Special Section on Pharmacokinetics and ADME of Biological Therapeutics–Minireview

A Cross Company Perspective on the Assessment of Therapeutic Protein Biotransformation

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ABSTRACT

Unlike with new chemical entities, the biotransformation of therapeutic proteins (TPs) has not been routinely investigated or included in regulatory filings. Nevertheless, there is an expanding pool of evidence suggesting that a more in-depth understanding of biotransformation could better aid the discovery and development of increasingly diverse modalities. For instance, such biotransformation analysis of TPs affords important information on molecular stability, which in turn may shed light on any potential impact on binding affinity, potency, pharmacokinetics, efficacy, safety, or bioanalysis. This perspective summarizes the current practices in studying biotransformation of TPs and related findings in the biopharmaceutical industry. Various TP case studies are discussed, and a fit-for-purpose approach is recommended when investigating their biotransformation. In addition, we provide a decision tree to guide the biotransformation characterization for selected modalities. By raising the awareness of this important topic, which remains relatively underexplored in the development of TPs (Bolleddula et al., 2022), we hope that current and developing practices can pave the way for establishing a consensus on the biotransformation assessment of TPs.

SIGNIFICANCE STATEMENT

This article provides a comprehensive perspective of the current practices for exploring the biotransformation of therapeutic proteins across the drug development industry. We, the participants of the Innovation and Quality therapeutic protein absorption distribution metabolism excretion working group, recommend and summarize appropriate approaches for conducting biotransformation studies to support internal decision making based on the data generated in discovery and development.

Historical Perspective and Introduction

Biotransformation refers to the structural modification of a drug either chemically or enzymatically, which may ultimately impact its efficacy or safety. Investigating the metabolism of new chemical entities is a well-established and critical activity in the pharmaceutical industry

(Roffey et al., 2007; Penner et al., 2009; FDA, 2020a,b). In contrast, the risks associated with the biotransformation of therapeutic proteins (TPs) were considered relatively low. Consequently, biotransformation has not been routinely required as part of the regulatory filings for TPs. In fact, no biotransformation data were included in the US Food and Drug Administration (FDA) submission packages for the majority of TPs approved between 2011 and 2020 (Bolleddula et al., 2022).

The diversity and complexity of TPs have grown considerably in the past decade (Hall, 2014; Walsh, 2018). For instance, bispecific antibodies were introduced for cancer immunotherapy, and various antibody fragments were designed to achieve better drug penetration into target.

ABBREVIATIONS: ADC, antibody-drug conjugate; ADME, absorption distribution metabolism excretion; BP, biotransformation product; CDR, complementarity-determining region; Fc, fragment crystallizable region; FcRn, neonatal Fc receptor; FDA, US Food and Drug Administration; LC-MS/MS, liquid chromatography–tandem mass spectrometry; mAb, monoclonal antibody; MMAE, mono methyl auristatin E; mPEG, methoxy PEG; PEG, polyethylene glycol; PK/PD, pharmacokinetic/pharmacodynamic; PTM, post-translational modification; PYY, peptide YY; TP, therapeutic protein.
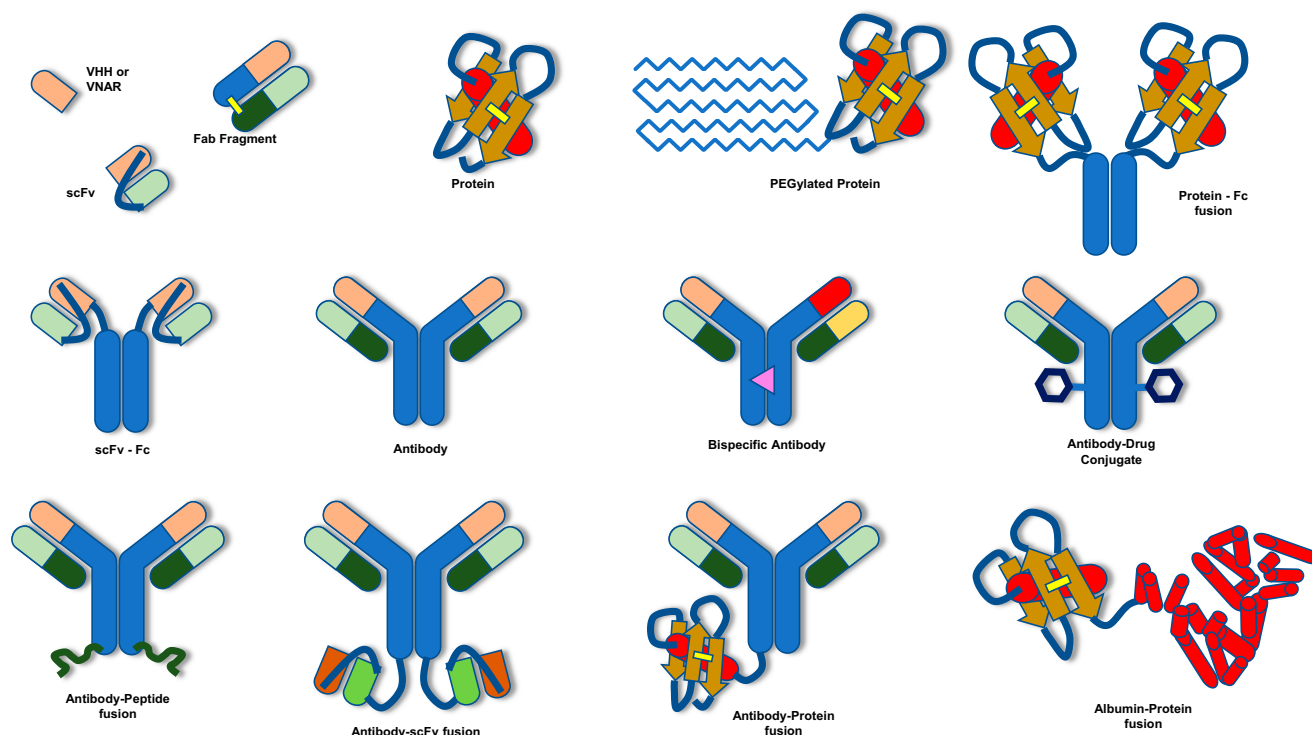
VHH – camelid antibody; VNAR – variable new antigen receptors; scFv – single chain variable fragment;
Fab – antigen binding fragment; Fc – crystallisable fragment

Fig. 1. Examples of therapeutic proteins. Reproduced with permission from (Bolleddula et al., 2022) and (Ball et al., 2022).

Recombinant versions of endogenous proteins, peptide hormones, or their analogs were fused or conjugated to large proteins, such as monoclonal antibodies (Rangwala et al., 2019; Camacho et al., 2020), fragment crystallizable region (Fc) domains (Glaesner et al., 2010; Hall et al., 2010; Hecht et al., 2012; Jafari et al., 2017; Lee and Lee, 2017) human albumin (Baggio et al., 2004; Matthews et al., 2008), or polyethylene glycol (PEG) (Jevsevar et al., 2010; Jevsevar et al., 2012; Freches et al., 2017) to mitigate fast clearance. In addition, antibody-drug conjugates (ADCs) were explored for targeted delivery of cytotoxic payloads (Kraynov et al., 2016; Thomas et al., 2016; Beck et al., 2017; Drago et al., 2021). A schematic overview of these modalities is shown in Fig. 1.

The increasing complexity of TPs could potentially make them more susceptible to in vivo biotransformation than a typical monoclonal antibody (mAb). Depending on their specific structure, TPs may undergo various categories of biotransformation, such as proteolysis, post-translational modifications (PTMs), or other chemical changes (Walsh and Jefferis, 2006; Ezan et al., 2014; Tibbitts et al., 2016; Schadt et al., 2019a). This differs from the metabolism of new chemical entities, which is primarily driven by oxidative/reductive and conjugative chemical reactions (Almazroo et al., 2017).

Biotransformation may affect the potency, pharmacokinetic/pharmacodynamic (PK/PD), efficacy, immunogenicity or safety of TPs. For instance, PTMs in complementarity-determining regions (CDRs) of an mAb can reduce its binding affinity, functional potency, and pharmacokinetics (Bults et al., 2016; Li et al., 2016; Xu et al., 2019). Similarly, proteolysis within a fusion protein may lead to compromised pharmacokinetics and/or safety. Modifications occurring in vivo may trigger immunogenic responses not seen with the original TPs (Mamula et al., 1999; Doyle et al., 2007). Additionally, biotransformation of the payload or linker of an ADC could lead to a profound impact on stability, efficacy, and toxicity (Shen et al., 2012; Su et al., 2018).

The influence of biotransformation products (BPs) on bioanalytical assay performance has also been documented (Bults et al., 2016; Liu et al., 2018).
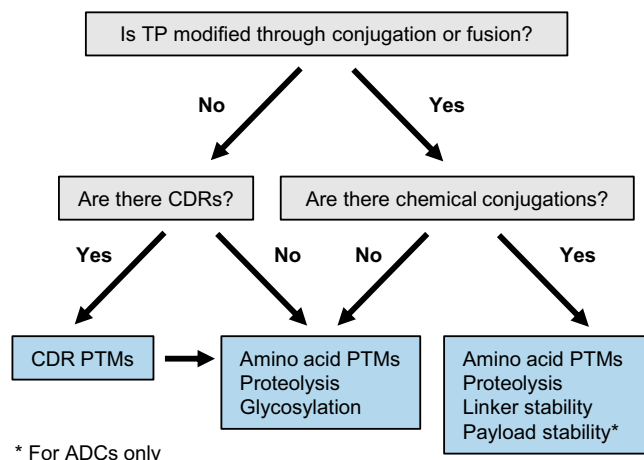
Fig. 2. Proposed general decision tree for the biotransformation assessment of TPs. It is recommended that appropriate analyses should be conducted based on the specific modality of the TP. For TPs without modifications through conjugation or fusion (or modified TPs with CDRs), the investigation should be initiated on any potential CDR PTMs for the molecules including mAb, fragment antigen binding, single-chain variable fragment, or other antibody fragments, followed by checking other amino acid PTMs outside the CDRs, proteolysis or glycosylation. For unmodified TPs containing no CDRs such as replacement TPs, possible amino acid PTMs, proteolysis or glycosylation should be verified. For modified TPs, such as fusion proteins, similar biotransformation analyses discussed earlier should still be applicable. For TPs involved chemical conjugations, e.g., PEGylated TPs or ADCs, evaluation of the linker stability should be prioritized. In addition, screening of the payload stability may be needed for ADCs as well. Please note that the types of evaluations suggested are not listed in the order of priority. Fab, antigen binding fragment; scFv, single chain variable fragment; VHH, camelid antibody; VNAR, variable new antigen receptors.

This review summarizes approaches used to explore the biotransformation of TPs. We propose a general decision tree, depicted in Fig. 2, for assessing biotransformation of different modalities of TPs. In the following sections, proteolysis and amino acid modifications/PTMs of TPs are discussed in detail, as well as necessary assessments for ADCs and PEGylated proteins. The scope of this perspective is focused on TPs as exemplified in Fig. 1. Peptides, oligonucleotides, vaccines, and gene and cell therapies were deemed out of scope for this review. In addition to understanding what may trigger the investigation of biotransformation, we also evaluated bioanalytical technologies that allow the effective characterization of BPs. We also suggest how biotransformation information can better enable lead molecule selection and optimization, and guide translation into humans. The technical challenges and knowledge gaps are also discussed. We acknowledge that a unique and fit-for-purpose approach will remain as the main path forward for studying biotransformation of TPs due to their complexity and diversity. Continued data accumulation and discussion within the industry may lead to a general consensus on when and how to conduct biotransformation evaluations for TPs in the future.

TP Proteolysis

Over the last decade, there has been a steady increase in the number of modalities and complexity of TPs (Hall, 2014; Walsh, 2018), and this has led to an increasing need for understanding proteolytic stability and its implications for safety, tolerability, and potential immunogenicity (Hamuro and Kishnani, 2012). One challenge, due to the complexity of new drug modalities, is the impact that in vivo proteolysis can have on altering the PK/PD relationship. Characterizing PK/PD in preclinical species is an important tool to guide translating TP pharmacology to humans, and proteolysis can negatively affect both exposure and potency. As demonstrated by the decision tree in Fig. 2, it is now considered essential to evaluate the proteolytic stability of modern biologic drugs. This is particularly true for fusion formats or conjugated TPs during early discovery to understand potential safety risks, interpret structure-activity-relationships, and guide protein engineering efforts.

A representative example that highlights the need to investigate proteolysis can be found with half-life extended protein fusion modalities, which are typically engineered to increase their exposure time in circulation. A case study is the fragment crystallizable region (Fc)-FGF21 fusion protein, in which the Fc domain of a human IgG1 was fused to FGF-21 to extend its half-life and provide a treatment of metabolic disorders. In vivo proteolysis of Fc-FGF21 was studied extensively, and specific cleavage sites on FGF-21 that led to loss of potency, plasma exposure, and PD effects were identified. This knowledge was leveraged to eliminate the proteolytic sites, to address other liabilities, and to improve Fc-FGF21 half-life while maintaining potency similar to wild-type FGF21 (Hecht et al., 2012; Hager et al., 2013).

For biotherapeutics that undergo cellular internalization and recycling, it is not always feasible to correlate in vitro and in vivo stability experiments due to the complexity and interplay of soluble, endothelial-associated, and intracellular proteases. Schadt et al. used two cell-based systems and ex vivo incubations with rabbit, rat, and cynomolgus monkey plasma to predict a major N-terminal proteolytic BP of tetranectin-apolipoprotein A1 fusion protein that was generated by the exopeptidase dipeptidyl peptidase-4 (Schadt et al., 2019b). However, as the authors reported, a secondary metabolite (i.e., residues 29–270) that was identified by Zell et al. during in vivo experiments run in rabbits was not detected in vitro (Zell et al., 2016). Additionally, there can be differences in in vivo biotransformation in preclinical species depending on the presence or absence of disease-state biology. For cancer indications, tumor type and state of progression can lead to increased proteolytic activity at

the target site. Similarly, proteolytic enzymes associated with autoimmune diseases can create additional proteolytic activity (Mason and Joyce, 2011; Herszenyi et al., 2014; Tabrez et al., 2020). Modern, novel TPs often contain linker sequences or non-IgG-like domains that are substrates for proteolysis, and almost all observed biotransformation through cleavage has been in fusion protein or conjugate formats (Hall et al., 2010; Hager et al., 2013; Zell et al., 2016; Kang et al., 2017). These issues are often observed during preclinical PK studies but may also be noted during clinical trials. Typical triggers for investigating biotransformation are: (1) unusually rapid clearance, (2) mismatches between bioanalytical assay formats that target different regions of a TP (Heinrich et al., 2015), (3) or a mismatch in the PK/PD relationship in which the expected pharmacological effect does not correlate with the concentration of circulating drug. Some of the scenarios described above are illustrated by Fig. 3, which shows a conceptual protein constructed by fusing a labile/active region with a stable region. As a result of proteolytic biotransformation, PK measured by different assays could lead to a discrepancy. For example, an assay that measures the stable region of a protein could show sustained exposure while a different assay, which measures a labile region, could reflect the loss of active analyte from the system over time (an intact assay that measures the entire molecule versus a bioassay that measures activity, e.g.). A real-life example can be found in the case of Fc-FGF21 fusion protein (Hager et al., 2013).

The mechanisms responsible for specific proteolytic sites are not always understood but can be differentiated from lysosomal degradation; the key difference is that BPs generated through extra-lysosomal proteolysis may circulate in blood where they can be detected. That means they must be formed either directly in plasma or close to the cell surface. One exception is proteolysis that occurs after cellular uptake, which may be detectable in circulation if the BP is recycled back to the cell surface and secreted into the blood stream, for example via the neonatal Fc receptor recycling pathway (Roopenian and Akilesh, 2007).

Analytical Tools for Investigating Proteolysis and Migration Strategies. For small molecule drug candidates, in vitro systems, such as recombinant CYP enzymes, liver microsomes, or hepatocytes from different species are typically used as models to investigate metabolic stability, generate metabolite profiles, and to establish in vitro-in vivo correlation for prediction of human metabolism. In comparison, there is a significant knowledge gap and lack of mature and established tools for TPs. Buffer systems, such as PBS or plasma/serum incubations under stressed conditions have been used to understand potential biotransformation of TPs. However, BPs are typically limited to amino acid level modifications, such as oxidation, deamidation, pyroglutamate formation, and asparagine isomerization, while proteolysis is not readily observable in these systems. A significant discrepancy in proteolytic stability can be observed between in vitro plasma/serum incubation and in vivo animal studies (Yin et al., 2013). This is because proteolytic enzymes involved in the biotransformation of a TP usually reside in tissue, on the surface of endothelial cells, and/or intracellularly. For half-life extended modalities (Kontermann, 2016) containing a neonatal Fc receptor (FcRn) binding site such as Fc-peptide fusion proteins, mAb-peptide conjugates or albumin fusion proteins, the biotransformation cannot only take place in a circulating system, but is also expected to take place intracellularly in endosomes/lysosomes when the protein is going through the FcRn-mediated pathway. Therefore, lysosomes can potentially be used as an in vitro system for prediction of in vivo biotransformation of TPs (Pearson and Rock, 2015). However, unlike microsomes, lysosomes are not mature products that can be easily and cost-effectively acquired, nor are there well-established protocols and controls for characterizing enzyme activity. More importantly, it may not contain all the proteases that the protein drug will encounter in vivo, such as proteases in extracellular
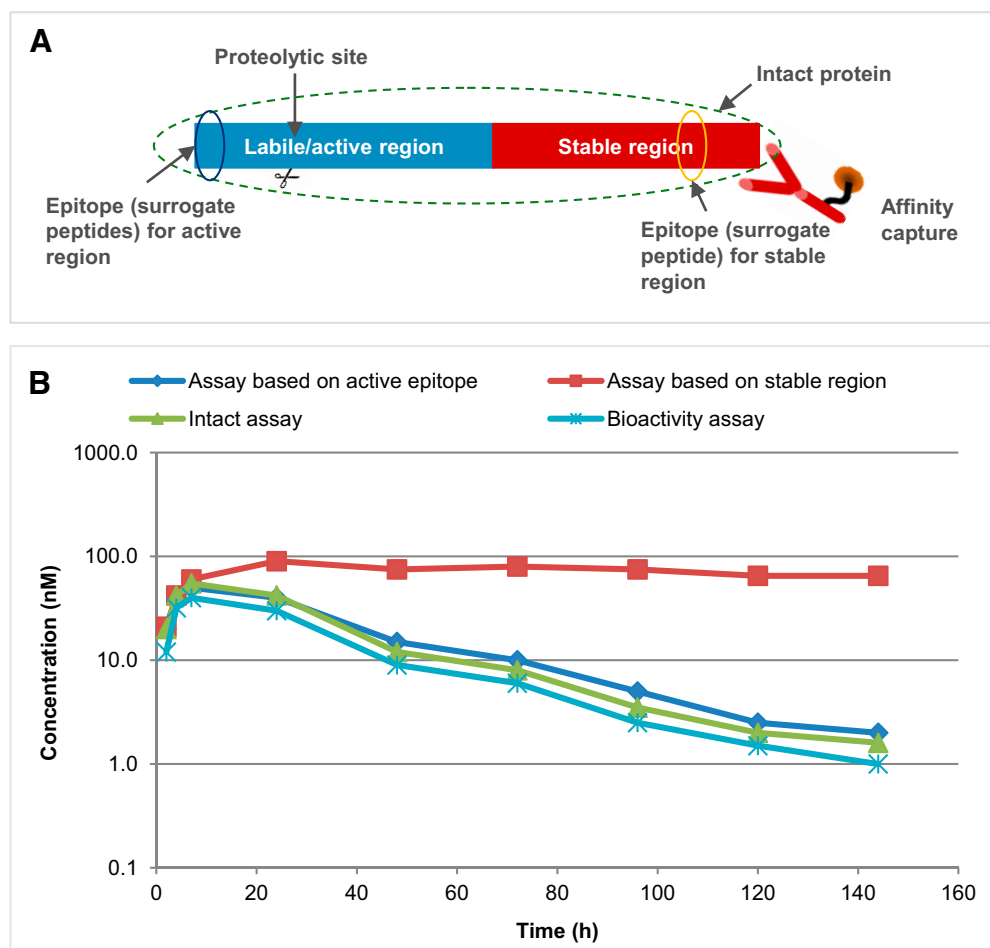
Fig. 3. Typical assay platforms for protein therapeutics that are susceptible to proteolytic biotransformation illustrated by a conceptual protein constructed by fusing a labile/active region with a stable region. (A) The scheme of the protein structure, the epitopes (surrogate peptides) for different assays, and proteolytic site; (B) hypothetical in vivo PK data based on different assay platforms showing the discrepancy in PK due to proteolytic biotransformation.

matrices or in cytosol. Alternatively, incubation with tissue homogenate may expose the protein to more proteases than it would encounter in vivo and therefore leads to overestimation of instability and altered metabolite profile. In addition, choice of relevant tissue could be a challenge if there is no clear understanding which tissue is the main location of biotransformation in vivo. Overall, significant knowledge gaps exist for in vitro prediction of proteolysis and in vivo studies should still be the main source of information. Comparison across in vitro, preclinical in vivo, and eventually clinical in vivo will continue to provide insight into the choice of relevant matrix and biologic systems for evaluation of proteolytic liability of TPs.

For analysis of proteins, both "bottom-up" and "top-down" (intact) approaches by mass spectrometry analysis are the most commonly used techniques (Fig. 4). Alternatively, a "middle-down" approach can be applied to improve resolution and sensitivity of the intact analysis by reducing the inter-chain disulfide bonds or cleaving the heavy chain of the antibody using hinge digestion enzymes, such as IdeS. In all the cases, immuno-affinity capture for cleanup of the sample and enrichment of the analytes is the key step. They will not be elaborated in this paper as the bioanalytical techniques and challenges for elucidating biotransformation of protein therapeutics have been previously reviewed by Schadt et al. (Schadt et al., 2019a). Several approaches have been used to mitigate the proteolysis for TPs mainly including protein engineering, incorporation of non-natural amino acids, such as rare synthetic and D-amino acids, along with cyclization.

Protein engineering is one of the most effective tools to enhance the catabolic stability of recombinant TPs. TPs can be truncated by various proteases and peptidases. The cleavage site generating the major BP is called the soft-spot, and this is comparable to the soft-spot of a small molecule, where the predominant metabolism occurs. Similar to the strategy to improve the metabolic stability of a small molecule, the soft spot of a TP can be modified to enhance the stability while maintaining its biologic activity. The modification of the soft-spot is typically implemented via site-directed mutagenesis for recombinant TPs. For example, in the case of Fc-FGF21 fusion protein (Hecht et al., 2012) a single-point mutation P171G introduced to ablate the cleavage at 171–172 while retaining the potency (Hecht et al., 2012). Another example is GLP-1-based TPs for the treatment of type 2 diabetes. The native GLP-1(7–37) has a very short half-life (<2 minutes in human) due to rapid cleavage between alanine 2 and glutamine 3 by dipeptidyl peptidase-4 (Mentlein et al., 1993; Matthews et al., 2008). To extend the half-life, the GLP-1(7–37) peptide was fused to human serum albumin (albiglutide) (Baggio et al., 2004; Matthews et al., 2008; Lee and Lee, 2017) or Fc domain of IgG4 (dulaglutide) (Glaesner et al., 2010; Lee and Lee, 2017). In both these recombinant proteins, Ala at position 2 was replaced with glycine to mitigate the DDPIV degradation, which eventually contributed to a 5-day half-life in humans for both proteins (Lee and Lee, 2017).

Incorporation of non-natural amino acids is a popular strategy to mitigate the proteolysis of peptides (Werle and Bernkop-Schnürch, 2006; Di, 2015). The strategy was also used for the conjugated biotherapeutics, particularly protein-peptide conjugates, which are produced by the conjugation of a peptide to the half-life extender. Santoprete et al. replaced Ser2 with aminoisobutyrate in oxyntomodulin for potential
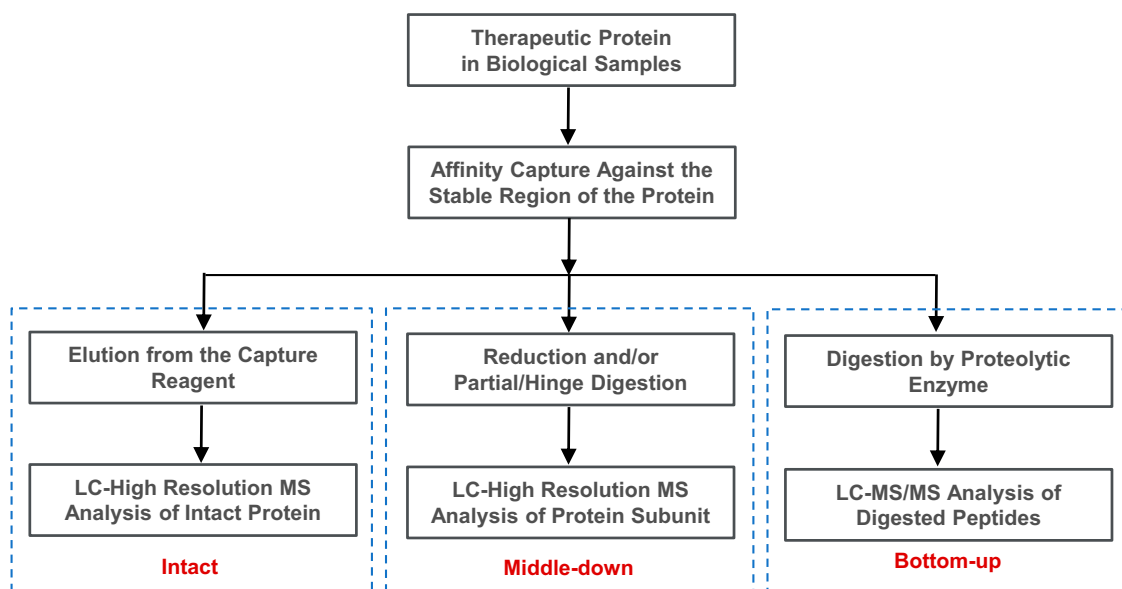
Fig. 4. Typical mass spectrometry-based workflows for identification of biotransformation products of protein therapeutics.

obesity treatment (Santoprete et al., 2011). This substitution alleviated the dipeptidyl peptidase-4-mediated proteolysis with increased half-life and potency. The same substitution was adopted to the oxyntomodulin moiety of a mAb oxyntomodulin conjugate (Camacho et al., 2020).

A cyclic peptide is more rigid than a linear peptide and more resistant to proteolysis. Therefore, cyclization has been widely used to enhance the catabolic stability of the therapeutic peptides (Werle and Bernkop-Schnürch, 2006; Di, 2015). The same technique was also used to extend the half-life for TPs. Gut hormone peptide YY (PYY)$_{3-36}$, a potential treatment of obesity (Gantz et al., 2007), has a very short half-life due to glomerular filtration and proteolytic degradation. To increase the half-life, PYY$_{3-36}$ was conjugated to a functionally silent mAb to escape the glomerular filtration (Rangwala et al., 2019). Moreover, the proteolysis of PYY$_{3-36}$ moiety of the mAb PYY conjugate was mitigated by two approaches: cyclization between position 31 and the amino-terminus and incorporation of a reduced peptide bond. The mAb cyclized PYY conjugate demonstrated greater stability in rhesus monkeys with a half-life of 6.5 days.

Other strategies to mitigate the proteolysis of peptides should also be applicable to TPs. For example, modification of N- or C-terminus is an efficient way to prevent the hydrolysis of a peptide by exopeptidases (Werle and Bernkop-Schnürch, 2006). This approach can be used for the peptide moiety of a protein peptide conjugate.

Amino Acid Modifications and PTMs

Biotransformation leading to modifications on single residues (Fig. 2), such as deamidation, isomerization, and oxidation impacts primary sequence and potentially higher order structure. A simple illustration of deamidation from asparagine to aspartic and iso-aspartic acid is shown in Fig. 5. Such degradations are generally most concerning when occurring on antigen binding domains, such as CDRs on mAbs, binding domains from cytokine or enzyme therapeutics, or other critical regions for function, such as FcRn and Fc gamma receptors The aforementioned liabilities can lead to loss of efficacy/potency, poor pharmacokinetics (Shah et al., 2018) and toxicity/immunogenicity (Yang et al., 2018). Specifically, in vivo deamidation within the CDR can result in loss of antigen binding due to disruption of antibody tertiary structure, and consequently, loss of potency (Tran et al., 2016). Depending on the rate and extent of modification, this can have a large

impact on the relative forms (active versus inactive) in circulation, complicating pharmacokinetic assessment and interpretation. While the active, unmodified form contributes to activity/potency through antigen binding or other targeted means, any off-target toxicity may be exacerbated through the accumulation of the inactive, modified form that clears through target-mediated distribution (TMDD). Thorough characterization and identification of modifications, which result in biologic consequences, is an important process of therapeutic development. Not only do modifications need to be identified, but the impact, if any, on disposition, potency, and potential toxicological effects should be considered. Not all modifications have downstream impact, so it is important to conduct a thorough evaluation.

Several residues are well known to be the most susceptible for single residue biotransformation. For example, deamidation most commonly occurs on asparagine, particularly if preceding a glycine residue, resulting in a conversion to aspartic and isoaspartic acids with a mass shift of +1 Da (Fig. 5). To a lesser extent, glutamine can also undergo an analogous degradation pathway. Alternatively, for residues such as aspartic acid (and to a lesser extent glutamic acid), isomerization leads to isobaric mass shifts. Oxidation most commonly occurs on residues of cysteine, methionine and tryptophan (and to a lesser extent on histidine and tyrosine) and leads to a mass shift of +16 Da. While deamidation and isomerization can be stressed in vitro by temperature and pH, oxidation has multiple stress attributes, including oxidizing agents (peroxides), UV irradiation, and also elevated temperatures. In
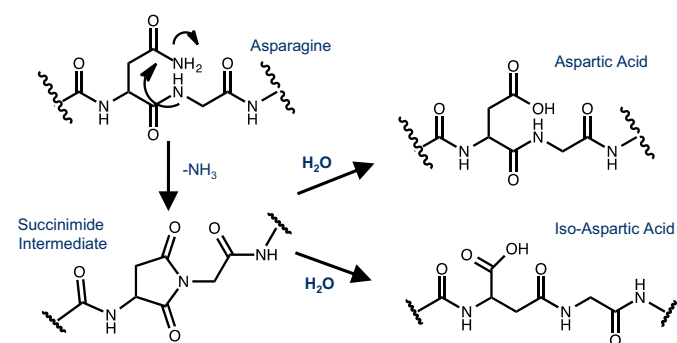
Fig. 5. Illustration of deamidation from asparagine to aspartic and iso-aspartic acid.

general, such degradations can occur during the production stage of therapeutic antibodies, in storage, and post administration in vivo.

Many companies perform in vitro stress assays to predict potential liabilities which occur during manufacturing as part of the drug development effort. For deamidation and isomerization, temperature and pH stress are used, whereas 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and peroxide chemical stress are commonly implemented to predict oxidation. While there have been reports suggesting a good correlation between in vitro stress and in vivo systematic degradation for deamidation and isomerization, more research is needed to have good translations for in vivo oxidation. While the downstream impact from an amino acid modification may be similarly independent of mechanism, upfront investigation and prediction between chemical modifications (e.g., deamidation) versus enzymatically catalyzed biotransformation can be much more challenging for the latter which require in vivo characterization.

Analytical Tools for Investigating Amino Acid Modifications and PTMs. Due to the complexity of in vivo samples (including blood and tissue samples and other biologic matrices), targeted peptide-based mass spectrometry analyses with the combination of immuno affinity capture offers unparalleled specificity and unambiguous identification (Fig. 4). While there have been reports of monitoring amino acid biotransformation using intact protein or top-down mass spectrometry, in general, characterization at the peptide level (using enzyme digestion) is preferred because of enhanced mass resolution and sensitivity. Unfortunately, the affinity capture and digestion procedures can lead to artificial modifications; therefore, there is a balance in ensuring high capture and digestion efficiency through optimizing parameters, such as digestion pH, time, and temperatures.

If a residue is determined to be susceptible for biotransformation, it is important to discuss a mitigation strategy. In addition to time and financial costs, a major challenge associated with reengineering the liable residue site is that it may lead to a decrease in potency/efficacy. Therefore, it is important to consider whether the degradation is a risk to the therapeutic pharmacological effect and safety. While it is ideal to identify any biotransformation liabilities early on in the development process, it is not always feasible.

Chemical Conjugation/Tethered TPs. The study of biotransformation increases in complexity with ADCs, or with PEGylated TPs, as the linker chemistry and chemical liability of the linked toxin or PEG may provide another source of modification.

Linker and conjugation chemistries for both ADCs and PEGylation can be via unspecific or site-specific conjugation using cleavable or non-cleavable linkers depending on their mode of action (Dubowchik et al., 2002; Toki et al., 2002; Senter, 2009; Ivens et al., 2015; Rao et al., 2015; Birdsall et al., 2016; Tumey and Han, 2017; Walles et al., 2017; Bargh et al., 2019). A comprehensive overview of linkers (including nature of linker and mechanism of cleavage) and payloads used in registered ADCs is shown in Table 1, while Table 2 gives a comprehensive overview of registered PEGylated biotherapeutics.

Two main features of ADC or PEGylated TP design can impact the biotransformation regarding changes in drug (or PEG) loading in vivo: stability of the linkage to the TP, and mechanism for drug release (i.e., cleavable or non-cleavable linkers). Therefore, we recommend in Fig. 2 to investigate the linker stability for both ADCs and Pegylated TPs. The linker stability investigations are usually part of the design. Nevertheless, change of drug/PEG loading over time could occur in vivo and should also be characterized similarly to other Biotransformation investigations.

Linker Chemistry for ADCs and PEGylated TP. For both tethered TPs, one of the main approaches used for conjugation is via maleimide-thiol chemistry where the product of such reactions results in thiosuccimide species (Fontaine et al., 2015; Ravasco et al., 2019).

TABLE 1
List of approved ADCs with linker and payloads used

Nonproprietary	Year of Approval (FDA or EMA)	Antibody	Conjugation and Linker Chemistry		Payload	Reference
Gemtuzumab ozagamicin	2000, 2017	mAb	Hydrazone	Cleavable (pH)	Calicheamycin	(Ali et al., 2019)
Brentuximab vedotin	2011	mAb	Valine-Citrulline	Cleavable (enzymatically)	MMAE	(Gravanis et al., 2016)
Trastuzumab emantansine	2013	mAb	succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate	Noncleavable	thiol-containing maytansinoid toxin	(Chen et al., 2016)
Inotuzumab ozagamicin	2017	mAb	Hydrazone	Cleavable (pH)	Calicheamycin	(Lamb, 2017)
Moxetumomab pasudotox	2018	mAb/fusion protein	recombinant fusion, no linker	Cleavable	38 kDa fragment of Pseudomonas exotoxin A	(Dhillon, 2018)
Polatuzumab vedotin-piiq	2019	mAb	Valine-Citrulline	Cleavable (enzymatically)	MMAE	(Deeks, 2019)
Enfortumab vedotin	2019	mAb	Valine-Citrulline	Cleavable (enzymatically)	MMAE	(Maas et al., 2021)
Trastuzumab deruxtecan	2019	mAb	Tetrapeptide	Cleavable (enzymatically)	Topoisomerase I inhibitor conjugate deruxtecan	(Narayan et al., 2021)
Sacituzumab govitecan	2020	mAb	Carbonate	Cleavable (pH)	SN38	(Wahby et al., 2021)
Belantamab mafodotin	2020	mAb	Maleimidocaproyl (mc) linker	Non cleavable	Monomethyl auristatin F	(Markham, 2020)
Loncastuximab tesirine	2021	mAb	Valine-Alanine	Cleavable (enzymatically)	Pyrrolobenzodi-azepine (PBD)	(Mullard, 2021)

TABLE 2
List of approved PEGylated proteins

Nonproprietary	Year of Approval (FDA or EMA)	PEG form/size (MW)	Conjugation and Linker Chemistry	References
Pegademase bovine	1990	Multiple 5 kDa	Random NHS (mPEG succinimidyl succinate) displacement to lysines.	(Ramos-de-la-Peña and Aguilar, 2019)
Pegaspargase	1994	Multiple (69-82) linear 5 kDa	Random NHS (mPEG succinimidyl succinate) displacement to lysines, serines, histidines.	(Belen et al., 2019)
Peginterferon alfa-2b	2000	Linear 12 kDa	mPEG succinimidyl carbonate to histidines	(Ramos-de-la-Peña and Aguilar, 2019)
Peginterferon alfa-2a	2001	2 × 20 kDa	mPEG succinimidyl carbonate to lysines	(Ramos-de-la-Peña and Aguilar, 2019)
Pegvisomant	2002	Multiple linear 5 kDa	mPEG succinimidyl succinate displacement to lysines	(Ramos-de-la-Peña and Aguilar, 2019)
Pegfilgrastim	2002	20 kDa	N-terminal aldehyde	(Ramos-de-la-Peña and Aguilar, 2019)
Pegaptanib	2004	2 × 20 kDa	mPEG amino	(Ramos-de-la-Peña and Aguilar, 2019)
Methoxy PEG epoetin beta	2007	linear 30 kDa	mPEG succinimidyl succinate displacement to lysines	(Ramos-de-la-Peña and Aguilar, 2019)
Certolizumab pegol	2008	2 × linear 20 kDa	mPEG maleimide to cysteine	(Ramos-de-la-Peña and Aguilar, 2019)
Pegloticase	2010	multiple linear 10 kDa	mPEG-nitrophenyl carbonate to lysine	(Ramos-de-la-Peña and Aguilar, 2019)
pegvaliase	2010	9 × 20 kDa	mPEG succinimidyl succinate displacement to lysines	(Ramos-de-la-Peña and Aguilar, 2019)
Peginesatide	2012	Branched 40 kDa	Lysine branched based (iminodiacetic acid) Methylpropionaldehyde	Eur J Endocrinol. 2017
Peginterferon alfa-1a	2014	20 kDa	(mPEG) moiety at the N-terminus	(Ramos-de-la-Peña and Aguilar, 2019)
PEG-rhGH	2014	40 kDa	Amino groups	FDA Approval letters 2014
Rurioctocog alfa pegol	2016	1 × 20 kDa branched (2 × 10)	Amide bond to lysine	(Ramos-de-la-Peña and Aguilar, 2019)
Nonacog beta pegol	2017	40 kDa	mPEG 2 selective N-linked glycoPEGylation	(Ramos-de-la-Peña and Aguilar, 2019)
Elapegademase	2018	80 kDa	mPEG succinimidyl carbamate	(Ramos-de-la-Peña and Aguilar, 2019)
Calaspargase pegol	2018	31-39 × 5 kDa	mPEG succinimidyl carbonate	(Ramos-de-la-Peña and Aguilar, 2019)
Damoctocog alfa pegol	2018	2 × 30 kDa	mPEG maleimide at a site specific cysteine	EMA assessment report (2018)
Turoctocog alfa pegol	2019	40 kDa	GlycoPEGylation.	EMA assessment report (2019)

Examples of this linker chemistry can be found in most approved products such as Traztuzumab Emantansine, Brentuximab vedotin (Table 1) or Damoctocog alfa pegol and Certolizumab pegol (Table 2).

The thiosuccimide species could undergo deconjugation through a retro-Michael pathway in plasma, leading to the loss of linker-payload. The resulting maleimide-payload conjugate can then be bound to other plasma protein thiols (e.g., human serum albumin, Fig. 6) leading to off-site toxicity and reduced efficacy (Wei et al., 2016). The retro Michael reaction of maleimides resulting in the cleaved PEG and therapeutic protein (Fig. 6) is a possible, but as yet unproven route.

Post-conjugation hydrolysis of the maleimide to the corresponding succinamic acid on the other hand (Fig. 6) eliminates the retro-Michael deconjugation pathway and results in more effective/stable antibody drug conjugates (Shen et al., 2012). As described before, proteolysis of the peptide-based linkers could also release payload (Salomon et al., 2019) and should be investigated as well as described in Fig. 2.

Analytical and In Vitro Tools to Investigate Linker Stability for ADCs. The traditional mechanism of action of an ADC involves antibody–antigen binding on the target cell surface, internalization by endocytosis and lysosomal processing to release the cytotoxic payload. Therefore, appropriate in vitro systems to study the linker stability include lysosomes, microsomes, cancer cells, hepatocytes and S9 fractions. The pros and cons of these systems to assess the linker stability have been reviewed recently (Kraynov et al., 2016). S9 fractions seem to have an advantage over the other matrices for the following reasons: the liver S9 fraction contains all major drug-metabolizing enzymes, does not depend on the permeability of the drug to reach the metabolizing enzymes, is transporter independent, is less susceptible to cytotoxic agents, and can be used at either pH 7.4 (to study metabolism of the drug) or acidified to mimic the pH of the lysosomal environment, which is the site of degradation of an ADC. However, all the described in vitro matrices including the S9 fractions generally lack abundant expression and activity of proteases which the protein drug will encounter in vivo. Therefore, stability testing of ADCs in vitro in plasma or serum (which contain much more proteases) of humans and the toxicology species, is in general recommended (EMA, 2016) in addition to support first in human trials.

Although the in vitro plasma stability of certain mono methyl auristatin E (MMAE) ADCs translates well with in vivo outcomes, there have been instances with other linkers or drugs in which the in vitro and in vivo plasma stability did not correlate well (Fourie-O'Donohue et al., 2020). In these cases, the discrepancy was either due to the amount of deconjugation of the drug from the antibody or a modification to the drug that was not detected with the plasma incubation but occurred in vivo. Similarly, stability screening in a different matrix like human liver S9 fractions showed good correlation with in vivo results for some ADCs evaluated, but this approach presented limitations related to chemical concerns for ADCs containing acid-labile linkers, as well as possible disulfide bond reduction, due to the incubation conditions (Fourie-O'Donohue et al., 2020). Recent investigations have shown that the translatability of the in vitro stability for certain ADCs to the in vivo situation can be significantly improved when the in vitro assay is performed in fresh whole blood as opposed to plasma due to higher enzyme activity (Fourie-O'Donohue et al., 2020).

The payload stability investigates whether payloads could undergo biotransformation in vivo while still attached to antibody. For example, acetate cleavage in tubulysin and amide hydrolysis in monomethyl auristatin D (MMAD) of site-specific conjugates resulted in a significant loss of potency (Su et al., 2018), and, therefore, we recommend the payload stability assessment for ADCs in Fig. 2. One way to assess the payload stability is through drug to antibody ratio determinations in vivo.

The most common high-throughput way is the direct comparison of conjugated drug [determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS)] to total antibody (measured by ELISA) (Sanderson et al., 2016). To further characterize each drug load species,
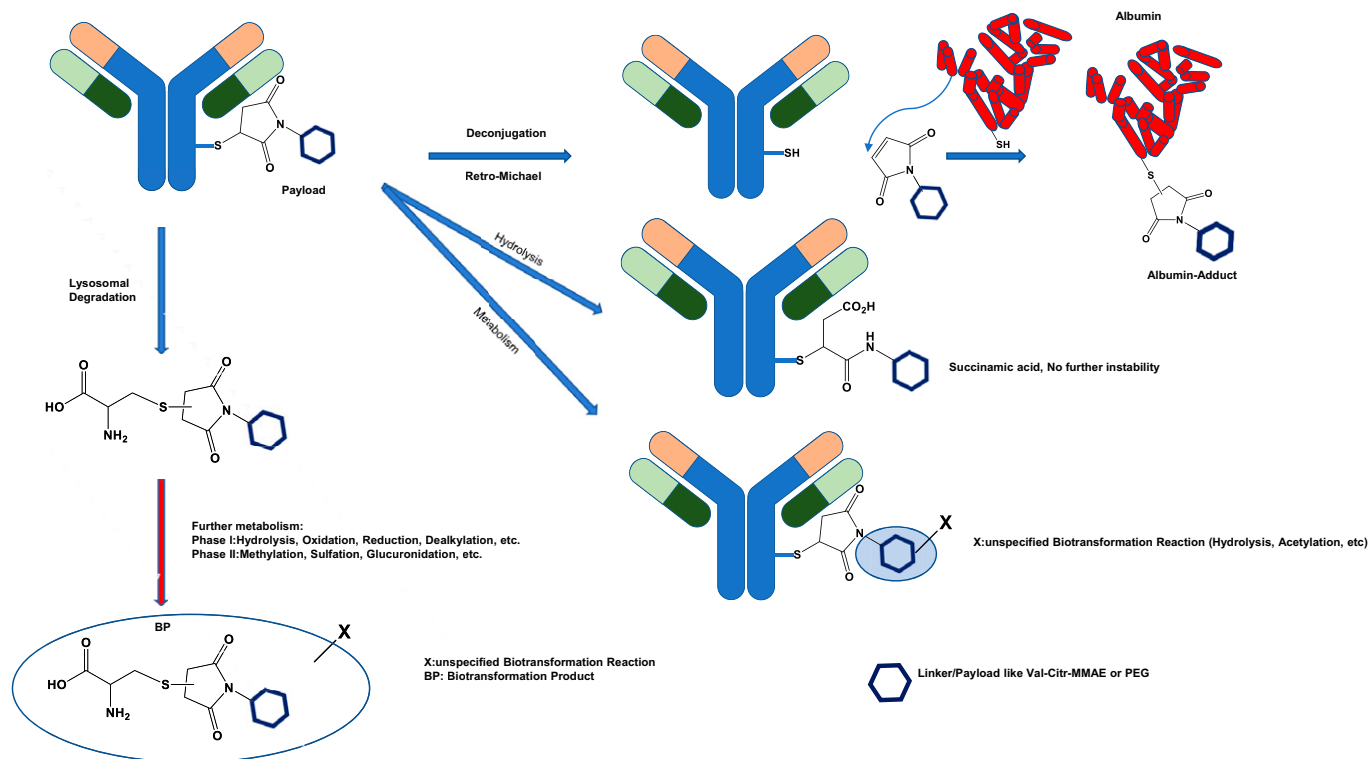
Fig. 6. Typical liabilities of tethered biotherapeutics, such as ADCs and PEGylated proteins, conjugated via maleimide linkage.

it is ideal to analyze the ADC in the intact form with minimal treatment so that its structural information can be preserved as much as possible (Valliere-Douglass et al., 2012; He et al., 2017). This approach is particularly suited for ADCs in which the payload is randomly distributed as for lysine-conjugated ADCs as thiol-containing maytansinoid toxin. However, the detection sensitivity for intact mass analysis is usually limited with the current mass spectrometry instruments.

To overcome these limitations in sensitivity, several methodologies have been developed to cleave the ADC in smaller parts by reducing either the inter-chain disulfide bonds or cleaving heavy chain of antibody using hinge digestion enzymes, such as IdeS as outlined in the proteolysis section. The former methodology is particular suited to determine the drug to antibody ratio of conventional cysteine-conjugated ADCs (Wei et al., 2016) and to determine both in vitro and in vivo ADC plasma stability for both conventional conjugated ADC and site-specific conjugated ADC (Grafmuller et al., 2016). The latter approach is beneficial if the payload is conjugated via site-directed conjugation to a specific part of the antibody, like the fragment antigen binding region of the ADC (Su et al., 2016).

Lastly, the metabolic fate of the attached synthetic moieties needs to be determined to derive the information of the forms of the small molecule entity that will require monitoring in preclinical and clinical efficacy and safety studies, as well as for drug-drug interaction assessments (Fig. 2 and 6). General recommendations for the assessment of the absorption, distribution, metabolism, and excretion properties of ADCs (Kraynov et al., 2016), as well as for their bioanalytical and drug-drug interaction properties (Li et al., 2021) (online), have been recently published and will not be further discussed here.

Further Biotransformation Considerations of PEGylated TPs. PEGylation continues to be adopted as a means to improve various characteristics of the TP or peptide, such as improved pharmacokinetic properties, selective distribution, and increased stability toward metabolizing enzymes (Turecek et al., 2016) and is already present in over 30 approved drugs (Table 2).

The process of PEGylation is typically based on a limited number of linker chemistries, such as N and O-glycation, succimidyl, lysine, serine, and hydrazide (Ramos-de-la-Peña and Aguilar, 2019), such as with Pegaspargase and Pegvisomant (Table 2). Although conjugation of linear PEG is the most conventional approach, use of forked, multi-arm or branched PEGs is also employed (Santos et al., 2018).

The major metabolite of PEGylated proteins is the intact unconjugated PEG and is the design strategy behind PEGylated TPs that require cleavage for activation, such as turoctocog alfa pegol (Bjornsdottir et al., 2020). The actual structure(s) of cleaved PEGylated species in vivo, from the range of the linker chemistries employed has yet to be determined and may well differ depending on the linker technology employed as this will play a role in its metabolism and the structure of this PEG species. The fate of the PEGylated species needs to be understood, although it is known that the release of the free PEG from the conjugated form in vivo is similar to other TPs, via cellular internalization through endocytosis, and then into endosomes and lysosomes. Here, under acidic conditions, the PEG linker is cleaved, resulting in the release of free PEG from the cell (Baumann et al., 2014). The release of the PEG is of interest in context of the subsequent formation of PEG-containing vacuoles with the major route of elimination of the free PEG being mostly renal (Nesbitt et al., 2007; Bjornsdottir et al., 2020).

As with ADCs, the stability of PEGylated TPs in serum can be determined by profiling acidic and basic species, with a particular emphasis on stability of the succinimide linker, using cation exchange chromatography and intact mass spectrometry. Other approaches have been employed to determine the stability of intact PEGylated protein in plasma and other biofluids such as ELISA, (Mahadevan et al., 2013) gel electrophoresis, and NMR (Elliott et al., 2012).

Metabolism of short-chain PEG is mediated by alcohol dehydrogenase, which oxidizes terminal alcohol groups to carboxylic acids, and with increasing molecular weight, the metabolic clearance of PEG becomes less prevalent (Herold et al., 1989; Webster et al., 2007; Webster et al., 2009). Longer PEG chains used for PEGylation of proteins and liposomes are not subjected to enzymatic degradation but are eliminated through a mechanism which is dependent on its molecular mass. While PEG can be metabolized, the formation of toxic metabolites from PEG is unlikely given the high molecular weight of the PEGs routinely used, and the relatively low amounts of PEG administered (Webster et al., 2007).

Unlike ADCs, in which the conjugated moiety is of a single structure, PEGylation is almost universally through conjugation of a heterogeneous population of PEG species, reported as having an average molecular weight of, for example, 40 kDa. The 40 kDa PEG will actually have a mass range of between approximately 35–45 kDa. This could therefore be considered to represent over 200 individual species which puts PEGylated drugs and their respective metabolites in a somewhat unique class. To profile the individual entities would not be possible nor particularly relevant, and they are therefore classified as a single entity. However, if the PEG itself underwent a significant biotransformation, rendering it as a significantly lower molecular weight species, then this in turn may result in its pharmacokinetics being different, and may require further profiling.

Quantitative profiling of PEGylated TPs is complicated by virtue of the heterogeneous nature of the PEG they are attached to, but advances have been made whereby NMR or liquid chromatography mass spectrometry are employed to quantify the PEGylated TP (Nesbitt et al., 2007; Alvares et al., 2016; Belen et al., 2019). As it is difficult to identify whether the PEG moiety is still conjugated or not using standard quantification methods, the fate of the PEG needs to be profiled separately as proposed by Ivens (Ivens et al., 2015).

Perspective on Future Directions

Scientists are gaining more insights into the importance of biotransformation characterization for the discovery and development of biologics in the context of efficacy, PK properties, and safety. As scientists are working on more challenging biologic targets, they have been evolving and expanding the available toolbox for modulation and target engagement at a rapid pace. This perspective provides a glimpse of only a small sample of therapeutic biologic modalities evolving from conventional antibodies or proteins/enzymes. In an attempt to identify a lead drug candidate, there are instances in which multiple modalities for engaging a challenging target are considered. For sustainability, in vitro assays would be beneficial for both throughput and reducing animal studies in selecting potential candidates with reduced biotransformation liabilities. Further, understanding potential species to species (including humans) translatability is usually more easily implemented with in vitro versus in vivo assays. Indeed, more data will continue to accumulate in the scientific community for in vitro/in vivo correlations for different types of biotransformation and modalities among different species. Our insights into biotransformation from diseased animal models and human patients will continue to evolve.

Since the properties and the engagement process involved with biologics are different, a fit for purpose strategy for studying biotransformation will likely be required for the near future for most case studies. We provide a decision tree (Fig. 2) to guide the biotransformation characterization for selected modalities. While the decision tree, which will constantly evolve, may work in certain instances, agility is required for

proper optimization of the unique molecule and therapeutic strategy. For example, much of the strategy in this review involves BPs identified in circulation. However, with the novel delivery mechanism focusing on tissues, characterization at the tissue level may become critical and requires a unique characterization strategy. With increasing complexity of the modalities, it is expected that the biotransformation scientists will play an important role to address the related biotransformation and analytical challenges. With these increasing demands being placed on the analytical technologies, it is not just the biotransformation scientists that are evolving, but also the instrument vendors are recognizing this and are focusing their engineering on characterizing biomolecules for biopharmaceutical applications.

With the demand to handle increasingly complex data, automated data processing from software becomes increasingly critical. There will be continued efforts in improving sample preparation, such as separation and affinity purification to augment the detection capability. In particular, having the ability to recover the BP analyte of interest from complex matrices is critical.

Conclusions

TPs have evolved toward a variety of modalities, including traditional mAbs, antibody fragments, fusion proteins, ADCs and PEGylated proteins with increasing diversity and complexity. Although the biotransformation data are not yet typically included in regulatory filings of TPs due to minimal corresponding safety concerns in general, there is increasing evidence that its analysis at various stages in the drug discovery and development pipeline may help not only streamline the lead candidate selection and optimization but also gain insights into translation from animals to humans. This perspective discusses various scenarios which may prompt the investigation of biotransformation of TPs and proposes a decision tree (Fig. 2) as a starting point in the investigation of possible BPs from proteolysis, PTMs, and degradations, such as linker deconjugations with ADCs and PEGylated TPs. Relevant examples are presented to show potential impact on functional potency, pharmacokinetics, immunogenicity, and safety, as well as bioanalytical assay performance. The authors recognize that a fit-for-purpose strategy will remain as the common approach when studying the biotransformation of a new TP based on its specific modality, complexity, and intended usage. Opinions are also provided on possible future directions in biotransformation assessment of TPs. We believe that with continued collaborative efforts, via platforms such as the Innovation and Quality consortium, the drug development community is on the path to reach a consensus on this emerging topic.

Acknowledgments

Authorship Contributions

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