA in the C-terminal heavy chain and GGLLQGA in the C-terminal light chain that were engineered into an antibody molecule. The study found that the site of attachment of the peptide tags impacts not just pharmacokinetics but also conformational stability. This approach has the ability to produce ADCs with optimal *in vitro* and *in vivo* antitumor activities [51,66].

## Tuning Nanoparticles for ADC-NP Synthesis and Conjugation

Nanoparticles (NPs) have been used in a wide range of applications including drug delivery. Various types of nanomaterials can be synthesized in different sizes [84–86]. Each type of NP has its advantages and disadvantages. NPs fall into one of three classes: inorganic (e.g., gold, silver, iron oxide, silica, or quantum dots), polymeric (e.g., dendrimer, polymer micelle or nanosphere), and lipid-based (e.g., liposome, lipid NP or emulsion) [30]. They are used as delivery agents that either attach to or encapsulate therapeutic drugs to ultimately deliver them to the target site. They are characterized using various analytical techniques such as transmission electron microscopy (TEM), dynamic light scattering (DLS), ultraviolet-visible spectroscopy (UV-Vis), surface plasmon resonance (SPR), and fourier transform infrared (FT-IR) spectroscopy [24,87,88].

An important factor to consider in the design of a nanoparticle is its size [89]. It is known that larger nanoparticles tend to have poor cell and tissue penetration but better retention abilities while smaller nanoparticles can easily penetrate the tumor but have poor retention [24]. To allow both optimal penetration and retention, tailoring the size of the nanoparticle depending on the type of tumor has acquired great interest. It is imperative for the size of nanoparticles to be optimized so that it is small enough to penetrate the tumor but large enough to escape immediate clearance and thereby sustaining a higher concentration of the drug in the tumor to exert its therapeutic effect. The gaps between endothelial cells are usually between 100 to 800 nm. Nanoparticles can extravasate through these gaps to the malignant tissue. This is known as passive targeting also termed enhanced permeation and retention (EPR) effect [7,22,89–91].

More recent studies found that 97% of nanoparticles tend to enter endothelial cells through an active process that involves the functionalization of particles with targeting moieties such as antibodies, peptides or integrin ligands for efficient NP uptake into tumor cells [28,90]. The challenge is to overcome the obstacles in the tumor environment consisting of a dense matrix and high fluid pressure which hinders retention and penetration of the nanoparticle [22]. There are many factors such as particle size, surface coating (e.g., citrate or PEGylation (PEG)), charge and shape of particle that influence the clearance, tumor interaction and stability of the complex in the circulation [30,32,92]. Rod-shaped NPs tend to extravasate easily through leaky vasculature. In terms of surface coating, uncoated or positively charged NPs tend to get cleared very quickly by macrophages [30,93,94]. The concept of antibody conju-

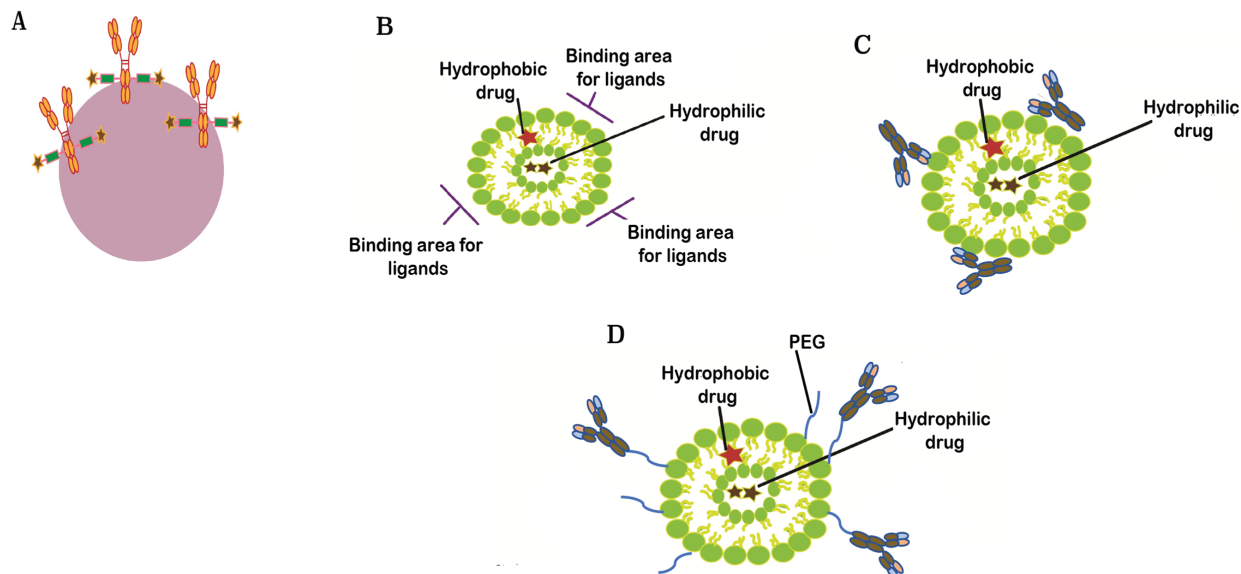


**Fig. 2. Antibody conjugated nanoparticles.** (A) Antibody conjugated inorganic nanoparticles (NPs): Nanoparticle (pink) with antibody-drug conjugates (ADCs) as targeting ligands comprised of an antibody (orange) linker (green) and cytotoxic small molecule (brown). (B) Basic schematic of an immunoliposome the lipid bilayer of the liposome is used to transport hydrophobic drugs (red) and the inner hydrophilic or aqueous layer is used to transport hydrophilic drugs (brown). The outer area of the liposome can be used to attach targeting ligands such as antibodies specific to the antigen on the tumor cell. (C) Random spatial orientation of monoclonal antibodies (mAbs) without a PEGylated (PEG) moiety. (D) PEGylated liposome representing monoclonal antibody (mAb) attachment to the PEG moiety via the fragment crystallizable (Fc) region, preferred method with the exposed antigen binding site.

gated nanoparticles (ACNPs) was introduced based on their potential to aid in ADC tumor penetration and subsequently delivery of the drug cargo. It is a synergistic approach to target tumors [95].

Furthermore, although various nanoparticle classes, such as inorganic, polymeric, and lipid-based types, are being explored, concerns like ADC solubility and toxicity necessitate further inquiry. Nanoparticles can be improved by integrating mAbs, potentially boosting target accuracy and tumor penetration. Liposomes, including immunoliposomes, have gained attention, offering advantages like versatile encapsulation of hydrophobic/hydrophilic drugs, surface modifications via PEG and targeting ligands, enhancing selectivity and binding. Prospective research should concentrate on antibody-oriented technologies for immunoliposomes, enhancing cell targeting and effectiveness.

## Antibody Conjugated Nanoparticles

The concept of ACNPs is an interesting approach based on the introduction of nanotechnology and the success of ADCs. It offers multiple advantages including the incorporation of higher drug or ligand concentrations depending on the size of the NPs and the use of payloads with lower potencies such as camptothecins [95]. Some of the main targets for ACNPs are human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), prostate-specific membrane antigen (PSMA) and transferrin receptor (TfR) [96]. A study evaluated the concept of ACNPs by investigating cellular uptake and *in vitro* potencies through the comparison of an ADC (Trastuzumab-Val-Cit-MMAE) and AuNP-ADC (gold nanoparticles conjugated to Trastuzumab-Val-Cit-MMAE). The findings suggested that the cytotoxic potency of the ADC was maintained even when bound to AuNP. The study also confirmed that the average number of mAbs that covered the surface of 50 nm AuNPs was approximately 25.6% higher than that of 20 nm subsequently affirming the significance of NP size [22]. Interestingly functionalized nanoparticles with a cell-penetrating peptide, HIV-TAT, with a PEGylated moiety demonstrated superior cellular uptake in breast cancer cell lines, SKBR3 and MCF-7 [22]. However, the penetration of antibodies into the solid tumor can be compromised due to the high interstitial fluid pressure in the tumor environment as a result of poor lymphatic drainage [29,97,98].

Functionalization of nanoparticles with targeting moieties such as antibodies or antibody fragments (Fig. 2), enhances the specificity of the conjugate and lowers the potential of toxic adverse or systemic toxicity [19,87,93].

### *Inorganic Nanoparticles*

Antibodies or other proteins and peptides can be bound to the surface of nanoparticles (Fig. 2A) in various spatial orientations via the Fc region, two major fragments (i.e., the two Fab fragments and the Fc region lying flat on

**Table 1. Antibody-nanoparticle conjugation strategies.**

| Conjugation strategies | Advantages | Disadvantages | References |
| --- | --- | --- | --- |
| Adsorption | Chemical modification is not required<br>Does not require activation of the functional groups | Non-covalent immobilization via ionic binding and physical adsorption compromises the stability of the complex | [19,99] |
| | Not laborious and straight forward | Can result in random orientation of the antibody which can impact its binding ability to the target | |
| Carbodiimide chemistry | Covalent binding approach. It has higher stability due to covalent binding between carboxyl and amine groups on the NP using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) linkers | Randomly immobilized mAb on the surface of the nanoparticle | [100] |
| | Chemical modification of the mAb is not required | Requires prior activation of the NP particularly for carboxyl present on the NP in order to react with amine groups | |
| Maleimide chemistry | Covalent binding approach<br>Either homobifunctional or heterobifunctional crosslinkers can be used | Requires binding through sulfhydryl (SH) groups on the antibody which are less abundant compared to amines. There is a risk of thiol exchange reactions | [101,102] |
| Click chemistry | Incorporates a series of simplified chemical reactions. It is performed at room temperature with mild and non-toxic aqueous solvents<br>Suitable for biomedical applications (i.e., site-specific conjugation for therapeutics) | Most common reaction is between an azide and alkyne in the presence of copper and subsequently a major disadvantage with this technique is copper-induced toxicity | [19,102,103] |
| Biotin-avidin | Strong non-covalent interaction | Costly technique | [19,102] |
| | Proper orientation on the functionalized NP due to binding at the Fc region of the functionalized mAb. The overall design ensures enhanced internalization and pharmacokinetics and lesser non-specific interactions | The stoichiometry between the mAb and NP is hard to control | |
| | Resistant to harsh conditions as a result of temperature, pH, or denaturants | Requires chemical modification of the mAb and functionalization of the NP | |

the surface of the NP), sideway orientation where the attachment is via one Fab and one Fc region, or via both Fab regions [19,23,99]. Out of these orientations, attachment via the Fc region attachment to the surface of the nanoparticle is recommended since this arrangement would not hinder the binding of the Fab region to the target antigen. The Fc region can be bound to the nanoparticle via Cys, Lys, or other amino acids or via sugar moieties [19], using a variety of different conjugation strategies including carbodiimide chemistry, maleimide chemistry, click chemistry, and biotin-avidin.

Adsorption is a simple reversible method of conjugation wherein mAbs are bound to NPs via hydrogen bonds, electrostatic forces, hydrophobic interaction, and van der Waal interactions [19,99].

Although adsorption is a simple technique, major disadvantages include the potential random orientation of the antibody that ultimately impacts the binding ability to the target site, and more critically conjugate disassembly before reaching the target as well as poor retention [99].

Therefore, covalent binding techniques such as carbodiimide chemistry is more stable and it involves covalent bonding between carboxyl and amine groups on the nanoparticle using linkers such as 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS). Chemical modification of these two techniques is not required as primary amines that are in abundance on the surface of antibodies can be utilized [100]. The drawback associated with carbodiimide chemistry is random immobilized mAbs on the surface of the nanoparticle, and prior activation of the NP particularly for carboxyl present on the NP to react with amine groups.

Maleimide chemistry involves the reduction of disulfide bonds on the antibody to expose the sulfhydryl groups followed by conjugation to the sulfhydryl groups on the antibody via primary amines on the nanoparticle using a heterobifunctional linker such a sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC), a crosslinker that is non-cleavable, membrane-impermeable and water-soluble. SMCC is water insoluble, hence the addition of sulfo moiety is needed. For conjugation involving maleimide chemistry, either a homobifunctional (homobifunctional refers to two similar functional groups) or heterobifunctional (heterobifunctional refers to two different functional groups) crosslinker can be used. The drawback of maleimide chemistry is the risk of thiol exchange reactions with thiol reactive groups such as glutathione in the serum albumin resulting in the deconstruction of the conjugate and consequently off-target toxicities [101,102].

Click chemistry is another method that can be employed for this purpose, which involves introducing new functional groups via site-specific mutation to enable conjugation between antibodies and nanoparticles. It incorporates a series of simplified chemical reactions performed under mild and non-toxic aqueous solvents. It differs from site-specific conjugation techniques which generally involve the introduction of site mutations on the mAb such as Cys or non-natural amino acids. This is because the reaction in click chemistry occurs between an azide and an alkyne in the presence of copper. However, a major disadvantage of this technique is copper-induced toxicity [19,102,103].

The biotin-avidin system entails site-specific biotinylation of antibodies at the Fc region which immobilizes the antibody. Avidin is a positively charged molecule used to functionalize NPs. Once the nanoparticle is functionalized with avidin, it is incubated with the biotinylated-antibody to produce the biotin-avidin complex. Although it has a strong interaction between the protein and ligand, it is an expensive technique with difficulty in controlling the stoichiometry between the antibody and nanoparticle. The overall design ensures enhanced internalization and pharmacokinetics and lesser non-specific interactions. Biotin-avidin is resistant to harsh conditions as a result of temperature, pH, or denaturants. Some drawbacks include the high cost of this technique, difficulty in controlling the stoichiometry between the mAb and NP, the impact on the structural integrity of the mAb and NP due to chemical modification of the mAb and functionalization of the NP [19,102]. Table 1 (Ref. [19,99–103]) summarizes the advantages and disadvantages of mAb-NP conjugation strategies.

### *Liposome Nanoparticles*

The use of liposomes as a drug cargo has increased in recent years due to its advantageous features such as tailored drug release profiles, feasibility of liposomal surface modifications for ligand attachment, ability to incorporate hydrophobic and hydrophilic drug cargos and building on the success of liposome nanoparticles such as Onivyde® (Merrimack Pharmaceuticals, Inc.), which is a liposome encapsulated topoisomerase I inhibitor, Irinotecan. It was approved in 2015 by the Food and drug administration (FDA) for the treatment of metastatic pancreatic cancer that is resistant to gemcitabine [104].

Liposomes were first discovered in the 1960s [105]. The first liposome formulation, PEGylated liposome containing Doxil® doxorubicin, was introduced in 1995. PEGylation increased the half-life of doxorubicin (Dox) and prevented rapid clearance of liposomes [106–108]. Liposomes are spherical vesicles composed of one or more aqueous cores enclosed in a single or multilamellar lipid bilayer, mimicking natural lipid bilayers. These structures are suitable for drug delivery because they have an amphiphilic nature and permit surface modifications through the incorporation of targeting ligands [105]. Liposomes can accumulate within the tumor tissue as a result of the enhanced permeability and retention (EPR) effect [109].

PEGylated liposomal drug formulations can provide longer drug half-lives, additionally their drug-release profiles can be tailored, reducing high peak plasma concentra-

**Table 2. Common features of antibody conjugated liposome nanoparticles.**

| Nanomaterial | Features | Liposomal Nanomaterials that have gained regulatory approval | References |
|---|---|---|---|
| Antibody-liposome nanoparticle conjugates in drug delivery | Target specificity is ensured due to mAb<br>Amphiphilic nature<br>Easy antibody surface modification<br>Longer serum half-lives due to site of nanoparticles<br>Tailored drug-release profiles, reducing high peak plasma concentrations | Doxil®, Onivyde®, Marqibo kit®, Myocet®, Mepact®, and Vyxeos® DaunoXome®, Lipusu®, Abraxane®, Genexol-PM®, PICN®, Paclical®, Hensify®, DHP107®, NanoTherm®, Nanoxel®, Depocyt®. | [96,97,105,111–113] |

tions [23,96,97,110]. Table 2 (Ref. [96,97,105,111–113]) summarizes the common features of antibody conjugated liposome nanomaterials that make liposome drug formulations suitable candidates for target specific and efficient payload delivery to the tumor site.

Although liposomes are effective drug carriers, their target specificity is enhanced through the addition of binding ligands such as antibodies termed immunoliposomes. Fig. 2B illustrates a basic schematic of a liposome and the ligand binding area as well as the inner aqueous phase and the lipid bilayer. The lipid bilayer of the liposome is used to transport hydrophobic drugs and the inner hydrophilic or aqueous layer is used to transport hydrophilic drugs. The outer area of the liposome can be used to attach targeting ligands such as antibodies [114] specific to the antigen on the tumor cell (Fig. 2B).

Studies have demonstrated that mAb-liposome conjugation can increase antitumor activity [114], overcome the limitation of drug loading wherein high DARs can compromise mAb stability and low DARs decrease the ADC efficacy [115,116].

Conjugation involves PEGylation of the liposome followed by surface attachment of ligands. Antibodies can be attached via varied spatial orientations illustrated in Fig. 2C,D. The preferred method of conjugation is antibody attachment to the end of PEG chains via sulfhydryl-maleimide bonds or amides wherein PEG moieties attach to the lysine or N-terminal residues via covalent bonding using crosslinkers such as *N*-hydroxysuccinimide (NHS) ester reaction [114,117]. Attachment via the Fc region allows the mAb to protrude from the liposome and exposes the antigen-binding site which eventuates in optimal antigen binding.

Alternatively, mAb Fab fragments can also be coupled to PEG moieties via maleimide-reactive sulfhydryl groups [117] producing actively targeted drug delivery systems [111,112]. Liposomal formulations allow for efficient targeting of the antibody-liposome bioconjugate to its matching antigen. The antibody-liposome biconjugate exerts its effects by docking at the site of the antigen and subsequently at the delivery of the payload [112,113]. It is an efficient drug delivery system that has the potential to minimize the off-target effects of the drug.

A study by Nikkhoi *et al.*, 2018 [20] explored the development of a bivalent, bispecific VHH-domain HER2-antibody fragment to attach to liposomes for the treatment of HER2-positive breast cancer. The VHH antibody fragments were attached to the surface of PEGylated liposomes using a thiol-maleimide reaction activated with NHS, a common technique for the attachment for antibodies [20]. The results suggested that their bivalent, bispecific antibody-liposomes demonstrated strong affinity towards HER2-positive cells in comparison with monovalent and bivalent, monospecific VHH antibody-liposome conjugates due to their ability to target the different HER2 epitopes [20].

One of the challenges with antibody-liposome attachment is the lack of control in the orientation of the antibody on the liposome surface and consequently the liposome-antibody conjugates contain many misaligned antibodies. This is believed to hinder the antigen-binding fragment (Fab) to bind to nearby antigens properly [112]. Shim *et al.* [21] showed that decorating the liposomes with an FcBP (Fc binding peptide) can address this issue [21]. The FcBP is found in Staphylococcus aureus and has the ability to differentiate between the Fab and Fc regions of the antibody. FcBP binds noncovalently to the Fc region of the antibody and as a result, the Fab portion of the antibody protrudes outwards, demonstrating that the use of antibody-orienting technologies is worth considering when conjugating antibodies to liposomes for improved cell targeting and enhanced potency.

## New Prodrug Conjugation Strategies

The human degradome system has approximately 600 proteases that fall into five categories: metalloproteinases, serine, cysteine, threonine, and aspartic proteases [118]. These proteases are responsible for regulating some physiological processes including proliferation, apoptosis, and other critical processes in the cell. Cancer progression dysregulates and disrupts expression of some of the proteases in tumor tissues [118]. Antibody prodrug conjugates (APDCs) are a new approach and class of targeted therapies that utilize proteases found within the tumor such as legumain to cleave specifically designed linkers. They are

**Table 3. Prodrug conjugates.**

| Prodrug conjugates | Features | Current research | References |
|---|---|---|---|
| Peptide drug conjugates | Peptide drug conjugates are suitable for cancers that tend to metastasize in the brain due to their advantageous small molecular size and excellent cell permeability. It has the ability to cross the blood-brain barrier (BBB). Current therapeutics can overcome multidrug resistance e.g., ANG1005. | Lutathera® was approved by the European medicines agency (EMA) in 2017 and by the Food and drug administration (FDA) in 2018 for gastroenteropancreatic neuroendocrine tumors (GEP-NETs). Additionally, Phase II clinical trials were completed for treatment of recurrent or relapsed high-risk neuroblastoma in children (NCT04903899).<br>ANG1005 is currently undergoing Phase III clinical trials (NCT03613181) for Leptomeningeal disease. Phase II clinical trials for breast cancer and recurrent brain metastasis have been completed (NCT02048059) for ANG1005.<br>Peptide fragment-doxorubicin (MAHNP-Dox) conjugate is another peptide drug conjugate (PDC) for the treatment of human epidermal growth factor receptor 2 positive (HER2+) breast cancer that has shown promising *in vitro* and *in vivo* results. | [38–41,101,119–122] |
| DEVD (Asp-Glu-Val-Asp) prodrug conjugates | Used in combination with radiotherapy to treat triple-negative breast cancers (TNBC). The DEVD moiety is cleavable by caspase-3. It is associated with low systemic toxicity upon payload delivery. | DEVD such as ApoPep-1-Asp-Glu-Val-Asp-S-doxorubicin (AP1-DEVD-SDox) is a potential therapeutic for triple-negative breast cancer after radiotherapy. | [110,123–126] |
| Legumain and Kinesin Spindle Protein inhibitor (KSPi) prodrug conjugates | Legumain-enzyme drug delivery systems are site-specific and effective in various solid tumors (ovarian, colon, breast, and prostate) that highly express legumain.<br>The KSPi payload molecules have attachment sites suitable for cleavable or non-cleavable linkers. Moreover, the moiety consists of the legumain cleavable peptide cap to prevent off-target toxicities. | Legumain and the KSPi is an antibody prodrug conjugate (APDC) that has high activity against tumor-associated antigen B7 homolog 3 (B7H3) is highly expressed in some solid tumors including breast cancer. | [37,127–133] |

DM
DISCOVERY MEDICINE 2001



subdivided into legumain and the Kinesin Spindle Protein inhibitor (KSPi) (Fig. 3A), PDCs (Fig. 3B) and the protease-sensitive peptide moiety Asp-Glu-Val-Asp (DEVD). The PDC and DEVD moieties are applicable in treating brain cancers and triple-negative breast cancers. These conjugates can integrate potent cytotoxic agents which are usually impeded by resistance mechanisms. However further research is necessary to enhance safety and gain deeper insights into active metabolites from APDCs in healthy organs. Table 3 (Ref. [37–41,101,110,119–133]) summarizes the various advantageous features of prodrug conjugates.

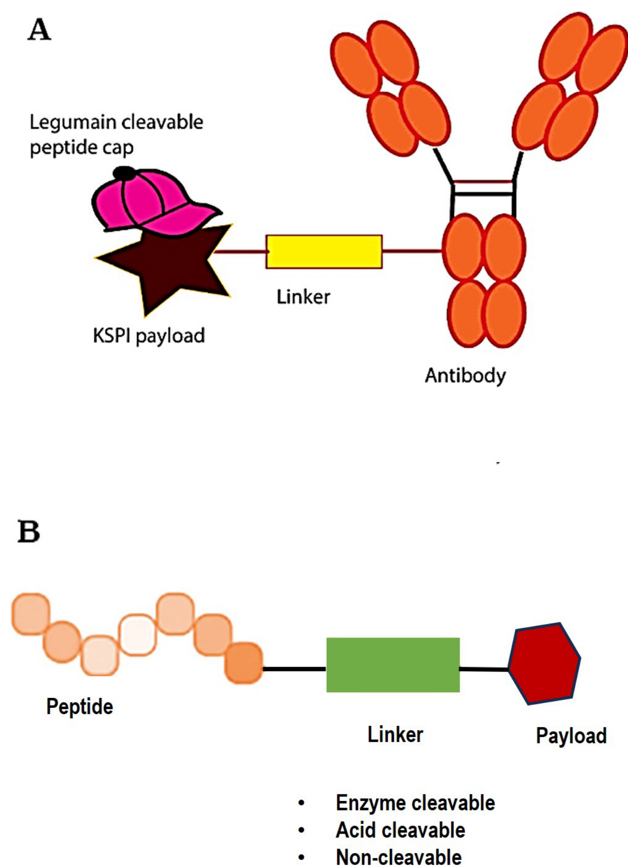


**Fig. 3. Antibody and peptide prodrug conjugates.** (A) Antibody-drug conjugate (ADC): the antibody (orange) is conjugated to the Kinesin Spindle Protein inhibitor (KSPi) payload (brown) via a linker (yellow). The KSPi payload is capped with a legumain cleavable peptide cap (pink) to prevent off-target toxicities. The cap is specifically cleaved by legumain proteases in the tumor followed by antibody degradation to form active metabolites and release the payload. (B) Basic structure of a peptide drug conjugate (PDC): the PDC is comprised of a peptide (orange), a linker (can be cleavable/acid-cleavable/non-cleavable) (green) and a cytotoxic small molecule (red).

The small size of the PDC modality and its excellent cell permeability makes it an ideal candidate for brain cancers. In fact, the first peptide receptor radionuclide therapeutic, Lutathera®, has been recently approved by the FDA and European medicines agency (EMA) [134,135]. In addition, PDC ANG1005 is currently undergoing late-stage Phase III studies however the results have not yet been published [72].

The DEVD peptide sequence is another prodrug model candidate that has the potential to target triple-negative breast cancers (TNBC) [123,124,136]. The use of the DEVD construct in combination with radiotherapy tends to upregulate caspase-3 activity and exposure which is a requirement for cleavage of the DEVD peptide sequence and subsequent payload release to generate a higher number of apoptotic cells. An exciting and appealing feature of the DEVD prodrug conjugate is that it is associated with low systemic toxicity upon payload delivery, particularly of significance for cancers such as TNBC that have radiotherapy as the main treatment option [123,124,136].

Legumain activatable APDCs are cleaved by the lysosomal endopeptidase legumain, and they are equally as potent as cathepsin B enzymes [137]. This APDC is optimized to have a unique cleavage site which means that it forms active metabolites in tumor cells compared to healthy cells. The effect of legumain cleavable linkers is still under scrutiny for other cancer types. The legumain protease is highly expressed in some solid tumors including ovarian, colon, breast, and prostate [37,127,129]. This makes the target specific legumain APDC a suitable candidate.

### *Legumain and the Kinesin Spindle Protein Inhibitor (KSPi)*

Legumain is a lysosomal asparaginyl peptidase. It has optimal activity in an acidic environment at a pH of 4–5 and is highly expressed in many solid tumors including ovarian, colon, breast, and prostate cancers compared to healthy tissues, and hence, this led to the design of legumain activatable APDCs [37,127,129].

The APDC is comprised of a mAb conjugated to a KSPi payload, via a linker with a legumain-cleavable peptide cap (Fig. 3A). Additionally, the KSPi molecules have attachment sites suitable for both cleavable and non-cleavable linkers.

As an extra measure of protection and to prevent off-target toxicities, the free amino group (NH2) in the KSPi molecule that is key to its cytotoxicity, can be blocked with a legumain cleavable peptide cap [128]. Antibody degradation and cleavage of the peptide cap to expose the free amino group precede the formation of active metabolites [131]. This construct has a larger therapeutic window unlike some antibody-drug conjugates which are involved in off-target toxicities and consequently, in clinical studies, fail to achieve an optimal therapeutic window [131,138]. The therapeutic window is generally a range within which drug dosages can effectively treat the disease without having toxic effects.

DM DISCOVERY MEDICINE 2001

The cathepsin B-activatable analogues legumain activatable APDCs are equally potent and had high activity against tumor-associated antigens Tweak receptor (TweakR) and B7 homolog 3 (B7H3) [131]. The transmembrane B7H3 is highly expressed in some solid tumors including breast cancer and is a potential therapeutic target [139,140]. Moreover, high legumain activity is linked to enhanced metastasis and cancer growth [130,132]. In comparison to other proteases, it has a high substrate specificity since its cleavage site is after the asparagine (Asn) residue [127,131,133,137]. Further studies need to be conducted to attain an improved safety profile and to understand more about the formation of active metabolites from APDCs in healthy organs with lower legumain activity [44,48].

*Peptide Drug Conjugates*

The design of PDCs including linker chemistries were adapted from ADCs. The peptide in a PDC can be up to 50 amino acids long [40]. The linker can be cleavable, acid-cleavable or non-cleavable (Fig. 3B) [40]. PDCs are a promising new targeted approach that has an advantage due to their small molecular size, which allows for delivering payloads at a high concentration that is known to have a short serum half-life. Similar to the mechanism of action of ADCs [12], the peptide in PDCs needs to be highly specific for its target in order to induce successful endocytosis, and the linker must be stable in circulation and release the payload only at the target site [38,39]. The cytotoxic payload can range from a small molecule cytotoxic agent such as doxorubicin (Dox) or radionuclides namely $^{177}$lutetium [135]. Different functional groups can be utilized on the linker to covalently attach to the peptide [40]. Enzyme cleavable linkers that are commonly used in ADCs can also be used for PDCs; for example, carbamate, ester, amide bond linkages, as well as acid-cleavable linkers that have carbonate and hydrazone linkages, and non-cleavable linkers that have trizole, oxime and thioether moieties [39].

The PDC $^{177}$Lutetium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-yrosine-3 octreotate (DOTA-TATE) (Lutathera®), was the first peptide receptor radionuclide therapeutic approved by the EMA in 2017 and by the FDA in 2018 for the treatment of somatostatin receptor-positive gastroenteropancreatic neuroendocrine tumors (GEP-NETs) [134,135]. The PDC is comprised of a cytotoxic radionuclide ($^{177}$Lutetium) attached via a bifunctional chelating agent (DOTA) to the peptide (octreoate) [135,141]. The $^{177}$Lutetium DOTA-TATE PDC targets somatostatin subtype 2 receptor found on the surface of GEP-NET cells [41,142,143]. The radionuclide $^{177}$Lutetium in this modality emits beta particles and the ionizing radiation subsequently elicits DNA damage leading to cell death [141]. The PDC has completed Phase II clinical trials for treatment of recurrent or relapsed high-risk neuroblastoma in children (NCT04903899) [72].

Small molecules Dox and paclitaxel are known to have low blood-brain barrier (BBB) permeability due to the multidrug resistance mechanism P-glycoprotein (P-gp) efflux pump, which is commonly found in tumor cells in the brain [119]. To overcome this low permeability, ANG1005 was developed. The PDC ANG1005 is comprised of three paclitaxel molecules covalently linked to a peptide (Angiopep-2) consisting of nineteen amino acids [120]. The peptide Angiopep-2 targets lipoprotein receptor-related protein 1 (LRP-1) that is found in abundance on the surface of capillary endothelial cells and glioblastoma cells [144]. Angiopep-2 enters BBB via LRP-1 meditated transcytosis [41,121]. Although paclitaxel is very potent in lung, glioblastoma, and ovarian carcinoma cell lines, in situ mouse brain perfusion, showed that in comparison to free paclitaxel, the uptake of the conjugate was 4.5-fold higher which reinforces its ability to bypass the P-gp and thereby penetrate the BBB [120]. Further Phase II clinical trials investigating the effect of ANG1005 in patients with breast cancer and recurrent brain metastasis (NCT02048059) have been completed [72]. In addition, it is currently undergoing Phase III clinical trials (NCT03613181) for Leptomeningeal disease [72].

The therapeutic potential of another PDC, peptide fragment-doxorubicin (MAHNP-Dox) conjugate, was firstly evaluated specifically for the treatment of human epidermal growth factor receptor 2 positive (HER2+) breast cancer [41,122]. The peptide fragment was acquired from the heavy chain 3 of the trastuzumab mAb that was termed anti-HER2/neu peptide (AHNP), which has a high affinity for the extracellular domain of the HER2 antigen. This peptide fragment was conjugated to the Dox payload via a matrix metalloproteinase 2 (MMP-2) sensitive linker through the ester/amide bond linkages to form the MAHNP-Dox conjugate. The *in vitro* results showed lower half-maximal inhibitory concentration (IC50) values against HER2+ breast cancer cells SKBR3 and BT474 compared to free Dox. Subsequently, *in vivo* studies using BT474 xenografted mice showed a reduction in tumor growth and less weight loss [122].

Due to their small size, PDCs are worth exploring as they possess the ability to increase the circulation time of the payload in the plasma, have lower toxicity, and enhance the efficacy of payload delivery to the tumor site, particularly for payloads that have poor permeability as a result of factors such as resistance mechanisms.

Another concept that follows a similar theme is human antibody fusion proteins with an aim of directing the ligand to the target site. The SNAP-tag fusion protein has a two in one function with the ability to play a role as a therapeutic and diagnostic tool specifically for breast and ovarian cancer. The human antibody fusion protein is a single construct that fuses antibody domains and proteins. It is an attractive method that allows the flexibility of incorporating multiple colors of fluorophores to enhance the visualization of the

tumor. According to this study, when injected in mice, tumors were distinctly visible and detectable [145].

*The Protease-Sensitive Peptide Moiety*

The protease-sensitive peptide or DEVD (Asp-Glu-Val-Asp), is used as a prodrug to target TNBC. One of the reasons triple-negative breast cancer is difficult to treat is because it lacks specific target antigens on the surface of tumor cells [146].

DEVD is a substrate sequence of caspase-3 where the amide bond following its last Asp residue is hydrolyzed when recognized by caspase-3. It can be conjugated to payloads via the para-aminobenzyl carbamate (PABC) spacer and is cleavable by caspase-3 [125]. Caspase-3 is upregulated following exposure to external stimuli e.g., radiation and thereby increases the chances of the prodrug to come into contact with caspase-3 [123,126,146].

Another modality wherein Dox was employed as the cytotoxic payload to construct ApoPep-1-Asp-Glu-Val-Asp-S-doxorubicin (AP1-DEVD-SDox) was prepared by conjugating ApoPep-1 (AP1) and Dox via DEVD peptide [123]. The AP1 cell binding peptide prolongs retention and overcomes rapid clearance of this prodrug construct within the irradiated tumor since it binds tightly to histone1 (H1) which is found on the surface of apoptotic cells. The outcome of this study demonstrated that the use of the DEVD-payload construct in combination with radiotherapy resulted in a greater number of apoptotic cells and an improved therapeutic outcome. The cytotoxic effect of the prodrug was assessed against MDA-MB-231 (TNBC cell line). Following cleavage of DEVD by caspase-3, the introduction of the prodrug AP1-DEVD-SDox results in the release of Dox to exert its cytotoxic effects on neighboring tumor cells. To deliver doxorubicin, importantly with low systemic toxicity, this construct would be particularly useful for triple-negative breast cancer after radiotherapy [123,124].

## Concluding Remarks and Outlook

Antibodies, particularly ADCs, have generated great interest over the years due to their unrivalled target specificity and efficacy. Several strategies have been devised to overcome some limitations including these induced by interstitial fluid pressure (IFP), P-gp, payload heterogeneity, poor structural stability, and inadequate therapeutic index that tend to hinder the efficacy of the ADC. Methods such as site-specific conjugation allow control of the binding sites and DAR in order to generate a more homogenous ADC that can improve ADC stability in the circulation, pharmacokinetics, and therapeutic index. Additionally, there are various classes of nanoparticles including inorganic, polymeric, and lipid-based nanoparticles undergoing research. However, some of the limitations including low solubility and toxicity concerns of ADCs, require further investigation.

Nanoparticle functionalization by incorporation of mAbs may improve the target specificity and tumor penetration of the nanoparticle antibody complexes. Since the discovery of liposomes, immunoliposomes have been an active area of research. Liposomes possess many desirable characteristics including their amphiphilic nature that allows the encapsulation of either hydrophobic or hydrophilic drug molecules and permits surface modifications through the incorporation of PEG moieties and targeting ligands which enhances its selectivity, performance, and binding ability. Future studies should focus on antibody-orienting technologies for immunoliposomes for improved cell targeting and enhanced potency.

Other upcoming methods that have the potential to yield a product include the development of prodrug conjugates that can be used to incorporate highly cytotoxic agents that are normally hindered by resistance mechanisms and makes use of newly discovered proteases that reside in the tumor that cleaves specifically designed linkers.

Prodrug conjugates are another emerging method where highly cytotoxic agents are conjugated to a mAb via a linker to form a prodrug such as PDC or DEVD. The linker is then cleaved by proteases that reside within the tumor. PDCs and DEVD can be used for brain cancers and triple-negative breast cancers. Further studies need to be conducted to attain an improved safety profile and to understand more about the formation of active metabolites from APDCs in healthy organs.

## Abbreviations

aaRS, aminoacyl-tRNA synthetase; ACNP, antibody conjugated nanoparticle; ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; ADCs, antibody-drug conjugates; AHNP, anti-HER2/neu peptide which is a peptide fragment from the heavy chain of trastuzumab; AP1, ApoPep-1; APDC, antibody prodrug conjugate; AuNP, gold nanoparticle; BBB, blood-brain barrier; CDC, complement-dependent cytotoxicity; CDCC, complement-dependent cellular cytotoxicity; CHO, Chinese hamster ovary; Cys, cysteine; DAR, drug-antibody-ratio; DEVD peptide sequence, Asp-Glu-Val-Asp peptide sequence; DLS, dynamic light scattering; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; Dox, doxorubicin; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; EGFR, epidermal growth factor receptor; EMA, European medicines agency; EPR, enhanced permeation and retention; F-HPA, F-hydroxypropylamide; FDA, Food and drug administration; $Fe_2O_3$, maghemite; $Fe_3O_4$, magnetite; FGE, formylglycine generating enzyme; FT-IR spectroscopy, fourier transform infrared spectroscopy; GalNAc, *N*-acetylgalactosamine; GEP-NETs, gastroenteropancreatic neuroendocrine tumors; GlcNAc, *N*-acetylglucosamine; H1, histone 1; HER2, human epidermal growth fac-

tor receptor 2; HER2+, human epidermal growth factor receptor 2 positive; IFP, interstitial fluid pressure; KSPi, Kinesin Spindle Protein inhibitor; LRP-1, lipoprotein receptor-related protein 1; Lys, lysine; mAbs, monoclonal antibodies; MAHNP, peptide fragment; MMP-2, matrix metalloproteinase 2; mTG, microbial transglutaminase; MUC16, Mucin 16; NaPi2b, sodium-dependent phosphate transport protein 2b; NHS, *N*-hydroxysuccinimide; NP, nanoparticle; NSCLC, non-small cell lung cancer; P-gp, P-glycoprotein; PABC, para-aminobenzyl carbamate; pAcF, p-acetylphenylalanine; pAMF, para-azidomethyl-l-phenylalanine; PDCs, peptide drug conjugates; PEG, PEGylation; PSMA, prostate-specific membrane antigen; SA, sialic acid; SPR, surface plasmon resonance; SrtA, sortase A; Sulfo-SMCC, sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; TATE, tyrosine-3 octreotate; TDC, THIOMAB™ drug conjugate; TEM, transmission electron microscopy; TfR, transferrin receptor; TNBC, triple-negative breast cancer; UAA, unnatural amino acid; UAG, amber stop codon; UV-Vis, ultraviolet-visible spectroscopy; VC-MMAD, valine-citrulline monomethyl auristatin D; VEGF, vascular endothelial growth factor; Ces1c, extracellular carboxylesterase 1c; EGCit, glutamic acid-glycine-citrulline.

## Author Contributions

Conceptualization, CMM and VK; writing—original draft preparation, CMM and VK; writing—review and editing, CMM and VK; supervision, VK; both authors have read and agreed to the published version of the manuscript. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

## Acknowledgment

Not applicable.

## Funding

This research received no external funding.

## Conflict of Interest

The authors declare no conflict of interest. VK is serving as one of the Editorial Board members of this journal. We declare that VK had no involvement in the peer review of this article and has no access to information regarding its peer review.

## References

[1] Cancer.org. Global Cancer Facts & Figures | American Cancer Society. 2021. Available at: https://www.cancer.org/research/cancer-facts-statistics/global.html (Accessed: 8 May 2021).

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a Cancer Journal for Clinicians. 2018; 68: 394–424.

[3] WHO. The top ten causes of death. 2020. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death (Accessed: 10 September 2021).

[4] Schirrmacher V. From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review). International Journal of Oncology. 2019; 54: 407–419.

[5] Elgundi Z, Reslan M, Cruz E, Sifniotis V, Kayser V. The state-of-play and future of antibody therapeutics. Advanced Drug Delivery Reviews. 2017; 122: 2–19.

[6] Seledtsov VI, Seledtsova GV. Attaining threshold antibody cytotoxicity for selective tumor cell destruction: an opinion article. Oncotarget. 2018; 9: 35790–35794.

[7] Cruz E, Kayser V. Monoclonal antibody therapy of solid tumors: clinical limitations and novel strategies to enhance treatment efficacy. Biologics: Targets & Therapy. 2019; 13: 33–51.

[8] Sifniotis V, Cruz E, Eroglu B, Kayser V. Current Advancements in Addressing Key Challenges of Therapeutic Antibody Design, Manufacture, and Formulation. Antibodies. 2019; 8: 36.

[9] Lu J, Jiang F, Lu A, Zhang G. Linkers Having a Crucial Role in Antibody-Drug Conjugates. International Journal of Molecular Sciences. 2016; 17: 561.

[10] Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR. Site-specific antibody drug conjugates for cancer therapy. mAbs. 2014; 6: 34–45.

[11] Mckertish CM, Kayser V. A Novel Dual-Payload ADC for the Treatment of HER2+ Breast and Colon Cancer. Pharmaceutics. 2023; 15: 2020.

[12] Mckertish CM, Kayser V. Advances and Limitations of Antibody Drug Conjugates for Cancer. Biomedicines. 2021; 9: 872.

[13] Anami Y, Yamazaki CM, Xiong W, Gui X, Zhang N, An Z, *et al*. Glutamic acid-valine-citrulline linkers ensure stability and efficacy of antibody-drug conjugates in mice. Nature Communications. 2018; 9: 2512.

[14] Ha SYY, Anami Y, Yamazaki CM, Xiong W, Haase CM, Olson SD, *et al*. An Enzymatically Cleavable Tripeptide Linker for Maximizing the Therapeutic Index of Antibody-Drug Conjugates. Molecular Cancer Therapeutics. 2022; 21: 1449–1461.

[15] McKertish C, Kayser V. Antibody Drug Conjugates: Fine tuning the targeted missiles. Asia-Pacific Journal of Clinical Oncology. 2021; 17: 38.

[16] Chudasama V, Maruani A, Caddick S. Recent advances in the construction of antibody-drug conjugates. Nature Chemistry. 2016; 8: 114–119.

[17] Junutula JR, Raab H, Clark S, Bhakta S, Leipold DD, Weir S, *et al*. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nature Biotechnology. 2008; 26: 925–932.

[18] Nejadmoghaddam MR, Minai-Tehrani A, Ghahremanzadeh R, Mahmoudi M, Dinarvand R, Zarnani AH. Antibody-Drug Conjugates: Possibilities and Challenges. Avicenna Journal of Medical Biotechnology. 2019; 11: 3–23.

[19] Marques AC, Costa PJ, Velho S, Amaral MH. Functionalizing nanoparticles with cancer-targeting antibodies: A comparison of strategies. Journal of Controlled Release. 2020; 320: 180–200.

[20] Nikkhoi SK, Rahbarizadeh F, Ranjbar S, Khaleghi S, Farasat A. Liposomal nanoparticle armed with bivalent bispecific single-

domain antibodies, novel weapon in HER2 positive cancerous cell lines targeting. Molecular Immunology. 2018; 96: 98–109.
[21] Shim G, Kim D, Lee S, Chang RS, Byun J, Oh YK. Staphylococcus aureus-mimetic control of antibody orientation on nanoparticles. Nanomedicine: Nanotechnology, Biology, and Medicine. 2019; 16: 267–277.
[22] Cruz E, Kayser V. Synthesis and Enhanced Cellular Uptake In Vitro of Anti-HER2 Multifunctional Gold Nanoparticles. Cancers. 2019; 11: 870.
[23] Moulahoum H, Ghorbanizamani F, Zihnioglu F, Timur S. Surface Biomodification of Liposomes and Polymersomes for Efficient Targeted Drug Delivery. Bioconjugate Chemistry. 2021; 32: 1491–1502.
[24] Yu W, Liu R, Zhou Y, Gao H. Size-Tunable Strategies for a Tumor Targeted Drug Delivery System. ACS Central Science. 2020; 6: 100–116.
[25] Yu W, Hu C, Gao H. Intelligent Size-Changeable Nanoparticles for Enhanced Tumor Accumulation and Deep Penetration. ACS Applied Bio Materials. 2020; 3: 5455–5462.
[26] Zhang YR, Lin R, Li HJ, He WL, Du JZ, Wang J. Strategies to improve tumor penetration of nanomedicines through nanoparticle design. Wiley Interdisciplinary Reviews. Nanomedicine and Nanobiotechnology. 2019; 11: e1519.
[27] Su YL, Hu SH. Functional Nanoparticles for Tumor Penetration of Therapeutics. Pharmaceutics. 2018; 10: 193.
[28] Sindhwani S, Syed AM, Ngai J, Kingston BR, Maiorino L, Rothschild J, *et al*. The entry of nanoparticles into solid tumours. Nature Materials. 2020; 19: 566–575.
[29] Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. Nature Biotechnology. 2015; 33: 941–951.
[30] Mitchell MJ, Billingsley MM, Haley RM, Wechsler ME, Peppas NA, Langer R. Engineering precision nanoparticles for drug delivery. Nature Reviews. Drug Discovery. 2021; 20: 101–124.
[31] Tarkistani MAM, Komalla V, Kayser V. Recent Advances in the Use of Iron-Gold Hybrid Nanoparticles for Biomedical Applications. Nanomaterials. 2021; 11: 1227.
[32] Sykes EA, Dai Q, Sarsons CD, Chen J, Rocheleau JV, Hwang DM, *et al*. Tailoring nanoparticle designs to target cancer based on tumor pathophysiology. Proceedings of the National Academy of Sciences of the United States of America. 2016; 113: E1142–E1151.
[33] Park H, Otte A, Park K. Evolution of drug delivery systems: From 1950 to 2020 and beyond. Journal of Controlled Release. 2022; 342: 53–65.
[34] Zhao B, Chen S, Hong Y, Jia L, Zhou Y, He X, *et al*. Research Progress of Conjugated Nanomedicine for Cancer Treatment. Pharmaceutics. 2022; 14: 1522.
[35] Zhong H, Chan G, Hu Y, Hu H, Ouyang D. A Comprehensive Map of FDA-Approved Pharmaceutical Products. Pharmaceutics. 2018; 10: 263.
[36] Anselmo AC, Mitragotri S. Nanoparticles in the clinic: An update. Bioengineering & Translational Medicine. 2019; 4: e10143.
[37] Vandooren J, Opdenakker G, Loadman PM, Edwards DR. Proteases in cancer drug delivery. Advanced Drug Delivery Reviews. 2016; 97: 144–155.
[38] Li Z, Krippendorff BF, Shah DK. Influence of Molecular size on the clearance of antibody fragments. Pharmaceutical Research. 2017; 34: 2131–2141.
[39] Ma L, Wang C, He Z, Cheng B, Zheng L, Huang K. Peptide-Drug Conjugate: A Novel Drug Design Approach. Current Medicinal Chemistry. 2017; 24: 3373–3396.
[40] Hoppenz P, Els-Heindl S, Beck-Sickinger AG. Peptide-Drug Conjugates and Their Targets in Advanced Cancer Therapies. Frontiers in Chemistry. 2020; 8: 571.
[41] Alas M, Saghaeidehkordi A, Kaur K. Peptide-Drug Conjugates with Different Linkers for Cancer Therapy. Journal of Medicinal Chemistry. 2021; 64: 216–232.
[42] Cancer.net. Brain Tumor: Types of treatment. 2021. Available at: https://www.cancer.net/cancer-types/brain-tumor/types-treatment (Accessed: 2 December 2021).
[43] Cancer.net. Brain Tumor: Statistics. 2021. Available at: https://www.cancer.net/cancer-types/brain-tumor/statistics (Accessed: 2 December 2021).
[44] Matsuda Y, Mendelsohn BA. An overview of process development for antibody-drug conjugates produced by chemical conjugation technology. Expert Opinion on Biological Therapy. 2021; 21: 963–975.
[45] Ebenig A, Juettner NE, Deweid L, Avrutina O, Fuchsbauer HL, Kolmar H. Efficient Site-Specific Antibody-Drug Conjugation by Engineering a Nature-Derived Recognition Tag for Microbial Transglutaminase. Chembiochem. 2019; 20: 2411–2419.
[46] Vollmar BS, Wei B, Ohri R, Zhou J, He J, Yu SF, *et al*. Attachment Site Cysteine Thiol $pK_a$ Is a Key Driver for Site-Dependent Stability of THIOMAB Antibody-Drug Conjugates. Bioconjugate Chemistry. 2017; 28: 2538–2548.
[47] Matsuda Y, Seki T, Yamada K, Ooba Y, Takahashi K, Fujii T, *et al*. Chemical Site-Specific Conjugation Platform to Improve the Pharmacokinetics and Therapeutic Index of Antibody-Drug Conjugates. Molecular Pharmaceutics. 2021; 18: 4058–4066.
[48] Matsuda Y, Robles V, Malinao MC, Song J, Mendelsohn BA. Comparison of Analytical Methods for Antibody-Drug Conjugates Produced by Chemical Site-Specific Conjugation: First-Generation AJICAP. Analytical Chemistry. 2019; 91: 12724–12732.
[49] Zhou Q. Site-Specific Antibody Conjugation for ADC and Beyond. Biomedicines. 2017; 5: 64.
[50] Park J, Lee S, Kim Y, Yoo TH. Methods to generate site-specific conjugates of antibody and protein. Bioorganic & Medicinal Chemistry. 2021; 30: 115946.
[51] Walsh SJ, Bargh JD, Dannheim FM, Hanby AR, Seki H, Counsell AJ, *et al*. Site-selective modification strategies in antibody-drug conjugates. Chemical Society Reviews. 2021; 50: 1305–1353.
[52] Zhou Q. Site-Specific Antibody Conjugation with Payloads beyond Cytotoxins. Molecules. 2023; 28: 917.
[53] Hamblett KJ, Senter PD, Chace DF, Sun MMC, Lenox J, Cerveny CG, *et al*. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. Clinical Cancer Research. 2004; 10: 7063–7070.
[54] Sadiki A, Vaidya SR, Abdollahi M, Bhardwaj G, Dolan ME, Turna H, *et al*. Site-specific conjugation of native antibody. Antibody Therapeutics. 2020; 3: 271–284.
[55] Strop P, Liu SH, Dorywalska M, Delaria K, Dushin RG, Tran TT, *et al*. Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. Chemistry & Biology. 2013; 20: 161–167.
[56] Kayser V, Reslan M. Pivotal Biology, Chemistry, Biochemistry, and Biophysical Concepts of Biologics and Biosimilars. In Iqbal R (ed.) Biologics, Biosimilars, and Biobetters: An Introduction for Pharmacists, Physicians, and Other Health Practitioners (pp. 89–107). 1st edn. John Wiley & Sons: Hoboken. 2021.
[57] Kayser V, Sen M. New Emerging Biotherapies: Cutting-Edge Research to Experimental Therapies. In Iqbal R (ed.) Biologics, Biosimilars, and Biobetters: An Introduction for Pharmacists, Physicians, and Other Health Practitioners (pp. 213–236). 1st edn. John Wiley & Sons: Hoboken. 2021.
[58] Hutchinson MH, Hendricks RS, Lin XX, Olson DA. Process development and manufacturing of antibody-drug conjugates. Biopharmaceutical processing (pp. 813–836). Elsevier: Amsterdam. 2018.

[59] Nunes JPM, Vassileva V, Robinson E, Morais M, Smith MEB, Pedley RB, *et al*. Use of a next generation maleimide in combination with THIOMAB™ antibody technology delivers a highly stable, potent and near homogeneous THIOMAB™ antibody-drug conjugate (TDC). RSC Advances. 2017; 7: 24828–24832.
[60] Voynov V, Chennamsetty N, Kayser V, Wallny HJ, Helk B, Trout BL. Design and application of antibody cysteine variants. Bioconjugate Chemistry. 2010; 21: 385–392.
[61] Yamada K, Ito Y. Recent Chemical Approaches for Site-Specific Conjugation of Native Antibodies: Technologies toward Next-Generation Antibody-Drug Conjugates. Chembiochem. 2019; 20: 2729–2737.
[62] Accessdata.fda. POLIVY™. 2019. Available at: https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/761121s000lbl.pdf (Accessed: 1 March 2021).
[63] Kaplon H, Muralidharan M, Schneider Z, Reichert JM. Antibodies to watch in 2020. mAbs. 2020; 12: 1703531.
[64] Hussain AF, Grimm A, Sheng W, Zhang C, Al-Rawe M, Bräutigam K, *et al*. Toward Homogenous Antibody Drug Conjugates Using Enzyme-Based Conjugation Approaches. Pharmaceuticals. 2021; 14: 343.
[65] Kim CH, Axup JY, Schultz PG. Protein conjugation with genetically encoded unnatural amino acids. Current Opinion in Chemical Biology. 2013; 17: 412–419.
[66] Behrens CR, Liu B. Methods for site-specific drug conjugation to antibodies. mAbs. 2014; 6: 46–53.
[67] Sochaj AM, Świderska KW, Otlewski J. Current methods for the synthesis of homogeneous antibody-drug conjugates. Biotechnology Advances. 2015; 33: 775–784.
[68] Axup JY, Bajjuri KM, Ritland M, Hutchins BM, Kim CH, Kazane SA, *et al*. Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109: 16101–16106.
[69] Zimmerman ES, Heibeck TH, Gill A, Li X, Murray CJ, Madlansacay MR, *et al*. Production of site-specific antibody-drug conjugates using optimized non-natural amino acids in a cell-free expression system. Bioconjugate Chemistry. 2014; 25: 351–361.
[70] Nagaraja Shastri P, Zhu J, Skidmore L, Liang X, Ji Y, Gu Y, *et al*. Nonclinical Development of Next-generation Site-specific HER2-targeting Antibody-drug Conjugate (ARX788) for Breast Cancer Treatment. Molecular Cancer Therapeutics. 2020; 19: 1822–1832.
[71] Skidmore L, Sakamuri S, Knudsen NA, Hewet AG, Milutinovic S, Barkho W, *et al*. ARX788, a Site-specific Anti-HER2 Antibody-Drug Conjugate, Demonstrates Potent and Selective Activity in HER2-low and T-DM1-resistant Breast and Gastric Cancers. Molecular Cancer Therapeutics. 2020; 19: 1833–1843.
[72] U.S. National Library of Medicine. Clinicaltrials. 2021. Available online: https://clinicaltrials.gov/ (Accessed: 30 September 2021).
[73] Kayser V, Chennamsetty N, Voynov V, Forrer K, Helk B, Trout BL. Glycosylation influences on the aggregation propensity of therapeutic monoclonal antibodies. Biotechnology Journal. 2011; 6: 38–44.
[74] Reslan M, Sifniotis V, Cruz E, Sumer-Bayraktar Z, Cordwell S, Prof, Kayser V. Enhancing the stability of adalimumab by engineering additional glycosylation motifs. International Journal of Biological Macromolecules. 2020; 158: 189–196.
[75] Qasba PK. Glycans of Antibodies as a Specific Site for Drug Conjugation Using Glycosyltransferases. Bioconjugate Chemistry. 2015; 26: 2170–2175.
[76] Duivelshof BL, Deslignière E, Hernandez-Alba O, Ehkirch A, Toftevall H, Sjögren J, *et al*. Glycan-Mediated Technology for Obtaining Homogeneous Site-Specific Conjugated Antibody-Drug Conjugates: Synthesis and Analytical Characterization by Using Complementary Middle-up LC/HRMS Analysis. Analytical Chemistry. 2020; 92: 8170–8177.
[77] Dean AQ, Luo S, Twomey JD, Zhang B. Targeting cancer with antibody-drug conjugates: Promises and challenges. mAbs. 2021; 13: 1951427.
[78] Fessler S, Dirksen A, Collins SD, Xu L, Lee W, Wang J, *et al*. XMT-1592, a site-specific Dolasynthen-based NaPi2b-targeted antibody-drug conjugate for the treatment of ovarian cancer and lung adenocarcinoma. 2020. Available at: https://aacrjournals.org/cancerres/article/80/16_Supplement/2894/642274/Abstract-2894-XMT-1592-a-site-specific-Dolasynthen (Accessed: 20 September 2021).
[79] Dennler P, Fischer E, Schibli R. Antibody Conjugates: From Heterogeneous Populations to Defined Reagents. Antibodies. 2015; 4: 197–224.
[80] van Berkel SS, van Delft FL. Enzymatic strategies for (near) clinical development of antibody-drug conjugates. Drug Discovery Today. Technologies. 2018; 30: 3–10.
[81] Lotze J, Reinhardt U, Seitz O, Beck-Sickinger AG. Peptide-tags for site-specific protein labelling in vitro and in vivo. Molecular BioSystems. 2016; 12: 1731–1745.
[82] Anami Y, Tsuchikama K. Transglutaminase-mediated conjugations. Antibody-Drug Conjugates (pp. 71–82). Springer: NY. 2020.
[83] Ohtsuka T, Sawa A, Kawabata R, Nio N, Motoki M. Substrate specificities of microbial transglutaminase for primary amines. Journal of Agricultural and Food Chemistry. 2000; 48: 6230–6233.
[84] Liu X, Chen Y, Li H, Huang N, Jin Q, Ren K, *et al*. Enhanced retention and cellular uptake of nanoparticles in tumors by controlling their aggregation behavior. ACS Nano. 2013; 7: 6244–6257.
[85] Auffan M, Rose J, Bottero JY, Lowry GV, Jolivet JP, Wiesner MR. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nature Nanotechnology. 2009; 4: 634–641.
[86] Anisuzzman M, Komalla V, Tarkistani MAM, Kayser V. Anti-Tumor Activity of Novel Nimotuzumab-Functionalized Gold Nanoparticles as a Potential Immunotherapeutic Agent against Skin and Lung Cancers. Journal of Functional Biomaterials. 2023; 14: 407.
[87] Sanità G, Carrese B, Lamberti A. Nanoparticle Surface Functionalization: How to Improve Biocompatibility and Cellular Internalization. Frontiers in Molecular Biosciences. 2020; 7: 587012.
[88] Selmani A, Kovačević D, Bohinc K. Nanoparticles: From synthesis to applications and beyond. Advances in Colloid and Interface Science. 2022; 303: 102640.
[89] Morales-Cruz M, Delgado Y, Castillo B, Figueroa CM, Molina AM, Torres A, *et al*. Smart Targeting To Improve Cancer Therapeutics. Drug Design, Development and Therapy. 2019; 13: 3753–3772.
[90] Adamo G, Campora S, Ghersi G. Functionalization of nanoparticles in specific targeting and mechanism release. Nanostructures for Novel Therapy (pp. 57–80). Elsevier: Amsterdam. 2017.
[91] Xu L, Xu M, Sun X, Feliu N, Feng L, Parak WJ, *et al*. Quantitative Comparison of Gold Nanoparticle Delivery *via* the Enhanced Permeation and Retention (EPR) Effect and Mesenchymal Stem Cell (MSC)-Based Targeting. ACS Nano. 2023; 17: 2039–2052.
[92] Mout R, Moyano DF, Rana S, Rotello VM. Surface functionalization of nanoparticles for nanomedicine. Chemical Society Reviews. 2012; 41: 2539–2544.
[93] Thiruppathi R, Mishra S, Ganapathy M, Padmanabhan P, Gulyás B. Nanoparticle Functionalization and Its Potentials for Molec-

ular Imaging. Advanced Science. 2016; 4: 1600279.

[94] Li X, Montague EC, Pollinzi A, Lofts A, Hoare T. Design of Smart Size-, Surface-, and Shape-Switching Nanoparticles to Improve Therapeutic Efficacy. Small. 2022; 18: e2104632.

[95] Johnston MC, Scott CJ. Antibody conjugated nanoparticles as a novel form of antibody drug conjugate chemotherapy. Drug Discovery Today. Technologies. 2018; 30: 63–69.

[96] Fay F, Scott CJ. Antibody-targeted nanoparticles for cancer therapy. Immunotherapy. 2011; 3: 381–394.

[97] Jasim A, Abdelghany S, Greish K. Current update on the role of enhanced permeability and retention effect in cancer nanomedicine. Nanotechnology-based approaches for targeting and delivery of drugs and genes (pp. 62–109). Elsevier: Amsterdam. 2017.

[98] Heldin CH, Rubin K, Pietras K, Ostman A. High interstitial fluid pressure - an obstacle in cancer therapy. Nature Reviews. Cancer. 2004; 4: 806–813.

[99] Lin X, O'Reilly Beringhs A, Lu X. Applications of Nanoparticle-Antibody Conjugates in Immunoassays and Tumor Imaging. The AAPS Journal. 2021; 23: 43.

[100] Polo E, Puertas S, Moros M, Batalla P, Guisán JM, de la Fuente JM, *et al*. Tips for the functionalization of nanoparticles with antibodies. Methods in Molecular Biology. 2013; 1051: 149–163.

[101] Beck A, Goetsch L, Dumontet C, Corvaïa N. Strategies and challenges for the next generation of antibody-drug conjugates. Nature Reviews. Drug Discovery. 2017; 16: 315–337.

[102] Juan A, Cimas FJ, Bravo I, Pandiella A, Ocaña A, Alonso-Moreno C. An Overview of Antibody Conjugated Polymeric Nanoparticles for Breast Cancer Therapy. Pharmaceutics. 2020; 12: 802.

[103] Shen M, Rusling J, Dixit CK. Site-selective orientated immobilization of antibodies and conjugates for immunodiagnostics development. Methods. 2017; 116: 95–111.

[104] Palchetti S, Caputo D, Digiacomo L, Capriotti AL, Coppola R, Pozzi D, *et al*. Protein Corona Fingerprints of Liposomes: New Opportunities for Targeted Drug Delivery and Early Detection in Pancreatic Cancer. Pharmaceutics. 2019; 11: 31.

[105] Aghebati-Maleki A, Dolati S, Ahmadi M, Baghbanzhadeh A, Asadi M, Fotouhi A, *et al*. Nanoparticles and cancer therapy: Perspectives for application of nanoparticles in the treatment of cancers. Journal of Cellular Physiology. 2020; 235: 1962–1972.

[106] Bulbake U, Doppalapudi S, Kommineni N, Khan W. Liposomal Formulations in Clinical Use: An Updated Review. Pharmaceutics. 2017; 9: 12.

[107] Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, *et al*. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Research. 1994; 54: 987–992.

[108] Allen TM, Hansen CB, Stuart DD. Targeted sterically stabilized liposomal drug delivery. In Lasic D, Papahadjopoulos D (eds.) Medical applications of liposomes (pp. 297–323). 1st edn. Elsevier Science: NY. 1998.

[109] Subhan MA, Yalamarty SSK, Filipczak N, Parveen F, Torchilin VP. Recent Advances in Tumor Targeting via EPR Effect for Cancer Treatment. Journal of Personalized Medicine. 2021; 11: 571.

[110] ten Hagen TLM, Seynhaeve ALB, van Tiel ST, Ruiter DJ, Eggermont AMM. Pegylated liposomal tumor necrosis factor-alpha results in reduced toxicity and synergistic antitumor activity after systemic administration in combination with liposomal doxorubicin (Doxil) in soft tissue sarcoma-bearing rats. International Journal of Cancer. 2002; 97: 115–120.

[111] Sercombe L, Veerati T, Moheimani F, Wu SY, Sood AK, Hua S. Advances and Challenges of Liposome Assisted Drug Delivery. Frontiers in Pharmacology. 2015; 6: 286.

[112] Almeida B, Nag OK, Rogers KE, Delehanty JB. Recent Progress in Bioconjugation Strategies for Liposome-Mediated Drug Delivery. Molecules. 2020; 25: 5672.

[113] Puri A, Loomis K, Smith B, Lee JH, Yavlovich A, Heldman E, *et al*. Lipid-based nanoparticles as pharmaceutical drug carriers: from concepts to clinic. Critical Reviews in Therapeutic Drug Carrier Systems. 2009; 26: 523–580.

[114] Koning GA, Kamps JAAM, Scherphof GL. Efficient intracellular delivery of 5-fluorodeoxyuridine into colon cancer cells by targeted immunoliposomes. Cancer Detection and Prevention. 2002; 26: 299–307.

[115] Hamamichi S, Fukuhara T, Umeda IO, Fujii H, Hattori N. Novel method for screening functional antibody with comprehensive analysis of its immunoliposome. Scientific Reports. 2021; 11: 4625.

[116] Lu L, Ding Y, Zhang Y, Ho RJ, Zhao Y, Zhang T, *et al*. Antibody-modified liposomes for tumor-targeting delivery of timosaponin AIII. International Journal of Nanomedicine. 2018; 13: 1927–1944.

[117] Makwana V, Karanjia J, Haselhorst T, Anoopkumar-Dukie S, Rudrawar S. Liposomal doxorubicin as targeted delivery platform: Current trends in surface functionalization. International Journal of Pharmaceutics. 2021; 593: 120117.

[118] Choi KY, Swierczewska M, Lee S, Chen X. Protease-activated drug development. Theranostics. 2012; 2: 156–178.

[119] He R, Finan B, Mayer JP, DiMarchi RD. Peptide Conjugates with Small Molecules Designed to Enhance Efficacy and Safety. Molecules. 2019; 24: 1855.

[120] Régina A, Demeule M, Ché C, Lavallée I, Poirier J, Gabathuler R, *et al*. Antitumour activity of ANG1005, a conjugate between paclitaxel and the new brain delivery vector Angiopep-2. British Journal of Pharmacology. 2008; 155: 185–197.

[121] Kumthekar P, Tang SC, Brenner AJ, Kesari S, Piccioni DE, Anders C, *et al*. ANG1005, a Brain-Penetrating Peptide-Drug Conjugate, Shows Activity in Patients with Breast Cancer with Leptomeningeal Carcinomatosis and Recurrent Brain Metastases. Clinical Cancer Research. 2020; 26: 2789–2799.

[122] You Y, Xu Z, Chen Y. Doxorubicin conjugated with a trastuzumab epitope and an MMP-2 sensitive peptide linker for the treatment of HER2-positive breast cancer. Drug Delivery. 2018; 25: 448–460.

[123] Cho YS, Chung SW, Kim HR, Won TH, Choi JU, Kim IS, *et al*. The novel strategy for concurrent chemoradiotherapy by conjugating the apoptotic cell-binding moiety to caspase-3 activated doxorubicin prodrug. Journal of Controlled Release. 2019; 296: 241–249.

[124] Chung SW, Lee BS, Choi JU, Kim SW, Kim IS, Kim SY, *et al*. Optimization of a Stable Linker Involved DEVD Peptide-Doxorubicin Conjugate That Is Activated upon Radiation-Induced Caspase-3-Mediated Apoptosis. Journal of Medicinal Chemistry. 2015; 58: 6435–6447.

[125] Poreba M. Protease-activated prodrugs: strategies, challenges, and future directions. The FEBS Journal. 2020; 287: 1936–1969.

[126] Lee BS, Cho YW, Kim GC, Lee DH, Kim CJ, Kil HS, *et al*. Induced phenotype targeted therapy: radiation-induced apoptosis-targeted chemotherapy. Journal of the National Cancer Institute. 2014; 107: dju403.

[127] Vidmar R, Vizovišek M, Turk D, Turk B, Fonović M. Protease cleavage site fingerprinting by label-free in-gel degradomics reveals pH-dependent specificity switch of legumain. The EMBO Journal. 2017; 36: 2455–2465.

[128] Dal Corso A, Pignataro L, Belvisi L, Gennari C. Innovative Linker Strategies for Tumor-Targeted Drug Conjugates. Chemistry. 2019; 25: 14740–14757.

[129] Dall E, Brandstetter H. Mechanistic and structural studies on legumain explain its zymogenicity, distinct activation pathways, and regulation. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110: 10940–10945.
[130] Guo P, Zhu Z, Sun Z, Wang Z, Zheng X, Xu H. Expression of legumain correlates with prognosis and metastasis in gastric carcinoma. PLoS ONE. 2013; 8: e73090.
[131] Lerchen HG, Stelte-Ludwig B, Sommer A, Berndt S, Rebstock AS, Johannes S, *et al*. Tailored Linker Chemistries for the Efficient and Selective Activation of ADCs with KSPi Payloads. Bioconjugate Chemistry. 2020; 31: 1893–1898.
[132] Liu C, Sun C, Huang H, Janda K, Edgington T. Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. Cancer Research. 2003; 63: 2957–2964.
[133] Poreba M. Recent advances in the development of legumain-selective chemical probes and peptide prodrugs. Biological Chemistry. 2019; 400: 1529–1550.
[134] Bushnell DL, Bodeker KL. Overview and Current Status of Peptide Receptor Radionuclide Therapy. Surgical Oncology Clinics of North America. 2020; 29: 317–326.
[135] Cooper BM, Iegre J, O' Donovan DH, Ölwegård Halvarsson M, Spring DR. Peptides as a platform for targeted therapeutics for cancer: peptide-drug conjugates (PDCs). Chemical Society Reviews. 2021; 50: 1480–1494.
[136] Chung SW, Cho YS, Choi JU, Kim HR, Won TH, Kim SY, *et al*. Highly potent monomethyl auristatin E prodrug activated by caspase-3 for the chemoradiotherapy of triple-negative breast cancer. Biomaterials. 2019; 192: 109–117.
[137] Lerchen HG, Stelte-Ludwig B, Berndt S, Sommer A, Dietz L, Rebstock AS, *et al*. Antibody-Prodrug Conjugates with KSP Inhibitors and Legumain-Mediated Metabolite Formation. Chemistry. 2019; 25: 8208–8213.
[138] Hinrichs MJM, Ryan PM, Zheng B, Afif-Rider S, Yu XQ, Gunsior M, *et al*. Fractionated Dosing Improves Preclinical Therapeutic Index of Pyrrolobenzodiazepine-Containing Antibody Drug Conjugates. Clinical Cancer Research. 2017; 23: 5858–5868.
[139] Loo D, Alderson RF, Chen FZ, Huang L, Zhang W, Gorlatov S, *et al*. Development of an Fc-enhanced anti-B7-H3 monoclonal antibody with potent antitumor activity. Clinical Cancer Research. 2012; 18: 3834–3845.
[140] Dong P, Xiong Y, Yue J, Hanley SJB, Watari H. B7H3 As a Promoter of Metastasis and Promising Therapeutic Target. Frontiers in Oncology. 2018; 8: 264.
[141] Hennrich U, Kopka K. Lutathera®: The First FDA- and EMA-Approved Radiopharmaceutical for Peptide Receptor Radionuclide Therapy. Pharmaceuticals. 2019; 12: 114.
[142] Das S, Al-Toubah T, El-Haddad G, Strosberg J. $^{177}$Lu-DOTATATE for the treatment of gastroenteropancreatic neuroendocrine tumors. Expert Review of Gastroenterology & Hepatology. 2019; 13: 1023–1031.
[143] Breeman WAP, Chan HS, de Zanger RMS, Konijnenberg MK, de Blois E. Overview of Development and Formulation of $^{177}$Lu-DOTA-TATE for PRRT. Current Radiopharmaceuticals. 2016; 9: 8–18.
[144] Ji X, Wang H, Chen Y, Zhou J, Liu Y. Recombinant expressing angiopep-2 fused anti-VEGF single chain Fab (scFab) could cross blood-brain barrier and target glioma. AMB Express. 2019; 9: 165.
[145] Padayachee ER, Biteghe FAN, Malindi Z, Bauerschlag D, Barth S. Human Antibody Fusion Proteins/Antibody Drug Conjugates in Breast and Ovarian Cancer. Transfusion Medicine and Hemotherapy: Offizielles Organ Der Deutschen Gesellschaft Fur Transfusionsmedizin Und Immunhamatologie. 2017; 44: 303–310.
[146] Chung SW, Choi JU, Lee BS, Byun J, Jeon OC, Kim SW, *et al*. Albumin-binding caspase-cleavable prodrug that is selectively activated in radiation exposed local tumor. Biomaterials. 2016; 94: 1–8.