Review article

Antibody conjugation and formulation

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ABSTRACT

In an era where ultra-high antibody concentrations, high viscosities, low volumes, auto-injectors and long storage requirements are already complex problems with the current unconjugated monoclonal antibodies on the market, the formulation demands for antibody-drug conjugates (ADCs) are significant. Antibodies have historically been administered at relatively low concentrations through intravenous (IV) infusion due to their large size and the inability to formulate for oral delivery. Due to the high demands associated with IV infusion and the development of novel antibody targets and unique antibody conjugates, more accessible routes of administration such as intramuscular and subcutaneous are being explored. This review will summarize various site-specific and non-site-specific antibody conjugation techniques in the context of ADCs and the demands of formulation for high concentration clinical implementation.

> **Statement of Significance:** Antibody-drug conjugates utilize a variety of site-specific and non-site-specific conjugation techniques. This review will detail some of the issues that may arise as heterogeneous antibody conjugate mixtures are formulated at high concentrations for use in clinical applications.

INTRODUCTION

Antibodies are produced in a variety of forms, from full-length naked antibodies to conjugated antibodies or antibody fragments. Antibodies themselves are capable of activating or inhibiting a target *in vivo* through either competitive binding, sequestration or providing a means of foreign body tagging inherit in the antibody Fc backbone structure. The first unmodified antibody was a murine anti-CD3 antibody (Muromonab) developed as a reversal agent to mitigate kidney transplant rejection and was approved in the US in 1986. To date there have been >60 Food and Drug Administration (FDA)-approved unmodified monoclonal antibodies. Pharmaceutical antibodies consistently are ranked among the highest grossing pharmaceuticals with the top five having combined annual sales that exceed $40 billion/yr [1]. Antibodies have come a long way since 1986 with the advent of chimeric and humanization processing that help minimize host immune activation against the administered antibodies themselves. With >550 monoclonal antibodies currently in the clinical pipeline, in both cancer and non-cancer indications, the number of monoclonal antibodies with unique clinical targets

will continue to rise [2–4]. There is no question whether or not these first-generation antibodies will continue to be developed; however, next-generation antibody-drug conjugates (ADCs) have already begun to gain a clinical footing with four currently approved ADC formulations with cancer-specific indications [4, 5]. This review will detail some of the issues that may arise in the field as heterogeneous antibody conjugate mixtures are formulated at high concentrations for clinical use [6].

ANTIBODY FORMULATION

After a clinical target has been determined and an antibody has been selected, it is necessary to formulate the antibody therapeutic prior to clinical use. Antibody formulation is a complex optimization process utilizing unique pharmaceutical additives to address the varying demands of storage, freeze–thaw and route of administration necessary for the clinical application. There has been an ever-growing trend to increase the concentration of antibody-based therapeutics for clinical applications making the formulation process more difficult. This is largely driven by the desire to

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decrease the volume of injection while still providing for the same dose of drug to be administered. The primary reason for this industry push is to enhance clinical outcomes and to allow for more flexible routes of administration.

Most antibody therapeutics are given systemically through an intravenous (IV) requiring a healthcare professional to first attain venous access and then set up the infusion. IV is an excellent route of drug administration for large volume drugs but the complications and difficulties that arise with venipuncture make IV less accessible than comparable intramuscular (IM) and subcutaneous (SC) injection modalities. It is important to note that there is a limit to the volume (<1.5 ml) that can be injected SC which is why antibody formulations are being pushed toward the 100's of mg/ml concentrations to allow for smaller volumes while still being able to attain a therapeutically relevant delivery of the pharmaceutical agent [6, 7]. IM and SC administration also provides for different release kinetics, bioavailability and extended half-life in some instances that can allow the periods between drug administrations to potentially be extended [8]. With the development of antibodies for more chronic diseases, having an SC injection route would allow for self-administration of a drug, similar to insulin, which would greatly increase accessibility. For these reasons, it is not surprising that antibody formulation has been pushing the boundaries of concentration. To add to the complexity, it is not simply sufficient to formulate an antibody at a very high stable concentration as the resulting formulation must also fall within physical parameter boundaries of viscosity [9, 10]. This is particularly important for auto-injectors but also plays a role in syringe-based injection with various syringe geometries and loading assemblies helping to improve tolerance of high viscosity formulations. At ultra-high viscosities, it is nearly impossible to force the high antibody concentration formulation through a small bore needle making administration, regardless of stability, unreasonable [11, 12].

Antibody formulation is best addressed early and often through the entirety of the antibody development lifecycle with the final antibody formulation for clinical use being finalized as early as possible in the development process. It is a mistake to not consider the demands of formulation upon selection of a lead compound early in development as not every antibody can be formulated in any way to achieve a specific outcome. Each unique delivery method (IV, IM, SC, auto-injector), unique molecular entity (anti-body, protein, nanoparticle, small molecule) and unique target (cancer, non-cancer) require different formulation conditions to ensure that maximum stability and shelf life can be attained [13–15]. The process of formulating an antibody therapeutic is complex requiring first an understanding of how the protein handles exposure to stressors such as freeze/thaw, agitation, thermal stability and pH/buffer response followed by the addition of excipients to mitigate undesirable protein instabilities [16, 17]. An excipient is any additive that is included in a formulation that is used to stabilize the formation that is not considered an active ingredient. Example excipients include fillers, extenders, diluents, solvents, preservatives, absorption enhancers and sustained release matrices. There is a relatively short list of buffers and excipients that are currently FDA approved for formulation of antibodies which greatly limits the space for high concentration optimization. Pharmaceutical companies are hesitant to introduce new excipients due to the high regulatory burden associated with getting a new excipient approved in the context of the already high regulatory burden in getting the antibody, and its subsequent clinical formulation, approved as well.

Most pharmaceutical antibodies are monoclonal antibodies; meaning, that each antibody molecule is identical to all other antibody molecules in solution. Despite this fact, formulating high concentrations of antibodies that retain a clinically significant self life is not a trivial task. Antibodies are large proteins made up of >1 200 amino acids and have a propensity to aggregate at high concentrations making formulation optimization difficult [18, 19]. With the onset of polyclonal antibodies, ADCs and mixtures of monoclonal antibody therapeutics, the already difficult task of antibody formulation becomes much more complicated as formulation difficulty is often correlated to sample complexity (Fig. 1).

To address the high demands for stability and concentration and the limited number of approved stabilizing compounds, a large amount of effort has been placed on the sequence of optimization steps used to produce the most stable final antibody formulation. An example of this is to first test a series of buffers and buffer concentrations and select for the most stable buffer composition. Following buffer selection excipients are then added sequentially, optimizing at each step, to selectively mitigate instabilities observed as concentration is increased modulating pH, ionic strength, surfactants, cryoprotectants
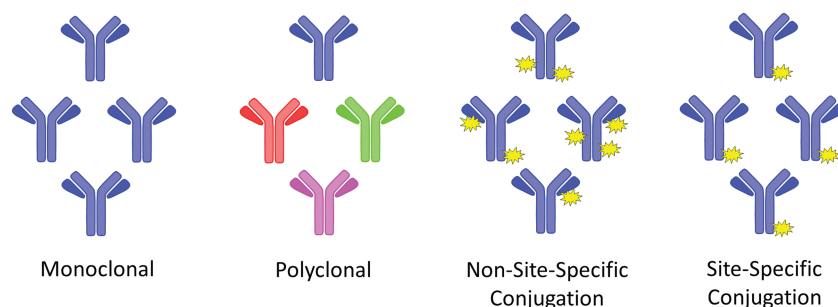

| Monoclonal | Polyclonal | Non-Site-Specific Conjugation | Site-Specific Conjugation |

Figure 1. Formulation difficulty increases comparing monoclonal antibodies to polyclonal antibodies and ADCs utilizing non-site-specific and site-specific conjugation strategies.

and other stabilizing agents working toward a final clinical formulation. There are a number of techniques utilized to determine the optimal formulation for each unique monoclonal antibody or ADC. Common characterization techniques that assess the antibody stability and activity following exposure to diverse stressed conditions include size exclusion chromatography to detect aggregate formation, ion-exchange chromatography or isoelectric focusing to detect charge variants generated by chemical instability, capillary electrophoresis sodium dodecyl sulfate for detection of fragmentation, mass spectrometry and potency assays for biological activity [20, 21]. Typical methodologies for assessing the antibody stability in its native formulation state include molecular modeling to assess surface characteristics, dynamic light scattering to assess self-association propensity and pH effects, isothermal chemical denaturation to assess optimal buffer conditions, differential scanning calorimetry to assess thermal stability and particulate formation characterization [22]. There are a number of proprietary formulation platforms that automate some of the processing but it is important to note that the ultimate formulation determination does depend on the route of optimization as different routes infrequently result in the same final optimized formulation.

ANTIBODY CONJUGATION

While the capabilities of unconjugated, first-generation antibodies are impressive, there is a growing trend toward endowing the native antibody with unnatural capabilities (immuno-conjugates) to enhance therapeutic efficacy. This comes in the most common form as a treatment of cancer utilizing ADCs. ADC formulations possess conjugated cytotoxic drugs that have enhanced localized effects through targeted delivery utilizing the antibody to provide specificity and the cytotoxic payload as an active agent. There are several different methods (both site specific and non-site specific) to conjugate functional ligands to antibodies. Conjugation options for antibody modification can be split into three primary categories: naturally occurring sites of modification that exist in the native antibody structure (natural), non-naturally occurring sites that can be added to the antibody backbone through genetic manipulation (non-natural) and unique (specialty) antibody modification modalities that do not fall neatly in either the natural or non-natural modification categories (Fig. 2). The categories and highlighted techniques detailed below are not intended to be an exhaustive list rather representative examples of commonly utilized conjugation strategies [3, 5, 23, 24]. Selection of an optimal conjugation technique is dependent on a number of factors including the payload to be attached, the intended target, the amount of conjugations needed to attain therapeutic efficacy, the ability to conjugate without negatively affecting antibody specificity and the tolerance for heterogeneity across the conjugated antibody population [25]. An important measure of heterogeneity is the drug-antibody ratio (DAR). Minimizing the variability in DAR is important as higher heterogeneity can result in varied pharmacokinetics, reduced half-life, increased plasma clearance, increased

toxicity and will increase the difficulty to attain a stable clinical formulation.

Natural

Antibodies are relatively large ~150 kDa glycoproteins that have many naturally occurring sites for conjugation. There are chemistries for conjugating to many of the 20 canonical amino acids [26, 27]. Despite the numerous available chemistries, the most common site for conjugation to proteins in general is through the ε-amine lysine side chain (Fig. 2.**1**). Due to the high abundance of lysine residues (>80 in the native antibody backbone), its positive charge, its relatively long extension into solution and its readily accessible aqueous conjugation techniques, it remains the most utilized technique despite not being site specific [28, 29]. While the high lysine abundance is useful for attaining high levels of conjugation to the antibody surface there is limited-to-no control over the number of conjugations per antibody (DAR) or their relative locations on the antibody surface [30, 31]. For this reason, it is not uncommon to have a highly heterogeneous conjugated antibody population with some of the conjugations occurring to the Fab, the Fc and even to the antigen-binding complementarity-determining region (CDR) resulting in partial inactivation of the conjugated antibody population. A modification to this amine conjugation technique is carried out under stringent conditions allowing for selective conjugation to the N-terminus of the antibody heavy and light chains taking advantage of the differing pKa value of the lysine ε-amine vs the N-terminus α-amine (Fig. 2.**2**) [32, 33]. Due to the proximity of the N-terminus to the antigen-binding region, there are some issues associated with steric interference to antigen binding following conjugation.

Another common naturally occurring amino acid that can be utilized for conjugation is the cysteine side chain. It is uncommon for the cysteine thiol group to be in its free-reduced form in nature as they are often found to be conjugated to another cysteine residue through a disulfide bond and this is no different in antibodies. Most antibodies have four inter-chain disulfide bonds that hold together the antibody heavy chains in the hinge region and the heavy and light chains in the Fab region [34, 35]. Due to the significantly reduced number of reactive sites, compared to lysine conjugation, there is a corresponding greatly reduced heterogeneity observed in the resulting antibody conjugate. The disulfide bonds in the hinge region can be selectively reduced providing for a site-specific location to conjugate a thiol reactive linker, such as maleimide, which results in the formation of a thioether bond (Fig. 2.**3**) [36, 37]. These disulfide bonds can also be utilized as targets for disulfide bond exchange allowing for an alternate site-specific conjugation methodology utilizing the canonical cysteine residues [38, 39].

Antibodies undergo post translational modifications, including glycosylation, which provide for an additional unique naturally occurring site for conjugation (Fig. 2.**4**). A reactive aldehyde group can be created at the carbohydrate locations through an oxidization reaction via the addition of sodium periodate allowing for further reaction with hydrazide functionalized linkers [40, 41]. Due to the
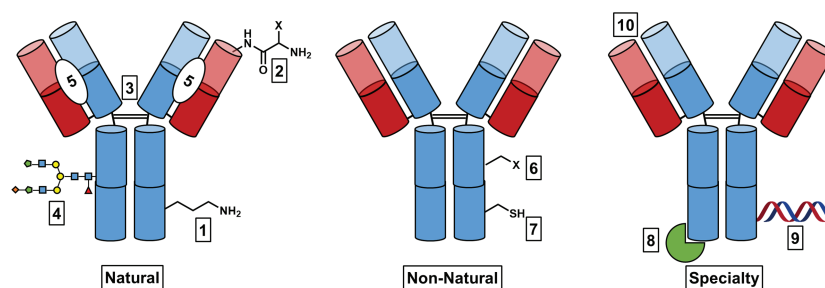
Figure 2. (**1**) Natural amino acids, (**2**) N-terminus heavy and light chain, (**3**) Interchain disulfide bonds, (**4**) Carbohydrate moiety, (**5**) NBS photoaffinity labeling, (**6**) Non-natural amino acids, (**7**) Engineered cysteines, (**8**) Protein-protein interactions, (**9**) Engineered tags, (**10**) Catalytic activity.

side reactions associated with periodate exposure and the varied nature and composition of post translational protein modifications, this technique requires significant optimization from antibody-to-antibody [42–44]. It is important to note that neither the carbohydrate nor the N-terminal heavy and light chain modification strategies have been utilized in any late-stage ADC formulations.

Relatively recently an alternate strategy for conjugation was described utilizing a highly conserved binding site present in nearly all antibody isotypes and across species between the heavy and light chains within the antibody variable region known as the nucleotide binding site (NBS, Fig. 2.**5**) [45, 46]. This conjugation strategy provides for two sites of conjugation, lending to the native antibody symmetry, and is achieved through UV activation of an NBS targeting small molecule such as an indole. [45, 47] While this conjugation strategy is appealing it has yet to be validated in animal studies due to its recent development and has therefore not been utilized clinically.

Non-natural

There are two primary non-natural antibody modification techniques that require utilization of genetic engineering to either insert a non-natural amino acid (Fig. 2.**6**) or place an engineered cysteine residue (Fig. 2.**7**) in the antibody backbone for subsequent conjugation. By adding a non-native cysteine, the inter-chain disulfide bonds that maintain the antibody tertiary structure can remain intact while still allowing for utilization of the highly developed thiol-specific chemistries and linkers [48]. These conjugation strategies result in the most homogenous modified antibody population but require the most intensive upfront development [49, 50]. The addition of non-canonical amino acids has also been explored to take advantage of alternate, orthogonal, chemical ligation strategies to improve conjugation homogeneity [51–54]. As a non-naturally occurring antibody modification each antibody would need to be specifically expressed with the backbone modification, making this technique less desirable as off-the-shelf antibodies cannot be readily modified. The backbone modification sites must be carefully tested to ensure that the resulting conjugated antibody maintains its other desirable engineered features such as extended serum half-life and immune activation capabilities, whenever relevant for each unique application [55, 56].

Specialty

Other antibody modification strategies include protein–protein interactions (Fig. 2.**8**), engineered tags (Fig. 2.**9**) and antibodies that possess catalytic activity (Fig. 2.**10**). The most common example of a protein–protein interaction (Fig. 2.**8**) is the use of an Fc-specific protein to bind an antibody such as the ZZ-domain which was engineered from the IgG binding domain of Staphylococcal protein A [57]. All other examples in this list of antibody modification techniques result in a covalent bond between the antibody and a functional linker other than this protein–protein interaction category. Engineered tags (Fig. 2.**9**) include sortase, split-proteins, coiled-coils, spy-tag/spy-catcher or other affinity tags in which the primary antibody is modified with an engineered tag that is complimentary to a secondary tag or protein that upon association or enzymatic reaction endows the antibody with a non-natural capability [52, 58–61]. Antibodies that have catalytic activity (Fig. 2.**10**) are unique in that their antigen specificity is also considered their substrate [62–64]. In some instances an antibody can be bi-specific in that one Fab may possess catalytic activity while the other has a differing antigen target or both CDRs can have catalytic activity. Antibodies with catalytic activity are rare and the *de novo* development of catalytic domains at the antibody terminus is non-trivial making this specialty category relatively uncommon.

ADC FORMULATION CHALLENGES

Unique challenges exist when formulating an ADC compared to a naked mAb. As detailed earlier, sample complexity plays a major role in making formulation of a non-site-specific ADC exceptionally difficult due to the diverse array of post conjugation species. In addition, the drugs that are attached to ADCs and the linker by which the drug is attached also instill added complexity to formulate an ADC [65, 66]. The drugs associated with ADCs often have less than desirable aqueous solubility characteristics themselves. These drugs have the propensity to cause inter-antibody aggregation through either exposure of protected aggregation-prone regions within the antibody backbone upon conjugation or through drug–drug mediated interactions [67–69]. This greatly limits longitudinal stability as well as high concentration formulation capabilities. The drugs that are conjugated to form ADCs are

also not the same from ADC to ADC making generalizable formulation protocols difficult to establish [3, 4].

The means by which the drugs are attached to the antibody is also a unique hurdle that needs special attention when considering formulation of an ADC compared to a naked mAb. Linkers vary in chemical composition providing the ADC with differing cleavable capabilities. Cleavable linkers fall into two primary categories that allow for cleavage either through enzymatic digestion or exposure to pH extremes [70–76]. There is an ever growing list of linker chemistries with distinctive capabilities that are being tested in diverse ADC applications [77]. These desirable delivery characteristics subsequently make the drug and linker susceptible to premature degradation and cleavage during storage which limits the potential formulation compositions considerably when compared to that of formulation of a naked mAb [75, 78]. Depending upon the selected linker and the method of conjugation of the linker to the antibody, critical antibody characteristics that directly affect solubility, including surface charge and zeta-potential, can be negatively impacted. For these reasons, drug, linker composition and antibody target are all critical components specifically selected to address the unique demands of the intended therapeutic application and unfortunately result in significantly more complex formulation demands.

CURRENT ADC FORMULATION

As of the writing of this review, there were only four FDA-approved ADCs: brentuximab vedotin (2012), ado-trastuzumab emtansine (2013), inotuzumab ozogamicin (2017) and gemtuzumab ozogamicin (2000/2017) (Table 1) [79–82]. All of these ADCs are formulated for IV infusion and have cancer-specific indications for use. Three of the four approved ADC formulations utilize non-site-specific conjugation of the cytotoxic agents to the abundant lysine residues found throughout the antibody surface (Fig. 2.**1**). These antibodies possess a variety of cleavable and non-cleavable linkers that are either susceptible to enzymatic degradation or are acid-labile, depending on each unique application [83]. Brentuximab vedotin utilizes a selective disulfide bond reduction and subsequent thiol conjugation strategy to site-specifically conjugate cytotoxic auristatins

to a monoclonal antibody (Fig. 2.**3**). It is not surprising that despite the high degree of heterogeneity associated with the non-site-specific lysine conjugation technique that it remains the dominant means of ADC production for clinical use due to its exceptional ease of use. All of these currently approved ADC formulations are administered via IV infusion at final antibody concentrations that are <20 mg/ml. Thus far there has been no high concentration ADC formulation produced and due to the relatively low antibody concentrations necessary for IV infusion formulation issues that are associated with non-site-specific antibody conjugation techniques have been less significant.

CONCLUSION

Despite there being only four currently approved ADC formulations, we will continue to see growth in the area of next-generation modified antibodies for a variety of applications as there are currently >60 novel ADC formulations in clinical trials, >50% in phase I. While the demand for high concentration ADCs has been thus far limited, the rapidly increasing next-generation antibody conjugates that are on the horizon may cause formulation to become a large issue that will limit routes of administration or drive more site-specific antibody modification techniques to be utilized. Polyclonal antibodies and non-site-specific conjugation techniques result in very heterogeneous mixtures of antibodies which may make high concentration formulation more difficult to attain. Site-specific conjugation should likely allow for the most optimal formulation and highest concentration attainable due to less conjugation variability which directly reduces sample complexity. Successful high concentration antibody formulation will open the door to more accessible routes of administration such as IM and SC injections which will expand the potential uses of antibodies and antibody conjugates in the treatment of diseases that currently remain out of reach due to the need for IV infusion. While the current chemotherapeutic and cytotoxic drug conjugates will continue to rise, we should see an increase in alternate drug conjugates for cancer and non-cancer indications such as peptides, diagnostic labels, immune-mediated disorders, cardiovascular/hemostasis, neurological, ophthalmic, infectious disease,

Table 1. Currently FDA-approved ADC formulations

Antibody	Antibody	Linker	Conjugation	Drug	Indication	Excipients
Brentuximab Vedotin (CD30)	Chimeric IgG1	MC-VC	Cysteine	MMAE	Hodgkin Lymphoma (HL)	Trehalose, sodium citrate, citric acid, polysorbate 80.
Ado-Trastuzumab Emtansine (HER2)	Humanized IgG1	MCC	Lysine	DM1	Metastatic Breast Cancer	Sodium succinate, sucrose, polysorbate 20.
Inotuzumab Ozogamicin (CD22)	Humanized IgG4	AcBut	Lysine	Calicheamicin	Acute Lymphoblastic Leukemia (ALL)	Sucrose, sodium chloride, tromethamine, polysorbate 80.
Gemtuzumab Ozogamicin (CD33)	Humanized IgG4	AcBut	Lysine	Calicheamicin	Acute Myeloid Leukemia (AML)	Sucrose, sodium chloride, sodium phosphate, dextran 40.

Abbreviations: MC-VC: maleimidocaproyl-valine citrulline; MCC: maleimidomethyl cyclohexane1-carboxylate; AcBut: 4-(4-acetylphenoxy)butanoic acid; MMAE: monomethyl auristatin E; DM1: N2′-Deacetyl-N2′-(3-mercapto1-oxopropyl)-maytansine.

metabolic disorders, respiratory diseases, immunotoxins and immunocytokines, to list a few [84, 85]. With the increased diversity in antibody conjugates, it is also likely that more novel excipients, and excipient combinations, will be submitted for FDA clearance as the variety and complexity of next-generation antibody conjugates continues to rise.

FUNDING

REFERENCES

1. Ecker, DM, Jones, SD, Levine, HL. The therapeutic monoclonal antibody market. *mAbs* 2015; **7**: 9–14.
2. Kaplon, H, Reichert, JM. Antibodies to watch in 2018. *mAbs* 2018; **10**: 183–203.
3. Diamantis, N, Banerji, U. Antibody-drug conjugates-an emerging class of cancer treatment. *Br J Cancer* 2018; **114**: 362–7.
4. Dempke, WCM, Fenchel, K, Uciechowski, P *et al.* Second- and third-generation drugs for immuno-oncology treatment—the more the better? *Eur J Cancer* 2017; **74**: 55–72.
5. Beck, A, Goetsch, L, Dumontet, C *et al.* Strategies and challenges for the next generation of antibody drug conjugates. *Nat Rev Drug Discov* 2017; **16**: 315–37.
6. Shire, SJ, Shahrokh, Z, Liu, J. Challenges in the development of high protein concentration formulations. In: Shire, S, Gombotz, W, Bechtold-Peters, K, Andya, J. (eds) *Current Trends in Monoclonal Antibody Development and Manufacturing. Biotechnology: Pharmaceutical Aspects*, vol 11. New York, NY: Springer, 2010, 131–147.
7. Viola, M, Sequeira, J, Seica, R *et al.* Subcutaneous delivery of monoclonal antibodies: how do we get there? *J Control Release* 2018; **286**: 301–14.
8. Zhao, L, Ji, P, Li, ZH *et al.* The antibody drug absorption following subcutaneous or intramuscular administration and its mathematical description by coupling physiologically based absorption process with the conventional compartment pharmacokinetic model. *J Clin Pharmacol* 2013; **53**: 314–25.
9. Yadav, S, Liu, J, Shire, SJ *et al.* Specific interactions in high concentration antibody solutions resulting in high viscosity. *J Pharm Sci* 2010; **99**: 1152–68.
10. Liu, J, Nguyen, MDH, Andya, JD *et al.* Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. *J Pharm Sci* 2005; **94**: 1928–40.
11. Neergaard, MS, Kalonia, DS, Parshad, H *et al.* Viscosity of high concentration protein formulations of monoclonal antibodies of the IgG1 and IgG4 subclass—prediction of viscosity through protein–protein interaction measurements. *Eur J Pharm Sci* 2013; **49**: 400–10.
12. Connolly, BD, Petry, C, Yadav, S *et al.* Weak interactions govern the viscosity of concentrated antibody solutions: high-throughput analysis using the diffusion interaction parameter. *Biophys J* 2012; **103**: 69–78.
13. Shire, SJ. Formulation and manufacturability of biologics. *Curr Opin Biotechnol* 2009; **20**: 708–14.
14. Uchiyama, S. Liquid formulation for antibody drugs. *BBA-Proteins Proteomics* 2014; **1844**: 2041–52.
15. Daugherty, AL, Mrsny, RJ. Formulation and delivery issues for monoclonal antibody therapeutics. *Adv Drug Deliv Rev* 2006; **58**: 686–706.
16. Mitragotri, S, Burke, PA, Langer, R. Overcoming the challenges in administering biopharmaceuticals: formulation and delivery strategies. *Nat Rev Drug Discov* 2014; **13**: 655–72.
17. Kiese, S, Papppenberger, A, Friess, W *et al.* Shaken, not stirred: mechanical stress testing of an IgG1 antibody. *J Pharm Sci* 2008; **97**: 4347–66.
18. Mahler, HC, Friess, W, Grauschopf, U *et al.* Protein aggregation: pathways, induction factors and analysis. *J Pharm Sci* 2009; **98**: 2909–34.
19. Wang, W, Nema, S, Teagarden, D. Protein aggregation—pathways and influencing factors. *Int J Pharm* 2010; **390**: 89–99.
20. Wakankar, A, Chen, Y, Gokarn, Y *et al.* Analytical methods for physicochemical characterization of antibody drug conjugates. *mAbs* 2011; **3**: 161–72.
21. Fekete, S, Guillarme, D, Sandra, P *et al.* Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals. *Anal Chem* 2016; **88**: 480–507.
22. Harris, RJ, Shire, SJ, Winter, C. Commercial manufacturing scale formulation and analytical characterization of therapeutic recombinant antibodies. *Drug Dev Res* 2004; **61**: 137–54.
23. McCombs, JR, Owen, SC. Antibody drug conjugates: design and selection of linker, payload and conjugation chemistry. *AAPS J* 2015; **17**: 339–51.
24. Panowski, S, Bhakta, S, Raab, H *et al.* Site-specific antibody drug conjugates for cancer therapy. *mAbs* 2014; **6**: 34–45.
25. Dennler, P, Fischer, E, Schibli, R. Antibody conjugates: from heterogeneous populations to defined reagents. *Antibodies* 2015; **4**: 197–224.
26. Hermanson, GT. (2008) *Bioconjugate Techniques*. 2nd edition. Cambridge, Massachusetts: Academic Press as an Imprint of Elsevier.
27. Sletten, EM, Bertozzi, CR. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. *Angew Chem Int Ed Engl* 2009; **48**: 6974–98.
28. Mueller, BM, Wrasidlo, WA, Reisfeld, RA. Determination of the number of epsilon-amino groups available for conjugation of effector molecules to monoclonal-antibodies. *Hybridoma* 1988; **7**: 453–6.
29. Kim, MT, Chen, Y, Marhoul, J *et al.* Statistical modeling of the drug load distribution on trastuzumab emtansine (Kadcyla), a lysine-linked antibody drug conjugate. *Bioconjug Chem* 2014; **25**: 1223–32.
30. Siegel, MM, Tabei, K, Kunz, A *et al.* Calichleamicin derivatives conjugated to monoclonal antibodies: determination of loading values and distributions by infrared and UV matrix-assisted laser desorption/ionization mass spectrometry and electrospray ionization mass spectrometry. *Anal Chem* 1997; **69**: 2716–26.
31. Adamczyk, M, Gebler, J, Shreder, K *et al.* Region-selective labeling of antibodies as determined by electrospray ionization-mass spectrometry (ESI-MS). *Bioconjug Chem* 2000; **11**: 557–63.
32. Scheck, RA, Francis, MB. Regioselective labeling of antibodies through N-terminal transamination. *ACS Chem Biol* 2007; **2**: 247–51.
33. Witus, LS, Netirojjanakul, C, Palla, KS *et al.* Site-specific protein transannination using N-Methylpyridinium-4-carboxaldehyde. *J Am Chem Soc* 2013; **135**: 17223–17229.
34. Sun, MMC, Beam, KS, Cerveny, CG *et al.* Reduction-alkylation strategies for the modification of specific monoclonal antibody disulfides. *Bioconjug Chem* 2005; **16**: 1282–90.
35. MaAuley, A, Jacob, J, Kolvenbach, CG *et al.* Contributions of a disulfide bond to the structure, stability, and dimerization of human IgG1 antibody C(H)3 domain. *Protein Sci* 2008; **17**: 95–106.
36. Liu, HC, Chumsae, C, Gaza-Bulseco, G *et al.* Ranking the susceptibility of disulfide bonds in human IgGs1 antibodies by reduction, differential alkylation, and LC-MS analysis. *Anal Chem* 2010; **82**: 5219–26.
37. McDonagh, CF, Turcott, E, Westendorf, L *et al.* Engineered antibody-drug conjugates with defined sites and stoichiometries of drug attachment. *Protein Eng Des Sel* 2006; **19**: 299–307.
38. Cal, P, Bernardes, GJL, Gois, PMP. Cysteine-selective reactions for antibody conjugation. *Angew Chem Int Ed Engl* 2014; **53**: 10585–10587.
39. Badescu, G, Bryant, P, Bird, M *et al.* Bridging disulfides for stable and defined antibody drug conjugates. *Bioconjug Chem* 2014; **25**: 1124–36.
40. Abraham, R, Moller, D, Gabel, D *et al.* The influence of periodate-oxidation on monoclonal-antibody avidity and immunoreactivity. *J Immunol Methods* 1991; **144**: 77–86.
41. Wolfe, CAC, Hage, DS. Studies on the rate and control of antibody oxidation by periodate. *Anal Biochem* 1995; **231**: 123–30.

42. Jefferis, R. Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* 2005; **21**: 11–6.

43. Raju, TS, Jordan, RE. Galactosylation variations in marketed therapeutic antibodies. *mAbs* 2012; **4**: 385–91.

44. Zauner, G, Selman, MHJ, Bondt, A *et al.* Glycoproteomic analysis of antibodies. *Mol Cell Proteomics* 2013; **12**: 856–65.

45. Alves, NJ, Champion, MM, Stefanick, JF *et al.* Selective photocrosslinking of functional ligands to antibodies via the conserved nucleotide binding site. *Biomaterials* 2013; **34**: 5700–10.

46. Pavlinkova, G, Rajagopalan, K, Muller, S *et al.* Site-specific photobiotinylation of immunoglobulins, fragments and light chain dimers. *J Immunol Methods* 1997; **201**: 77–88.

47. Alves, NJ, Mustafaoglu, N, Bilgicer, B. Conjugation of a reactive thiol at the nucleotide binding site for site-specific antibody functionalization. *Bioconjug Chem* 2014; **25**: 1198–202.

48. Stimmel, JB, Merrill, BM, Kuyper, LF *et al.* Site-specific conjugation on serine right-arrow cysteine variant monoclonal antibodies. *J Biol Chem* 2000; **275**: 30445–30450.

49. Bhakta, S, Raab, H, Junutula, JR. In Ducry, L (ed). *Antibody-Drug Conjugates*, vol. **1045**. Totowa, NJ: Humana Press, 2013, 189–203.

50. Junutula, JR, Raab, H, Clark, S *et al.* Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat Biotechnol* 2008; **26**: 925–32.

51. Hallam, TJ, Smider, VV. Unnatural amino acids in novel antibody conjugates. *Future Med Chem* 2014; **6**: 1309–24.

52. Kim, CH, Axup, JY, Schultz, PG. Protein conjugation with genetically encoded unnatural amino acids. *Curr Opin Chem Biol* 2013; **17**: 412–9.

53. Wang, Q, Parrish, AR, Wang, L. Expanding the genetic code for biological studies. *Chem Biol* 2009; **16**: 323–36.

54. Axup, JY, Bajjuri, KM, Ritland, M *et al.* Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. *Proc Natl Acad Sci U S A* 2012; **109**: 16101–16106.

55. Dall'Acqua, WF, Kiener, PA, Wu, HR. Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn). *J Biol Chem* 2006; **281**: 23514–23524.

56. Hinton, PR, Xiong, JM, Johlfs, MG *et al.* An engineered human IgG1 antibody with longer serum half-life. *J Immunol* 2006; **176**: 346–56.

57. Nilsson, B, Moks, T, Jansson, B *et al.* A synthetic IgG-binding domain based on staphylococcal protein A. *Protein Eng* 1987; **1**: 107–13.

58. Pakulska, MM, Miersch, S, Shoichet, MS. Designer protein delivery: from natural to engineered affinity-controlled release systems. *Science* 2016; **351**: aac4750-1–aac4750-9.

59. Parthasarathy, R, Subramanian, S, Boder, ET. Sortase A as a novel molecular 'stapler' for sequence-specific protein conjugation. *Bioconjug Chem* 2007; **18**: 469–76.

60. Ritzefeld, M. Sortagging: a robust and efficient chemoenzymatic ligation strategy. *Chem Eur J* 2014; **20**: 8516–29.

61. Alves, NJ, Turner, KB, Daniele, MA *et al.* Bacterial nanobioreactors—directing enzyme packaging into bacterial outer membrane vesicles. *ACS Appl Mater Interfaces* 2015; **7**: 24963–24972.

62. Barbas, CF, Heine, A, Zhong, GF *et al.* Immune versus natural selection: antibody aldolases with enzymic rates but broader scope. *Science* 1997; **278**: 2085–92.

63. Tanaka, F, Almer, H, Lerner, RA *et al.* Catalytic single-chain antibodies possessing beta-lactamase activity selected from a phage displayed combinatorial library using a mechanism-based inhibitor. *Tetrahedron Lett* 1999; **40**: 8063–6.

64. Wagner, J, Lerner, RA, Barbas, CF. Efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. *Science* 1995; **270**: 1797–800.

65. Schrama, D, Reisfeld, RA, Becker, JC. Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov* 2006; **5**: 147–59.

66. Wu, AM, Senter, PD. Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol* 2005; **23**: 1137–46.

67. Kern, JC, Dooney, D, Zhang, R *et al.* Novel phosphate modified cathepsin B linkers: improving aqueous solubility and enhancing payload scope of ADCs. *Bioconjug Chem* 2016; **27**: 2081–8.

68. King, HD, Dubowchik, GM, Mastalerz, H *et al.* Monoclonal antibody conjugates of doxorubicin prepared with branched peptide linkers: inhibition of aggregation by methoxytriethyleneglycol chains. *J Med Chem* 2002; **45**: 4336–43.

69. Beerten, J, Schymkowitz, J, Rousseau, F. Aggregation prone regions and gatekeeping residues in protein sequences. *Curr Top Med Chem* 2012; **12**: 2470–8.

70. Dubowchik, GM, Firestone, RA, Padilla, L *et al.* Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjug Chem* 2002; **13**: 855–69.

71. Elgersma, RC, Coumans, RGE, Huijbregts, T *et al.* Design, synthesis, and evaluation of linker-duocarmycin payloads: toward selection of HER2-targeting antibody-drug conjugate SYD985. *Mol Pharm* 2015; **12**: 1813–35.

72. Kern, JC, Cancilla, M, Dooney, D *et al.* Discovery of pyrophosphate diesters as tunable, soluble, and bioorthogonal linkers for site-specific antibody-drug conjugates. *J Am Chem Soc* 2016; **138**: 1430–45.

73. Li, Y, Gu, C, Gruenhagen, J *et al.* An enzymatic deconjugation method for the analysis of small molecule active drugs on antibody-drug conjugates. *mAbs* 2016; **8**: 698–705.

74. Saito, G, Swanson, JA, Lee, KD. Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities. *Adv Drug Deliv Rev* 2003; **55**: 199–215.

75. Simoes, S, Moreira, JN, Fonseca, C *et al.* On the formulation of pH-sensitive long circulation times. *Adv Drug Deliv Rev* 2004; **56**: 947–65.

76. Zhao, RY, Wilhelm, SD, Audette, C *et al.* Synthesis and evaluation of hydrophilic linkers for antibody-maytansinoid conjugates. *J Med Chem* 2011; **54**: 3606–23.

77. Ducry, L, Stump, B. Antibody-drug conjugates: linking cytotoxic payloads to monoclonal antibodies. *Bioconjug Chem* 2010; **21**: 5–13.

78. Shen, BQ, Xu, KY, Liu, LN *et al.* Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. *Nat Biotechnol* 2012; **30**: 184–9.

79. Hamann, PR, Hinman, LM, Hollander, I *et al.* Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem* 2002; **13**: 47–58.

80. Schumacher, FF, Nunes, JPM, Maruani, A *et al.* Next generation maleimides enable the controlled assembly of antibody-drug conjugates via native disulfide bond bridging. *Org Biomol Chem* 2014; **12**: 7261–9.

81. Wakankar, AA, Feeney, MB, Rivera, J *et al.* Physicochemical stability of the antibody-drug conjugate Trastuzumab-DM1: changes due to modification and conjugation processes. *Bioconjug Chem* 2010; **21**: 1588–95.

82. Flygare, JA, Pillow, TH, Aristoff, P. Antibody-drug conjugates for the treatment of cancer. *Chem Biol Drug Des* 2013; **81**: 113–21.

83. Jain, N, Smith, SW, Ghone, S *et al.* Current ADC linker chemistry. *Pharm Res* 2015; **32**: 3526–40.

84. Staben, LR, Koenig, SG, Lehar, S *et al.* Targeted drug delivery through the traceless release of tertiary and heteroaryl amines from antibody-drug conjugates. *Nat Chem* 2016; **8**: 1112–9.

85. Liu, RH, Wang, RES, Wang, F. Antibody-drug conjugates for non-oncological indications. *Expert Opin Biol Ther* 2016; **16**: 591–3.