Antibody-drug conjugates (ADCs) represent an exciting and promising therapeutic approach for the treatment of cancers. The US Food and Drug Administration’s recent approval of brentuximab vedotin (Adcetris®) in 2011 and trastuzumab emtansine (Kadcyla®) in 2013 has brought a proof of the ADC concept in hematologic and solid tumors, respectively. The number of ADCs or immunoconjugates undergoing clinical studies is rapidly increasing, driving much attention to understand the criteria for success. The ADC concept looks quite simple. The basic idea is to conjugate a potent cytotoxic drug to a monoclonal antibody (mAb) selected to target a tumor associated antigen. Both drug components are conjugated by a linker designed to be stable in the blood streamline and to properly release the drug into the targeted cancer cell. Tremendous progress has been made in each area (i.e., antibody, drug, linker, target), but many important parameters must be addressed simultaneously to make a successful ADC product. The aim of Antibody-Drug Conjugates, which was edited by Laurent Ducry (Lonza),¹ is to provide detailed protocols for many of the critical ADC techniques necessary to succeed in the field. Each chapter addresses a specific aspect and is written by scientists who have recognized expertise in the domain.

In the introductory chapter, Ingrid Sassoon and Veronique Blanc (Sanofi) review the current ADC clinical pipeline, which includes nearly 30 candidates in addition to the two approved products mentioned above. The clinical data for the most advanced clinical candidate, such as CMC-544, SAR3419, CDX-011, PSMA-ADC, BT-062, and IMGN901, are summarized. Notably, a table of discontinued ADCs is also provided and discussed. The current challenges in ADC development are clearly identified. These include the improvement of the therapeutic index, the careful selection of the targets, the better understanding of ADC mechanism of action and the understanding of ADC off-target toxicities. Last but not least, the selection of appropriate clinical settings, including the patient selection and the dosing regimen, is also discussed.

The critical factors for target selection that must be met to succeed in ADC development are then reviewed by Neil H Bander (Cornell University). The requirement for high level expression for current cytotoxic agents to be effective seems to be one of these criteria. Development of innovative ways to identify suitable targets expressed at lower levels, and more potent cytotoxics, may allow expanded use of ADCs.

In the next chapter, Jay Harper, Adeela Kama, and colleagues (MedImmune) discuss detailed protocols for selecting optimal antibodies for ADC therapies. Two variables of paramount importance, internalization and intracellular localization, are examined. A successful antibody needs to bind to the target antigen on the surface of tumor cells and then be internalized by the cell. Following internalization, the ADC must

**Keywords:** antibody-drug conjugate, cytotoxic drug, drug-to-antibody ratio, hydrophobic interaction chromatography, linker, mass spectrometry, site-directed conjugation
be transported to the lysosome to be processed, and the pharmacological active
drug must then be released to exert its
cytotoxic effects on tumor cells.

The crucial ADC components
discussed by Jan Anderl and colleagues
(Heidelberg Pharma) are the toxin
payloads, including maytansinoids (DM1,
DM4), auristatins (MMAE, MM4),
calicheamicin and amanitin. Thousands
of cellular toxins available from natural
sources or by chemical synthesis are
cytotoxic, but only a very few are suitable
as components for ADCs. First, the
cytotoxic potency of a payload has to
be extremely high, as a limited tumor
penetration of IgGs, low expression of
antigens, inefficient internalization,
and linker metabolism may result in
very low toxin concentrations in the
cell. Second, the target of the payload
must be located inside the cell because
most current ADC strategies depend
on internalization of the conjugates,
endocytosis of the ADC/antigen complex,
lysosomal degradation and, finally, release
of the payload into cytoplasm of the cell.
Third, the molecular structure of the
drug has to be small to reduce the risk
of immunogenicity. Finally, it should be
soluble in aqueous buffers to facilitate
conjugation to antibodies and to limit
aggregation of the resulting ADC.

As noted in the chapter by Birte
Nolting (Pfizer), one of the biggest
challenges in the development of ADCs
has been the generation of suitable linkers
for the conjugation of antibody and drug.
The role of the linker is fundamental
because, in addition to efficient delivery
of the cytotoxic drug, the stability of
the drug-antibody linkage is a crucial
factor in determining the efficacy and
toxicity of an ADC, and therefore its
therapeutic potential. There are several
important considerations regarding the
linker component, including the site of
attachment on the antibody, the average
number of attachment sites per antibody
molecule, the cleavability of the linker
(ability to disintegrate releasing the
drug), and the polarity of the linker. The
chapter provides an overview of linker
technologies currently used for ADCs
and advances that have resulted in linkers
with improved properties. In addition,
considerations for the conjugation of
antibody and drug linker, such as drug-
to-antibody ratio (DAR) and site of
conjugation, are discussed.

As described by Pierre-Yves Abecassiss
and Céline Amara (Sanofi), another
important part of the design and
optimization of ADCs is the in vivo testing
of the drug-linker stability. Monitoring in
vivo drug-linker stability is critical to the
linker selection process. It is performed
by identifying the pharmacokinetics
(PK) profiles. PK properties of ADCs are
measured by following the profiles of the
conjugate (mAb entity carrying at least
one drug), the total antibody (mAb entity
regardless of drug load), as well as the free
drugs and metabolites entities. The chapter
focuses on the key analytical methods,
such as ELISA immunoassays, turbulent
flow chromatography coupled to mass
spectrometry (TFC-MS/MS), and high-
resolution mass spectrometry (HRMS).
These methods are used to support the PK
profiles assessment of the three entities,
allowing the characterization of ADC
drug-linker stability.

PK and absorption, distribution,
metabolism and excretion (ADME)
characterizations of ADCs are reviewed in
a chapter by Kedan Lin, Jay Tibbitts,
and Ben-Quan Shen (Genentech).
Heterogeneity from production and in vivo
processing, the necessity of monitoring
multiple active ADC analytes, complex
PK/pharmacodynamic relationships, and
less understood catabolic and metabolic
species are among the considerations
in characterizing the unique PK and
ADME properties of ADCs. The
formation of cytotoxic drug-containing
products from ADCs may occur by two
concurrent processes, deconjugation and
catabolism. The deconjugation process
includes release of cytotoxic drug-
containing products from the ADC via
enzymatic or chemical processes and
unconjugated antibody, with preservation
of the antibody backbone. The catabolism
process includes proteolytic catabolism of
the antibody and formation of cytotoxic
drug-containing catabolites (free drug or
drug–amino acid conjugates).

Safe handling of cytotoxic compounds
in a biopharmaceutical environment is
also mandatory as discussed by Miriam
Hensgen and Bernhard Stump (Lonza)
in the next chapter. Handling cytotoxic
drugs such as ADCs represents a challenge
related to the potency of the compounds.
These derivatives are dangerous to humans
if they accidentally get in contact with the
skin, are inhaled or are ingested, either as
pure compounds in their solid state or as
a solution dissolved in a co-solvent. Any
contamination of people involved in the
manufacturing process must be avoided.
On the other hand, biopharmaceuticals
need to be protected simultaneously
against any contamination from the
manufacturing personnel. Therefore,
a tailor-made work environment that
includes appropriate technical equipment
to keep potential hazardous substances
contained is mandatory for ADC
manufacturing. In addition, clearly
defined working procedures based on risk
assessments and proper training for all
personnel involved in the manufacturing
process are needed to safely handle these
highly potent pharmaceuticals. The
amounts used in the clinic are typically
at low mg-scale range for the complete
ADC, resulting in a microgram quantity
of payload injected in patients. In the
manufacturing of an ADC, much larger
quantities of the cytotoxic component of
the ADC are handled. Manufacturing
these active compounds involves
safe standard operating procedures
(SOPs), containment strategies, and an
appropriate, rigorous training to minimize
the occupational risks for workers that
could come into contact with the cytotoxic
payload, the ADC, or related waste.

In the next chapter, James E Stefano
and colleagues (Sanofi) discuss micro- and
mid-scale maleimide-based conjugation of
cytotoxic drugs to antibody hinge region
thiols. All of the current ADC chemistries
generate populations of molecules that
differ in critical properties known to
affect efficacy, PK, and the therapeutic
window. This creates a need for the ability
to generate comparably high-quality
products early in development and at
sufficient scale for evaluating in vitro
potency and in vivo efficacy, as well as
the early identification of any deficiencies
in critical quality attributes, including
solubility and stability. Detailed protocols
using maleimide-based chemistry for the

www.landesbioscience.com  mAbs  31
conjugation to reduce hinge disulfides in antibodies by several cytotoxic drugs are provided. Initial characterization of the reduction/alkylation reaction using polyethylene glycol (PEG) as a drug surrogate can be performed at 5 mg scale, including cell proliferation assays. As an extension, 150 mg scale process is than done for performing efficacy studies in small animals.

Marie-Priscille Brun and Laurence Gauzy-Lazo (Sanofi) then report several methods for the preparation ADCs through conjugation of drugs to solvent-exposed ε-amino groups of lysine residues. These methods apply to various cytotoxic agents, both tubulin binders and DNA-targeting agents and different types of linkers, e.g., cleavable or not, peptidic or disulfide-based. Lysine residues exposed at the surface of antibodies are used as sites for drug conjugation because their side-chain amino groups are good nucleophiles. An IgG contains approximately 80–100 lysine residues and most of them are sufficiently exposed or accessible to be reactive. The main chemistry used for lysine conjugation involves a simple reaction, namely, the formation of a stable amide bond using activated esters of the drug to be conjugated, usually O-succinimide reagents like N-hydroxysuccinimidyl (NHS) or sulfo-NHS esters.

In the next chapter, Sunil Bhakta, Helga Raab, and Jagath R Junutula (Genentech) describe protocols for engineering and selection of THIOMABs, which are antibodies designed for site-specific conjugation of thiol-reactive inkers. Conventional conjugation methods result in heterogeneous antibody conjugate products with a mixture of different molar ratios of conjugated species to antibody linked at different sites. To create more homogeneously loaded ADCs better suited to clinical development, antibodies with engineered cysteines to enable thiol conjugation at these specific sites (THIOMABs) have been created. The resulting THIOMAB–drug conjugates (TDCs) are superior to conventional ADCs because these conjugates exhibit uniform distribution of linker-drugs and, being equivalently efficacious, they also display superior safety with respect to liver and bone marrow toxicity in rats and monkeys. THIOMABs have been successfully used to conjugate cytotoxic drugs for therapeutic applications and also to conjugate biotin, fluorophore, or radiolabels for antibody-based research and imaging applications.

As reported by Patrick Dennler, Roger Schibli, and Eliane Fischer (Paul Scherrer Institute), another site-directed technique is based on enzymatic antibody modification by bacterial transglutaminase. Enzymatic posttranslational modification of proteins may allow more precise control over conjugation sites than chemical modification of reactive amino acid side chains. The chapter provides a protocol for bacterial transglutaminase (BTGase)-mediated conjugation of cadaverine-derivatized substrates to an IgG1. As a result, a stable bond is formed between glutamine 295 of the antibody heavy chain and the substrate. This procedure requires enzymatic removal of N-linked glycans from the antibody and yields a defined substrate/antibody ratio of 2:1. Alternatively, a mutant aglycosylated IgG1 variant may be generated by site-directed mutagenesis. Interestingly, the mutation introduces an additional glutamine and yields a substrate/antibody ratio of 4:1 after coupling. To demonstrate the efficacy of the conjugation method, ESI-TOF mass spectrometry-based method is used to analyze the uniformity of the resulting conjugates and as quality control. The approach allows an easy generation of homogeneous antibody conjugates and can be applied to any IgG1, as well as to a wide range of cadaverine-derivatized substrates.

Formulation development of antibody-drug conjugates (ADCs) aims to ensure that stable, high-quality products are dosed to patients, as highlighted by William J. Galush and Aditya A. Wakankar (Genentech). This goal is common to all pharmaceutical formulations, but, compared with traditional therapeutic proteins, ADCs present a unique set of physicochemical properties that can affect safety, quality, and efficacy. While there is a body of literature that outlines which product attributes affect the safety, quality, and efficacy for conventional, unconjugated mAbs, an understanding of attributes important to ADCs is only now emerging. Quality attributes of ADCs include those associated with the antibody—small molecule conjugate form, the small molecule drug moiety, and the antibody. The DAR is a critical quality attribute. A high DAR could potentially affect the safety profile of the ADC, whereas a low DAR could lead to decrease in efficacy. Similarly, the release of free drug species is another attribute that affects safety and bioactivity. In addition to these ADC- and drug-specific attributes, those that are intrinsic to the mAb portion of the molecule must also be taken into consideration.

In the next chapter, Bernhard Stump and Jessica Steinmann (Lonza) review conjugation process development and scale-up. Manufacturing highly potent ADCs combines conventional organic synthesis with biotechnological processing. As a result, a series of new and unique engineering and chemistry challenges must be addressed to support clinical trials and commercial manufacturing. These include the development of reliable processes leading to uniform product properties, as well as establishment of ADC-specific analytical methods and safe strategies for handling cytotoxic compounds. The antibody has to be handled with special care because it is susceptible to degradation on exposure to high temperatures, temperature fluctuations and high stirring rates. The cytotoxic drug is usually very hydrophobic, which requires the use of organic co-solvents that must also be tolerable for the antibody portion.

Anil Wagh and Benedict Law (North Dakota State University) follow up with methods for conjugating antibodies to nanocarriers. Various approaches, including periodate oxidation, carbodiimide, maleimide, and heterofunctional linkers, for conjugating antibodies to different nanocarriers may be used. Specific examples are selected to demonstrate the experimental procedures and to illustrate the potential for use with other nanocarrier system. Based on the modifications of the functional groups, the conjugation methods can be categorized into carbohydrate modification, amine or carboxylic acid modification, and
conjugation via the sulfhydryl group. There is no universal method that is superior to the others. The differences in the amino acid composition and sequence in the polypeptide chains may affect the reactivity of the antibody. One important feature is preservation of the binding affinity of the antibody, and the chemistry should be selected in accordance to the type and the availability of the functional group on the carrier surface.

UV/Vis spectroscopy is a simple and convenient method to determine protein concentrations and the average number of drugs that are conjugated to the ADC, as discussed by Yan Chen (Genentech) in the next section. The average DAR can be determined using the measured absorbances of the ADC and the extinction coefficients of the antibody and the drug. This technique has been used widely for various ADCs with different drugs including the maytansinoids DM1 and DM4, methotrexate, CC-1065 analogs, Adriamycin, calicheamicin analogs and dipeptide-linked auristatins such as vc-MMAE and MMAF.

As described by Jun Ouyang (Genentech), drug-to-antibody ratio and drug load distribution can also be determined by hydrophobic interaction chromatography (HIC) and reversed phase high-performance liquid chromatography. HIC is the method of choice for determination of the DAR and drug load distribution for Cys-linked ADCs. The drug-loaded species are resolved based on the increasing hydrophobicity, with the least hydrophobic, unconjugated form eluting first and the most hydrophobic, 8-drug form eluting last. The area percentage of a peak represents the relative distribution of the particular drug-loaded ADC species. The weighted average DAR is then calculated using the percentage peak area information and the drug load numbers. RP-HPLC offers an orthogonal method to obtain DAR for Cys-linked ADCs. The method involves, first, a reduction reaction to completely dissociate the heavy and light chains of the ADC, then separation of the light and heavy chains and their corresponding drug-loaded forms on an RP column. The percentage peak area from integration of the light chain and heavy chain peaks, combined with the assigned drug load for each peak, is used to calculate the weighted average DAR.

DAR and drug load distribution can also be determined by LC-ESI-MS, as reported by Louiseisa Basa (Genentech). The method is suitable for lysine-linked ADCs. The ADC sample is desalted using a reversed-phase LC column with an acetonitrile gradient prior to online MS analysis. The MS spectrum is processed, deconvoluted and converted to a series of zero charge state masses that corresponds to the increasing number of drugs in the ADC. Integration of the mass peak area allows the calculation of the DAR and drug load distribution of ADCs.

Determination of charge heterogeneity and level of unconjugated antibody may be achieved using imaged capillary isoelectric focusing (icIEF), as illustrated in the next chapter by Joyce Lin and Alexandru C. Lazar (Immunogen). The electropherogram from icIEF can be integrated to quantitate the amount of unconjugated antibody present in a conjugate sample. A conjugate sample can first be prepared by mixing with appropriate ampholytes, pI markers, and additives. Then, the sample is focused in a fluorocarbon-coated fused silica capillary, where absorbance images were taken. Quantitation of the unconjugated antibody was achieved by using a calibration curve.

The book closes with a chapter by Weibing Ding (Pall Corporation), who discusses risk-based scientific approach for determination of extractables/leachables from biomanufacturing of ADCs. The desire to remove cleaning and cross-contamination issues from drug production have led to the introduction of single-use technologies. As a consequence, there is a risk of increased levels of extractables and leachables within a process. The risk-based approach in the chapter minimizes the amount of test work while meeting the regulatory requirements to ensure the drug safety and quality. The test design is optimized and the analytical methods (gas chromatography/mass spectrometry, liquid chromatography/mass spectrometry, and inductively coupled plasma/mass spectrometry) are shown to be suitable for quantifying and identifying the extracted chemical compounds. Application of this characterization method speeds up the filing process for qualification and validation of single-use systems used in bioprocesses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
1. Ducry, Laurent (Ed.) Antibody Drug Conjugates. In: Methods in Molecular Biology. Book 1045. New York (NY); Humana Press; 2013.