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Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars

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Abstract

Biologics such as monoclonal antibodies are much more complex than small-molecule drugs, which raises challenging questions for the development and regulatory evaluation of follow-on versions of such biopharmaceutical products (also known as biosimilars) and their clinical use once patent protection for the pioneering biologic has expired. With the recent introduction of regulatory pathways for follow-on versions of complex biologics, the role of analytical technologies in comparing biosimilars with the corresponding reference product is attracting substantial interest in establishing the development requirements for biosimilars. Here, we discuss the current state of the art in analytical technologies to assess three characteristics of protein biopharmaceuticals that regulatory authorities have identified as being important in development strategies for biosimilars: post-translational modifications, three-dimensional structures and protein aggregation.

The clinical and commercial success of biologics such as monoclonal antibodies and recombinant versions of endogenous proteins is transforming the pharmaceutical industry. In 2010, worldwide sales of all biologics approached the US$100 billion mark[1], and by 2015 it is expected that more than 50% of new drug approvals will be biologics[2], rising to more than 70% by 2025 (REF. 3). As these drugs begin to come off patent, substantial opportunities exist for other companies to make copies or ‘generic’ versions of these drugs.

For small-molecule drugs, abbreviated regulatory pathways for the development and introduction of generic versions of the drug (following the expiration of patent protection on the original product) have been established for more than 25 years. Rather than requiring generic versions to undergo the same level of evaluation as the original drug, including

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Competing interests statement
The authors declare competing financial interests.

FURTHER INFORMATION
Draft guidances for biosimilars on FDA website: http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/default.htm
EMA website (scientific guidance documents on biosimilar medicines): http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000408.jsp&mid=WC0b01ac058002958c
FDA website 27 October 2010 press release: http://www.fda.gov/Safety/Recalls/ucm231639.htm
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clinical trials, abbreviated approval for the same purposes is generally based on demonstrating that the generic drug is pharmaceutically equivalent (that is, it contains the same active ingredient in the same purity, strength, dosage form and route of administration) and bioequivalent (that is, it is absorbed into the body at a similar rate and extent) to the original drug[4]. Consequently, abbreviated approval is considerably less expensive to achieve, thus dramatically lowering the costs of generic drugs. This has led to the widespread use of generic versions and substantial cost savings for health-care systems; a recent paper noted that in 2009 almost 75% of small-molecule drug prescriptions dispensed in the United States were for generics, and the approval of a generic drug resulted in average savings of 77% of the original product's cost within 1 year[5].

However, for biologics, establishing a regulatory pathway for the introduction of follow-on versions of the original product (once its patent protection has expired) is much more challenging than for small molecules. Some simple biologics — for example, small peptides such as recombinant insulin and recombinant human growth hormone — can be well characterized by established analytical approaches, which has facilitated the regulatory approval of follow-on versions under abbreviated pathways (based in part on data from the original drug and in part on analytical data and limited clinical data in some cases)[4]; however, many biologics such as monoclonal antibodies and other recombinant therapeutic proteins are much larger and more complex. For such biologics, the extent to which existing analytical technologies can be used to support the likelihood of clinical comparability between a follow-on version and the original product is much more limited than for small-molecule drugs, and it is not possible to demonstrate that the two products are absolutely identical.

Consequently, a key question for the development and regulation of follow-on biologics — also known as biosimilars — is how much and what kind of data are needed to establish that the differences between similar (although not identical) products are not clinically important[4].

Clearly, the overall success of developing a biosimilar — as has been the case with generic small-molecule drugs — will depend on the ability of the biosimilar sponsor to offer a highly similar, safe and efficacious drug product at a cost saving that will encourage health-care providers to purchase it over the original product while still allowing the biosimilar sponsor to make an adequate profit. If the bar of comparability or similarity is set too high, the economics of biosimilar development may not be sufficiently attractive for companies to participate. However, if the bar of comparability or similarity is set too low, the drug's efficacy and the safety of patients could be in jeopardy. With the setting of this bar in the hands of government regulators (coupled with the recent or imminent expiration of patent protection for a growing number of commercially successful biologics), regulatory authorities globally have been developing pathways for the introduction of biosimilars that are intended to realize the ultimate desired benefits.

In Europe, the European Medicines Agency (EMA) introduced the first operating framework in 2005 (REF. 6) for a path towards developing and marketing biosimilars. Since then, European biosimilar guidelines have been described[7] (see the EMA website) and 13 biosimilars have been approved and are still on the market[8]. In the United States, the 2009 Biologics Price Competition and Innovation (BPCI) Act empowered the US Food and Drug Administration (FDA) to develop a pathway to introduce biosimilars within the United States, and the draft guidelines were announced on 9 February 2012 (see the FDA website) (BOXES 1,2). In developing this draft, a hearing by the FDA[9] was conducted in 2010 to seek input from stakeholders on four main areas related to biosimilars: "First, what scientific and technical factors should the agency consider in determining whether the biological

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product is highly similar to the reference product, notwithstanding minor differences in clinically inactive components? Second, what scientific and technical factors should the agency consider in determining the appropriate analytical, animal, and clinical study or studies to assess the nature and impact of actual or potential structural differences between the proposed biosimilar product and the reference product? Third, what range of structural differences between a proposed biosimilar product and the reference product is consistent with the standard "highly similar" and may be acceptable in a 351(k) application if the applicant can demonstrate the absence of any clinically meaningful differences between the proposed biosimilar product and the reference product? Fourth, under what circumstances should the agency consider finding that animal studies or a clinical study or studies are 'unnecessary' for submission of a 351(k) application?"

Box 1

Commentary on the FDA's proposed regulatory pathway for biosimilars in the United States

The 2009 Biologics Price Competition and Innovation (BPCI) Act empowered the US Food and Drug Administration (FDA) to develop a pathway to approve biosimilars. In February 2012 the FDA released three draft documents (see the FDA website) in support of developing the initial pathway that sponsors of biosimilars need to follow to achieve drug approval, and on 11 May 2012 the FDA held a 1 day public hearing to obtain input on the draft guidance documents. The new pathway outlined by the FDA, summarized pictorially in BOX 2, is based on a risk based approach using what the agency calls "totality of the evidence".

In principle, at first sight this approach is not novel. The approval of any biopharmaceutical product is based on the totality of the evidence provided to the FDA in the data package filing that any drug sponsor normally provides to support drug approval (whether that is for a completely new drug or for a change to an existing drug, including a second generation drug made by the same sponsor) or to gain approval to conduct a clinical trial. This "totality of the evidence" data package includes biochemical, biophysical, biological, toxicology and clinical data. However, for a biosimilar, it is already known that the original drug is sufficiently safe and efficacious from the work of the innovator (along with the drug's commercial history). The crucial question becomes: how similar or comparable to the innovator's drug does the biosimilar have to be in order to take advantage of the innovator's experience and the drug's long history (particularly with regard to the extent of clinical data needed to support biosimilar approval)?

One key conclusion emphasized by the FDA in its recent guidance and in prior publications[4,5] is that the answer to this question is not unique. Although there are clearly core features corresponding to information that is normally provided in a chemistry, manufacturing and controls (CMC) filing that will be needed in all biosimilar filings (for example, information on aggregation, impurities, and so on), several paths could be followed to achieve the goal of obtaining an abbreviated approval for a biosimilar, especially without the need to conduct clinical trials. The answer is therefore formulated on a case by case basis. Minimal clinical studies (or perhaps even no clinical studies) might be sufficient for the approval of the biosimilar, if biochemical, biophysical and biological data (structural and functional analysis) can demonstrate that the innovator drug (also known as the reference product) and its biosimilar are identical (or similar enough) and that there is no effect of any difference in the mode of formulation, container closure as well as handling and administration, so that equivalence in clinical performance (pharmacokinetics/pharmacodynamics (PK/PD) and immunogenicity) can be assured.

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An important feature in enabling the agency to make clear and accurate decisions as to what is needed for the approval of a biosimilar will rest on delivering a robust and comprehensive structural and functional analysis package for the molecule. Such a data package requires more extensive information than what is normally provided in drug filings for innovator products. In stating the requirement for more information, the agency has referenced the use of orthogonal, state of the art and fingerprint methods that, although not yet validated (or even very difficult to validate), are based on sound science.

For biologics, proving that two separate manufactured lots are identical is virtually impossible. Even an innovator company cannot manufacture its own biologic so that it is absolutely identical on a lot to lot basis owing to the inherent level of complexity of these molecules and the way in which they are made. The best a manufacturer of a biologic can do is to demonstrate consistency in manufacture, with attributes that fall within a set of acceptable specification criteria that regulators have agreed to through a history of testing and characterization. Such a history of information concerning the innovator's drug is not known by the sponsor of the biosimilar. However, as a result of filings and historical data provided to them by the innovator, known as "prior knowledge", regulatory agencies have this information or key subsets of this information. This prior knowledge may include unique biochemical, biophysical, biological and maybe even toxicology and clinical (including PK/PD and immunogenicity) data that the sponsor of the biosimilar may not be aware of, in addition to the normal standard filing information. Hence, to reduce development time and costs for the sponsor of the biosimilar, as well as achieve optimum utilization of FDA resources, a new paradigm for interaction would be needed between sponsors of biosimilars and the FDA. In such a paradigm, biosimilar sponsors would have early, effective and active dialogue (known as a stepwise approach) with the FDA to understand what this "totality of the evidence" data package should include, especially in terms of understanding what kind of toxicological and clinical data will be required. Even after a biosimilar receives approval, pharmacovigilance studies will need to be implemented to mitigate any additional unknown potential risks associated with biosimilars relative to the innovator drug.

Finally, the FDA documents indicate that clinical studies will be needed for a biosimilar to reach a level of identity — in terms of clinical performance and immunogenicity — that is considered to be close enough to that of the innovator drug for it to be used "interchangeably". Such interchangeability could be established before the approval of a biosimilar via clinical studies, post approval through additional clinical trials or possibly through pharmacovigilance studies. It should be noted that at present neither the FDA nor the European Medicines Agency (EMA) has provided a clear definition and draft guidance on "interchangeability" (see BOX 2 for the FDA's statement on interchangeability).

Box 2

Summary of the draft pathway for biosimilars in the United States

The figure below summarizes the key points in the draft documents released by the US Food and Drug Administration (FDA) (see the FDA website) that initiate its plans for the development of the path for obtaining approval of a biosimilar in the United States. Selected points of this process are listed below.

- Owing to the uniqueness of this process, the FDA recommends a stepwise approach involving a substantial level of interaction of the biosimilar sponsor with the FDA (step **a**).

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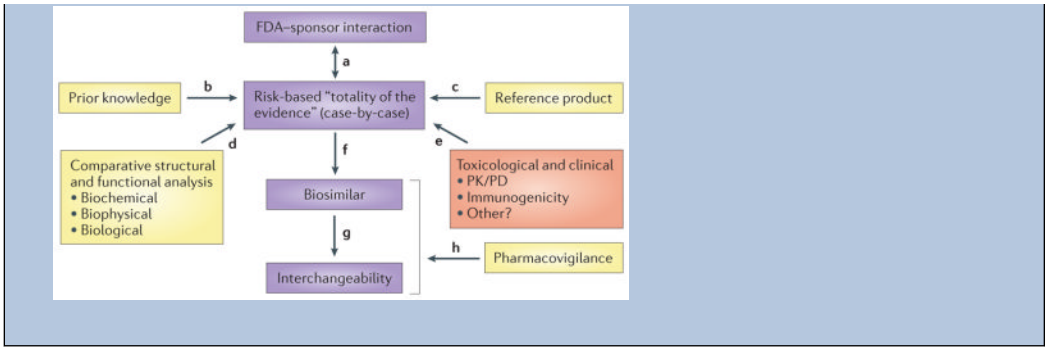
- Part of the uniqueness resides in the level of prior knowledge of the innovator drug (step **b**).
- Another part of the uniqueness of this process is in assessing the level of comparability or biosimilarity (usually referred to as 'highly similar') of the biosimilar to the innovator drug (also known as the reference product) (step **c**). In conducting this assessment, several different lots (of both the biosimilar and the reference product) should be used to understand the data space variability of the biosimilar and the innovator drug.
- The first experimental key step in the biosimilar approval process is to assess the comparative structural and functional analysis between the biosimilar and the reference product (step **d**). In performing this assessment, the FDA emphasizes the added use of "orthogonal methods" and "fingerprint like methods". These latter methods may represent more advanced or state of the art analytical characterization methods that have not been validated but must be scientifically sound. The biosimilar sponsor should realize at this stage that the more extensive, comprehensive and robust the comparability process, the lower the likelihood of requiring data from animal (toxicology) and clinical (human) studies (see below).
- Using prior information coupled with the outcome from step **d**, the level of toxicology and clinical data required will be assessed and determined (step **e**). At a minimum, pharmacokinetics/pharmacodynamics (PK/PD) and immunogenicity data will need to be provided in support of biosimilarity; however, other more extensive toxicology and/or clinical studies may be required, depending on prior knowledge (step **b**) and the results from step **d**, especially in reference to the need to address residual uncertainties regarding biosimilarity.
- Biosimilarity, as defined by the FDA, is when: "The biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components … [and] there are no clinically meaningful differences between the biological product and reference product in terms of safety, purity and potency of the product." In making this assessment, the biosimilar sponsor needs to realize that the assessment is made on a case by case basis (step **f**).
- A higher standard of biosimilarity is defined as "interchangeability" (step **g**). Although the FDA has not clearly defined this attribute, the agency did say that "an applicant must provide sufficient information to demonstrate biosimilarity, and also to demonstrate that the biological product can be expected to produce the same clinical result as the reference product in any given patient and, if the biological product is administered more than once to an individual, the risk in terms of safety or diminished efficacy of alternating or switching between the use of the biological product and the reference product is not greater than the risk of using the reference product without such alternation or switch (see section 351(i)(3) of the PHS [Public Health Service] Act)".
- Following the approval of either a biosimilar or a biosimilar that can be used under the status of interchangeability, a pharmacovigilance programme needs to be in place to ensure the safety and the effectiveness of the biosimilar therapeutic protein product, especially during the initial phase of its introduction into the public setting (step **h**).



Related to several of these areas, three properties of therapeutic proteins — in the opinion of the FDA[10] — cannot be sufficiently measured but are deemed to be important for understanding the behaviour of protein drugs: post-translational modifications, three-dimensional structures and protein aggregation. Given the need to rapidly improve our ability to measure these three properties, it is an opportune moment to survey the currently available analytical techniques used to generate information about these properties, which are discussed here in light of the guidance issued by the FDA and EMA. Before considering the three main areas identified for analytical improvement, however, it is important to discuss some of the general challenges in assessing comparability.

General challenges in assessing comparability

The first challenge in assessing the comparability of biologics is understanding exactly what is meant by the terms ‘comparable’, ‘similar’ and ‘highly similar’. During the development of innovative biologics, there are often numerous changes to the manufacturing process (for various reasons), sometimes including changes even after the drug is commercialized. As a result, the innovator will need to conduct comparability studies to show regulators that drug products before and after process changes are ‘comparable’ in order to be able to use these post-change drug products in any subsequent clinical trials or in existing commercial licensed products[11]. Here, we will refer to this type of comparison as an ‘internal’ comparison. In the case of developing a biosimilar version of an originator’s biologic, the manufacturer of the biosimilar must execute a far more challenging task of comparability in order to prove that its biosimilar is ‘similar’ (the term used by the EMA) or ‘highly similar’ (the term used by the FDA) to the innovator’s biologic. Here, we refer to this type of comparability as an ‘external’ comparison. It should be noted that the terms comparable, similar and highly similar are specific terms used by the EMA and the FDA for the above mentioned purposes[7] (see the FDA website), and so care should be taken in their use; see below.

The approach taken by the EMA to deal with the primary issues of comparability for biosimilars — defined here as external comparability — is a logical extension of the concept of internal comparability set forth initially by the FDA and conceptualized by the International Conference on Harmonisation (ICH) Q5E guidelines (BOX 3). In fact, the European Generic medicines Association (EGA) indicated in its response to the FDA’s public biosimilar meeting in 2010 (REF. 9) that: “It should be noted that the EMA never limited the application of comparability to a single company making manufacturing changes to its own product, instead it was held to be a scientific evaluation irrespective of the source (manufacturing change or biosimilar) to the two entities being compared. Thus, while we all recognize that ICH Q5E defines comparable as having ‘highly similar quality attributes’, and limits its use to a single company with their own product, the European Union guidelines use the terminology of similarity and comparability interchangeably within the same regulatory documents, and recognize that the two terms refer to the same scientific

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principles." The EGA further added: "As successfully used in the European Union, the scientific grounding of similarity and comparability is the same, and the regulators endeavour to apply these principles consistently to both, original biologics and biosimilars. A high degree of similarity forms the basis for abbreviated clinical programs, approvability, extrapolation of indications, interchangeability, and trust by patients and health-care providers."

Box 3

Existing characterization guidelines and relationship to comparability studies

Perhaps the most relevant existing industry standards in place to determine the comparability of biopharmaceuticals are the International Conference on Harmonisation (ICH)'s harmonized tripartite guidelines referred to as Q5E (Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process), which were issued by the ICH Working Group in November 2004 (REF. 143). These guidelines were developed for innovators of biopharmaceutical products to help them establish internal comparability between products before and after process changes in order to implement process changes for their products. These guidelines focus on four main criteria described in Q5E section 2.2.2:

Physicochemical properties (as defined in ICH Q6B)

The ICH Q6B guidelines on physicochemical properties cover comparability exercises of the molecular entity to assess the degree of molecular homo and/or heterogeneity. Higher order structural analyses are recommended, including the assessment of any changes in secondary, tertiary and quaternary structures. Dedicated guidelines are described in the ICH Q6B document. If higher order structural information cannot be obtained, it is suggested that relevant biological assays could be used to confirm or support conformational equivalency between the two products.

Biological activity/bold

Manufacturers are encouraged to provide meaningful and insightful bioassay data in order to highlight or confirm the absence of any effects that might be attributed to changes in process. These assays may also be used in some circumstances as surrogates to confirm higher order structures. In cases where physicochemical or biological assays are not sufficient to confirm that higher order structures of two products are maintained, non clinical and clinical studies may be necessary.

Immunochemical properties

In the case of antibodies and antibody based products, the manufacturer should confirm that specific attributes of the (two) products are maintained and/or comparable by appropriate assays. Given that small differences in glycosylation and/or deglycosylation are known to have immunogenic consequences, this is an area of special interest in the development of guidelines for biosimilars and interchangeable biologics.

Purity, impurities and contaminants

The guidelines on purities, impurities and contaminants, which are intended to ensure that isoforms and degradation products are detected, specify that a combination of analytical procedures should be used to confirm that the purity profile of the product (or products) has not changed. It is assumed that the manufacturer will take measures to prevent the formation of contaminants, and any that are discovered through this process are identified and characterized using appropriate methods.

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However, what is potentially the Achilles heel in the above EGA statement is that such an approach implies that the manufacturer of a biosimilar has: first, the same extensive knowledge base about the properties and behaviour of the innovator's drug as the innovator; and second, the availability of appropriate reference material for making comparisons. Regarding the second point, although there may be a perception that the sourcing of reference standard material could be solved by purchasing the innovator's product on the open market, this approach presents its own set of problems[12]. The formulation matrix of the innovator's product could interfere with necessary comparison studies between the innovator's drug and the biosimilar product. Some type of deformulation or extraction of the active drug material from its original drug product formulation may be required[13,14], and such sample processing could lead to alterations in the generated reference material that could in turn cause misleading comparison results[15,16]. This problem was recognized by both the EMA and the FDA, and hence a validated deformulation and extraction process of the active drug substance from the commercial drug product will be required[6] (see the FDA website).

A possible approach for achieving such a valid deformulation and extraction process may require the manufacturer of the biosimilar to place its biosimilar drug molecule into the same formulation as the innovator drug product and co-process it with the innovator drug product in order to conduct a potentially more valid comparability study.

A further interesting point related to appropriate reference material is the source of the reference material relative to the jurisdiction of the regulatory body reviewing the biosimilar. In the case of the EMA the reference material must be derived from its jurisdiction, whereas in the case of the FDA reference material outside its jurisdiction can be used if an acceptable bridging study of this material to a US-licensed reference product can be provided.

A second challenge is defining the acceptable level of comparability to confirm the claim that two or more biologics are in fact comparable, similar or highly similar. As already stated, the use of the term 'identical' is inappropriate owing to the inherent complexity of protein bio-pharmaceuticals and their manufacturing processes. For protein drugs, simple changes such as a single-amino-acid mutation or covalent modification (a post-translational modification (PTM)) may lead to small perturbations in higher-order structure. Such small perturbations might result in the drug not functioning or, worse, cause a drug to malfunction via aggregation or immunogenicity. Therefore, defining the meaning of the terms comparable, similar and highly similar as acceptability levels is a real challenge. In particular, as noted earlier in this article, in the biosimilar area the term 'similar' is specifically used by the EMA, whereas the term 'highly similar' is specifically used by the FDA. Such word usage unfortunately only further adds to the confusion, and hence it is worth repeating that care should be taken in determining how and when to apply these terms. In the case of the EMA, various product-specific biosimilar guidelines have been generated (see the EMA website) (for example, for recombinant erythropoietins and recombinant granulocyte colony-stimulating factor) and continue to be generated (for example, for interferon-β and monoclonal antibodies) to help guide applicants of biosimilars to more effectively formulate their comparability studies.

Below, we discuss the three properties of therapeutic proteins that, in the FDA's view, cannot be sufficiently measured (as described above), as well as the analytical tools that are available and being developed to assess these properties. We also discuss key challenges for the development of biosimilars in light of regulatory guidance related to these three properties.

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Post-translational modification

There are many types of PTMs of proteins[17,18]. Common PTMs are glycosylation (which includes galactosylation, fucosylation, high mannose derivatives and sialylation), oxidation, phosphorylation, sulphation, lipidation, disulphide bond formation and deamidation. In most cases these chemical changes occur *in vivo* but some chemical changes can also occur *in vitro*: for example, during various stages of manufacture such as purification and storage.

Changes to proteins as a result of PTMs can have a role in protein activity[19], and so there is a need to characterize and understand them when manufacturing biologics. In addition, PTMs may influence the immunogenicity of biologics. Several comprehensive reviews[20,21] have addressed the immunogenicity of biologics and include discussions on the potential contribution (or contributions) of PTMs to immunogenicity. In the case of several types of PTMs (for example, deamidation, oxidation and differential glycosylation), direct connections between the post-translationally modified biologics and immunogenicity have not been clinically demonstrated[22]. Nevertheless, it is known that PTMs can alter protein structure and cause aggregation, and that such changes can cause immunogenicity problems (glycosylation and amino acid isomerization are also discussed below). Therefore, it is crucial to be aware of PTMs and understand them for each biologic. Furthermore, to demonstrate the reproducible production of a biologic, the manufacturer needs to monitor PTMs at many steps during the manufacturing process[23], which requires the identification of PTMs, control of their levels and assessment of their impact on the protein. The levels of variability in PTMs that are allowed in a developmental or commercial biologic can vary and are generally determined by information concerning their importance and prior manufacturing history. In most cases, a definitive impact (on clinical outcome) of variability in the amount, level and forms of the different PTMs is not known[24].

Although PTM characterization is a very daunting task in proteomics studies (in which there are hundreds, if not thousands, of proteins to investigate all in the same sample)[25], biologics represent a much simpler case. For biologics, the sequence and identity of the protein is known and the various modified forms can generally be isolated, sometimes in large quantities. As a result, characterization of PTMs is more straightforward than it is in a global proteomics investigation. Software from several instrumentation vendors is designed to first identify whether modifications are present, in what quantity and, in some cases, where the modifications have occurred. The analysis of PTMs is not, however, without limitations. Sophisticated instrumentation, skilled analysts and time are all required and although computation and automation have eased the burden, there is still a long way to go.

Mass spectrometry (MS) is a valuable tool for detecting and investigating protein modifications (by monitoring mass differences), for determining where they occur on the protein (by analysing peptide fragments and their mass alterations) and for elucidating what causes them (by comparing different types of samples, storage conditions and formulations) (FIG. 1; see REFS 26–28 for examples). Several detailed reviews[17,29–33] on the topic of applying MS to PTM detection have highlighted a few trends. One recent trend is the increasing emphasis on understanding the nature of the sugars involved in glycosylation. Given that various cell lines, expression hosts and protocols can result in different glycosylation patterns, measuring and understanding glycosylation by MS is crucial. Furthermore, to understand batch-to-batch variability and to compare innovator and biosimilar proteins, it is necessary to determine where glycosylation occurs (which is relatively straightforward in peptide mapping experiments) as well as the structure and content of the individual sugars (which is much more challenging, even for MS).

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For example, a study comparing the glycosylation pattern of an innovator tissue-type plasminogen activator (tPA) and its biosimilar demonstrated a ~2.5-fold greater amount of glycosylation at one *N*-linked site in the innovator material versus the biosimilar material[34]. Differences between an innovator monoclonal antibody, trastuzumab (Herceptin; Genentech/Roche), and a bio-similar were readily detected with liquid chromatography–MS (LC–MS)[35], including changes in glycosylation and amino acid mutations in the heavy chain. Analysis of recombinant and human-derived factor IX glycans following enzymatic digestion and LC–MS[36] indicated that the fucosylation site on the human-derived protein and the recombinant version was different (FIG. 1). The clinical ramifications of these differences are not clear. It was recently reported[24] that although changes in glycosylation levels were observed for etanercept (Enbrel; Amgen/Pfizer), darbepoetin alfa (Aranesp; Amgen/Kyowa Hakko Kirin) and rituximab (Rituxan/Mabthera; Biogen Idec/Genentech/Roche) as a result of post-approval process changes, the clinical ramifications of the differences were unknown.

Differences involving one or two monosaccharides and/or their linkage specificity (mostly from sugars that are not made in humans but are instead made in the mammalian cells used to produce the recombinant protein) have been linked to immunological responses in only a few cases[37]. Galactose-α-1,3-galactose linkages or terminal-α-1,3-galactose have been connected to anaphylaxis associated with cetuximab (Erbitux; Bristol-Myers Squibb/Lilly/Merck Serono)[38] and immune response to bovine thrombin[39]. The sialic acid *N*-glycolylneuraminic acid (Neu5Gc; also known as NGNA) is known to be associated with immunogenicity issues[40], and reduction or elimination of this sugar in recombinant proteins is highly desirable[41]. A recent comparison[42] of Neu5Gc in cetuximab and panitumumab (Vectibix; Amgen) showed the presence of Neu5Gc in cetuximab but not in panitumumab, and showed that addition of Neu5Ac (*N*-acetylneuraminic acid) to the culture media reduces the incorporation of Neu5Gc. The ability to detect differences in glycans by MS is therefore valuable for biologics[43], including for the analysis of human glycosylation pathways through the discovery of non-consensus additions[44] and for the assessment of whether different glycosylation patterns have clinical relevance[19].

Another trend in the application of MS to the study of PTMs is the awareness that the modification itself could be altered by the analysis method. To correct this limitation, there has been an increasing role for alternatives to classical collision-induced dissociation MS fragmentation methods in recent years. The value of the bottom-up approach of digesting the protein into peptides and characterizing the mass of all the peptides is well established, but analysis in a top-down fashion is now also proving to be useful[29,45,46]. In a top-down analysis, characterization of the whole protein (rather than a collection of its digested pieces) is conducted inside the mass spectrometer, in which fragmentation methods such as electron transfer dissociation (ETD) and electron capture dissociation (ECD)[22,47,48] are used to fragment the protein into smaller pieces for more detailed analysis. ETD and ECD are not only able to preserve labile PTMs, but common labile structural elements of a protein (such as disulphide bonds) that may be scrambled in other fragmentation methods are also preserved[49].

The detection of amino acid isomerization is another important form of PTM that is benefiting from ongoing method development. One amino acid of particular concern is aspartic acid as it can isomerize readily to form isoaspartic acid (isoAsp), which has been reported to have some undesirable immunogenic consequences[50,51]. The formation of isoAsp can potentially occur either through direct isomerization of Asp or through deamidation of asparagine, proceeding through a common succinate intermediate. Such subtle isomeric differences (the mass difference is zero) obviously present a substantial challenge for detection and analysis, and this issue is compounded in large proteins. MS can

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be used to detect and characterize isoAsp, particularly with ETD[52,53] or ECD[54–56]. In one case involving β-amyloid protein[57], enzymatic cleavage at aspartic acid residues (using the endoproteinase AspN) allowed the isoAsp/Asp ratio to be quantified by ETD–MS to levels as low as 0.5%. However, considerable expertise and time may be required to achieve such analyses.

A good way to localize, detect and characterize *N*-linked glycans on proteins is to enzymatically release them and then carry out MS analysis. However, in the case of *O*-linked glycans[58] caution must be exercised as the chemical cleavage and release steps may often result in breakdown of the glycan itself, and so alternative strategies may need to be considered. Although the presence of *O*-glycosylated sites can be detected on intact proteins using new, softer ionizing MS techniques, information on the covalent structure at the linkage site is typically difficult to obtain by MS. One method that is well suited to *O*-glycan analysis is nuclear magnetic resonance (NMR) spectroscopy. Indeed, this was the breakthrough technique used in the well-publicized case of heparin contamination; here, the contaminant — over-sulphated chondroitin sulphate — was detected by 600 MHz NMR spectroscopy, which revealed the location, identity and orientation of the *O*-glycan chains[59].

One of the general limiting factors in traditional NMR analysis of proteins has been the large sample size required (up to ~20 mg) for meaningful data collection. However, recent advances in NMR technologies — including flow NMR and most recently microcoil NMR — have changed this outlook. In the latter case, it is possible — with limits of detection below 100 pmol — to obtain high-quality spectra. In a recent example[60] of an analysis of cyanobacterial cell extracts, it was possible to detect metabolic components representing 1% of a mixture (the total quantity injected was 30 μg) after LC separation. Coupling MS with NMR makes the analyses more powerful. LC–MS–NMR can accommodate the large disparities in the requirements (such as sample mass and analysis time) of the two techniques, and directs the more demanding NMR technique to ambiguities or gaps in the MS analysis[60]. An attractive extension of this for biologics could be to start with proteomic analyses of mixtures separated by high-performance LC (HPLC) and ultra-performance LC (UPLC), and to then split the effluent for both MS and MS–MS, followed by microcoil NMR analysis of selected features. Future developments in LC–MS–NMR technology could hopefully be applied to PTM characterization.

Higher-order structure

Although major advances have been made in developing tools for primary structure analysis, as discussed above, what is missing in these studies is an understanding of the impact of the PTM on the three-dimensional structure of the biopharmaceutical product. The higher-order structure of proteins — that is, the secondary, tertiary and quaternary structure — is what gives each protein its three-dimensional shape and ultimately affects the way that the protein functions. So, the ability to monitor the higher-order structure of intact proteins has obvious importance for the characterization of biologics[61–63]. Differences in higher-order structure can not only provide potential clues to any observed biological and/or immunological differences between proteins and variant forms (that is, proteins with PTMs) but can also serve as a means for assessing the lack of comparability between versions of an innovator product before and after process changes, as well as for establishing a lack of comparability between an innovator product and a biosimilar version.

Although characterization of the higher-order structure of biopharmaceutical proteins represents a substantial challenge, progress is being made on various fronts using specialized analytical methods[64]. The higher-order structure of proteins results from a collection of forces, many of which are weak when independently considered but strong when combined.

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These weak interactions have a major role in the overall conformation and conformational dynamics of all proteins. During the manufacture of biopharmaceutical proteins (which includes steps such as cell culture overexpression, purification, transportation, storage and handling), factors can be encountered that can perturb some of these weak forces in proteins, causing alterations to the three-dimensional structure without changing the primary structure of the protein. Such changes can effectively be referred to as 'silent' changes as they have no chemical covalent signature that one could detect as a fingerprint of the change. Without analytical tools to detect and characterize these conformational changes, their impact on structure–function relationships remains unknown. Finally, protein dynamics in solution (involving protein structure 'breathing', polypeptide chain bending, flexing and local protein structure unfolding) is another important attribute of protein behaviour that has — until now — been virtually unknown in biopharmaceutical analysis owing to the lack of appropriate practical routine analytical tools (as discussed below).

The two main techniques for protein structure determination are X-ray crystallography and NMR. Unfortunately, the application of these technologies for the purpose of higher-order structural comparability studies presents major problems. X-ray crystallography is impractical for routine testing as the sample must first be crystallized — something that may or may not be possible for structural reasons (that is, owing to the presence of PTMs and/or disordered regions in the protein). Structural analysis via X-ray crystallography is also generally too time-consuming and complex for routine biopharmaceutical analysis. In the case of NMR, the large size of protein biopharmaceuticals (with their complex array of structural elements), the relatively low sensitivity of the NMR signals and the low natural abundance of active nuclei (other than the ^{1}H isotope) in biopharmaceuticals all make this technique impractical for routine higher-order structure comparability studies. However, under certain circumstances where smaller protein biopharmaceuticals are being developed, simple one-dimensional ^{1}H NMR to produce NMR fingerprints may provide a very useful and practical comparability assessment[65].

The use of two-dimensional NMR for biopharmaceuticals has been reported[66,67]; however, these applications have again been for small protein biopharmaceuticals and have required lengthy data acquisition times just for one sample (especially when natural abundance levels of active nuclei are being used), also making them impractical for routine application where many samples need to be compared. We note that although present opportunities for using NMR in routine protein biopharmaceutical comparability analyses are limited, it appears that the use of this technology for polysaccharide biopharmaceuticals is more feasible[68,69] (see the brief discussion below concerning NMR and heparin).

Other classical biophysical techniques such as circular dichroism, fluorescence, differential scanning calorimetry, isothermal calorimetry, analytical ultracentrifugation (AUC), size exclusion chromatography (SEC) and various dye-binding assays can be used to characterize protein structure[15,70,71]. A major limitation of these methods is that they generally provide information that is derived from a sum of signal inputs that come from many different parts of the protein being probed. The information obtained from these types of measurements corresponds to a global average over the entire structure of the biopharmaceutical. For example, circular dichroism measurements indicate only the average percentage of each basic type of major secondary structural element (α-helix, β-sheet and random coil) that is present in a protein. If a protein with several α-helices is analysed and only part of one α-helix is modified in one of the two samples being compared, the biopharmaceutical scientist has the difficult task of trying to discriminate between two large signals that only differ by a small amount. Furthermore, the ability to detect this difference is also a function of the inherent noise level, which in many cases is large in comparison with the amplitude of the actual difference.

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Classical biophysical tools are thus not well suited for detecting small, subtle differences in protein conformation or even major changes in a biopharmaceutical product when those altered molecules represent only a small fraction (population) of the total ensemble of molecules present in solution. Even when changes are observed, these techniques provide little to no information about the location in the molecule where the change has occurred. Hence, other methods that could provide more information in a practical and routine way would be very desirable.

The use of protein labelling methods such as hydrogen deuterium exchange MS (HDX–MS)[72] and covalent labelling strategies[73] can be valuable in detecting small changes in the higher-order structure of a biopharmaceutical. Importantly, when changes are detected, these techniques can provide information as to where in the biopharmaceutical molecule the change has occurred. In HDX–MS, the protein is exposed to deuterated water (D_2O) and exchangeable hydrogen atoms become labelled with deuterium, thereby adding one additional measurable mass unit per amino acid. The exchange is a function of protein structure and dynamics. By conducting the exchange and analysis in real time under physiological conditions, information on the dynamics of the protein is obtained, as well as comparative information on the higher-order structure of the protein. HDX–MS provides information-rich data, is highly sensitive (requiring only 1–2 nanomoles of material for complete characterization), can be automated[74] and can localize where changes or differences exist in specific regions of the biopharmaceutical; furthermore, resolution at the single amino acid level is on the horizon[75]. Current limitations continue to be the analysis time required to interpret the data and the complexity introduced by solution conditions that are incompatible with MS analyses (for example, detergent-containing buffers); however, these have been substantially improved in recent years[72,76].

Recently, HDX–MS was used to study the conformation and conformational dynamics of a recombinant immunoglobulin G1 monoclonal antibody (FIG. 1), and was used to monitor the changes to its higher-order structure after removing its glycans, altering its oligosaccharide structure and following receptor interaction[77,78]. The ability to acquire these and similar data could have an instrumental role in building a comprehensive map of the structural aspects of a biopharmaceutical that are crucial for understanding its function, maximizing its stability and understanding how PTMs such as glycans influence local and global features and properties. Because HDX–MS can reveal details of the higher-order structure of proteins, as well as protein dynamics, the method has the potential to become an important tool for studies assessing comparability between innovator products and their biosimilars[79–81]. It should also be mentioned that HDX–MS can be used to monitor the effects of the interactions of biopharmaceuticals with target proteins that are deemed to be important to their biological function[82–84], so this technique could also have a key role in the discovery and development of biopharmaceuticals.

As many aspects of higher-order structure can be driven by the proper formation of disulphide bonds, knowing their location and verifying that they are formed correctly during protein manufacture and handling is crucial[85]. In recent examples[86,87], comprehensive characterization of the disulphide bonding pattern was performed on the blood-clotting regulator tPA and on therapeutic monoclonal antibodies using LC–MS. Key to analyses of these biopharmaceuticals was the use of carefully controlled enzymatic digests followed by soft ionization (electrospray ionization) and gentle fragmentation (ETD). Before the development of such methods, comparative homology models with existing crystallographic structures were widely used. The ability to make the measurements on the actual agent, no matter how complex, will become a standard tool in determining structural equivalence between protein samples involving disulphide bonds.

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Another method that can provide some information on higher-order structure is ion mobility spectrometry (IMS)[88]. In IMS, information about protein conformation is generated by probing the collisional cross-section of the molecule in the gas phase[88]. The utility of this information, however, is dependent on preserving the native state or important attributes of a protein's native-like structure during ionization and transition into the gas phase; this is something that is now well understood[88,89], especially from work on native MS of proteins[90] and protein complexes[91]. IMS can be used to characterize, among other things, the effects of pegylation of protein therapeutics[92], potential lead antibody products[93] and other aspects of antibody parameters[31], as well as diagnose the presence of aggregates[94] (FIG. 1).

One area where current analytical methods fall short in assessing the higher-order structure of biopharmaceuticals is in detecting the presence of small, conformationally altered populations of the active drug that represent about 10% (or less than 10%) of the total population of normal molecules in a given sample. Unfortunately, these minor conformational forms of the biopharmaceutical are part of a complex mixture of closely related structures in a dynamic equilibrium, which makes the task of characterizing them very challenging.

Using current technology, the best opportunities for addressing this problem could be based on analytical techniques in which a separation method such as chromatography or electrophoresis (conducted under conditions that maintain the native structural and conformational population distribution of the biopharmaceutical) is coupled with online structure analysis methods or other orthogonal separation methods. Such possibilities include the coupling of ion exchange chromatography (IEC) separation to MS (using an MS-friendly buffer system) to conduct IEC-native or native-like MS[64,90,91,94]. Here, charge-state distributions can be utilized to extract information on the various separated drug variants to assess their conformation and aggregation state. In addition, if the mass spectrometer has IMS or HDX–MS capability, tandem approaches such as IEC–IMS–MS or IEC–IMS plus gas-phase HDX–MS[95] could be conducted to dissect complex mixtures. Such separation–analysis systems should enable the quantification of biopharmaceutical variant components (resulting from covalent and non-covalent PTMs) even when these components are present at very low levels.

Aggregation

A major concern in manufacturing protein biopharmaceuticals is their propensity to form aggregates. These undesirable associated states of the monomeric form can be reversible or irreversible, and can range in size from a dimer to particles that may contain trillions (or more) of monomer units that can be visible to the naked eye. In general, aggregation can be a problem for any protein biopharmaceutical. Beyond the obvious detrimental impact of reducing the actual dosing concentration of the drug (as most aggregates have little or substantially reduced drug activity in comparison with the monomeric form of the drug), by far the greatest concern surrounding the presence of aggregates is their unpredictable ability to give rise to adverse toxicological and immunological responses, which in extreme cases can result in severe responses that can be life-threatening[96–98]. As a result, the area of aggregation has attracted considerable amounts of research attention. Weak evidence has mounted over the years pointing to factors such as the amount, size and native-like repeating array structure of these aggregates as potential key attributes associated with the adverse effects[97]. Hence, there is considerable scrutiny and interest in how the biopharmaceutical industry monitors and assesses protein biopharmaceutical aggregation in terms of its detection, quantification and characterization[99,100].

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There are some excellent recent reviews that cover many of the challenges associated with measuring and characterizing biopharmaceutical aggregates[100–105]. Recently, an entire issue of *Current Pharmaceutical Biotechnology* (volume 10, June 2009) was devoted to aggregation. Lists of analytical techniques that can detect, quantify or characterize aggregates have appeared in many technical papers, in which the advantages, limitations and specifics of each technique have been discus sed[20,96,99,100,102,103,106–111]. Unfortunately, there are limitations in the ability of all of these techniques to detect and quantify protein aggregates[100,102,112–114].

SEC has been, and will probably continue to be, the major method used to characterize protein aggregates owing to its simplicity, low cost, low amount of sample required, ease of use, high speed and therefore high sample throughput capability[100,102,115]. Although advances in SEC column development have led to a number of ultra-high-performance size exclusion columns with improved resolution, allowing measurements to be made with less sample and in a shorter time[116], other methods have exposed validity issues and limitations in the use of SEC[115,117,118]. Orthogonal analytical methods such as AUC and asymmetric flow field flow fractionation (AFFFF (or AF4); also known as field flow fractionation (FFF)) can provide a certain additional level of assurance that the data generated by SEC methods are accurate[115,118–120]. In fact, regulatory agencies are now beginning to ask for data from orthogonal aggregation assessment methods, such as AUC or AF4, to help assess SEC methods. Although AUC and AF4 may not be capable of quantifying aggregate formation as precisely and accurately as correctly functioning SEC[103,105,109,110,114,119–124], their orthogonal nature in the detection and quantification of aggregates in combination with SEC data reduces the possibility of overlooking gross errors encountered when using SEC. These errors include the inability to detect aggregates because of their removal by the SEC column or their dissociation during the SEC process. Thus, it is clear that at present no method is the best for the analysis of all forms of aggregates[102].

In light of immunogenicity concerns surrounding aggregation (as alluded to earlier in this section), more recent crucial insight into the entire scope of aggregation has led to concerns regarding the specific lack of monitoring of aggregates that have a size that falls between that which is analytically measured via SEC, AUC and AF4 (less than or equal to a few million daltons) and that which is measured via light obscuration and direct visual monitoring (greater than or equal to ~10 μm)[99,112,125–127]. Aggregates in this intermediate size range (from about 0.1 μm to almost 10 μm) include subvisible and submicrometre-sized particles that border on the edge of solubility and are present at very low concentrations. Techniques to assess such particles include dynamic or static light scattering, or recent new flow-imaging instruments that use light scattering or direct microscopic imaging. There is also the ability to interrogate the chemical composition of these intermediate aggregates using Raman spectroscopy[128] (see the rap.ID Particle Systems website) or Fourier transform infrared (FTIR)[129] spectroscopy to confirm whether they represent the actual protein drug product or some type of extraneous material[100,126].

In conclusion, although there are analytical tools to address those aggregates that fall into this range (approximately 0.1–10 μm), challenges still exist owing to inherent bias in the size range limits of each specific analytical tool, particle properties (for example, particle transparency), particle concentration, sample concentration and the way data are reported (number of particles in a specific size range per unit volume in comparison with mass concentration of particles in a specific size range per unit volume). Alternative approaches that improve or supplement appearance testing and light obscuration methods, which are commonly used to attempt to investigate such aggregates as well as visible particles, have not been agreed upon[112].

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Impact, adoption and legal ramifications

The methods described in this article provide powerful analytical tools for the analysis of key aspects of protein biopharmaceuticals. The difficulties facing regulatory agencies and manufacturers of biopharmaceuticals will be to determine (for both external and internal comparability studies) the measurements that should be conducted, the significance of the data obtained and the level of difference that is unacceptable. The precision of the data and the level of confidence in it are crucial in this endeavour. Ultimately, some correlation needs to be made between the parameters that are measured and the effects of the drug when it is administered to a patient. In other words, is measuring all of these analytical attributes really relevant to the drug's stability, clinical outcome or the functional activity of the active drug substance? In the case of biosimilars, one possible future decision — which would have obvious economic consequences — could be that the manufacturers are not required to run exhaustive clinical trials on the basis that their drug is deemed to be analytically highly similar to the innovator drug.

At some point in the process, it will also be important for both the innovators and the developers of biosimilars to determine which analytical methods are appropriate for inclusion with regulatory filings, and to what degree these analytical measurements contribute to assessing physicochemical stability and clinical relevance, especially given the concerns stemming from adverse clinical events involving biopharmaceuticals[4] (see the 27 October 2010 press release on the FDA website) and the vexing problem of assessing comparability in relation to immunogenicity. The properties of a potential biopharmaceutical that will elicit an immunological response in a patient are poorly understood and at present cannot be adequately predicted from *in vitro* or *in vivo* (animal) testing, structural knowledge or analytical measurements of the biopharmaceutical. It is known, however, that various factors can influence immunogenicity[20,130,131], including the drug itself[22,130], the process by which it is made[132–136], the genetic and health history of the patient[22,137] and the mode of drug delivery[22]. At present, only clinical trials can provide definitive data, and these data may only appear after the later stages of drug development or even after drug approval[20].

It is anticipated that advances in bioanalytical methodologies will continue at a rapid pace, but this will also be accompanied by multiple challenges. First, regulators will need to keep abreast of developments with these methodologies and require submitters of products to include analytical methods that are information-rich. In so doing, however, regulators will need to understand the capabilities and limitations of these advanced analytical tools and keep track of their improvement as these new tools are further developed. A second challenge will be in making these more advanced analytical technologies available to the numerous smaller biopharmaceutical companies that could have difficulty in covering the high costs associated with these methods, such as the necessary instrumentation and highly skilled staff to perform the testing and interpret the resulting data. A third challenge is the associated cost faced by the instrument manufacturers in the development, maintenance and improvement of these tools. Here, a balance is needed between development costs, potential limited profit (when the number of units that can be sold may be relatively low) and the importance of the characterization information that these tools are capable of providing for biopharmaceuticals.

To remain most viable, instrument manufacturers will probably focus their resources on areas where the greatest profits can be achieved. Further difficulties arise when the task of instrument improvement is raised where market saturation has been nearly reached and a new instrument or improvement itself does not mandate the end user to buy an entirely new instrument. For example, in AUC the present sole vendor of this very important technology

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(Beckman Coulter, which pioneered the commercial development of this instrument) has not provided substantial improvements to this instrument in about two decades, irrespective of major advancements in electronics, detectors, computer hardware and software as well as in the detection of product quality issues[121,122,124,138]. Such a situation potentially compromises the full capability of this technology in terms of precision, accuracy and confidence in what it can truly deliver.

An additional potential concern that can arise as these advanced instruments and methods are developed and become commonplace is related to the due diligence on the part of the drug manufacturers. The possibility of a safety-related issue being traced back to inadequate analytical characterization of a product is very real, as illustrated in the case of heparin contamination. Here, simple one-dimensional ^{1}H-NMR measurements were easily able to reveal the presence of the contaminant[59,139]. It is hoped that both the biopharmaceutical industry and regulatory agencies will fully realize and appreciate the ramifications of the emerging bioanalytical technologies available to them, as well as existing technologies whose potential has not been fully exploited, to help monitor the safety of biopharmaceutical products. Failure to adopt and/or incorporate such methods into future regulatory filings could render corporations liable to product recalls and patient-initiated litigations, but more importantly it could cause needless harm to patients. Furthermore, it is expected that the use of these new characterization tools will prove to be definitive in intellectual property and patent-related litigations regarding the equivalency of the composition of matter, for both innovators and the biosimilar industry.

Given the importance of effectively establishing the comparability and biosimilarity of biopharmaceuticals, it is expected that manufacturers of analytical instruments — working with industry, regulatory agencies and the academic community — will develop more sensitive and more specifically targeted analytical technologies. There are also merits to the establishment of truly independent reference laboratories (within either the government or the private sector) for conducting advanced bioanalytical testing. A recent US government accountability office report cited potential conflict of interest concerns with a biotechnology company that had been engaged by the FDA to assist in solving the heparin contamination issue[140]. Hence, this approach — if taken — needs to be carefully executed. In addition to reference laboratories, there is great merit in developing standardized assays[141] that can be utilized, especially for comparison purposes, to address the complex issues associated with developing biopharmaceuticals and biosimilars. The opportunity to obtain government funding for reference laboratories, instrumentation companies and academics in order to encourage them to develop, maintain and improve reference standards, instrumentation and technologies that are identified to be of key importance might also merit serious consideration in dealing with some of the challenges discussed in this article.

In closing, modern analytical technology is rapidly advancing the characterization of biopharmaceuticals. However, despite gaining an ever-expanding knowledge of biopharmaceuticals and their effects in patients, our understanding of how living systems work is still limited, as for every answered question there often seem to be many more new ones to answer. A sensible path forward for the characterization of biopharmaceuticals is to work to close the gap between what we know and what we do not; however, we cannot wait until these gaps are closed. We need to gather whatever information we can from the best analytical tools available to make the best decisions feasible. Using a “risk management plan”[20] that is understood and embraced by all stakeholders, including the public, we then need to move forward with the development process of biosimilars. The application of analytical technologies such as those described in this article will have an important role in this process by enhancing our understanding and reducing some of the risks associated with

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biopharmaceuticals, an approach that is well reflected by a quote from the late George Bernard Shaw[142]:

> "The only man who behaved sensibly was my tailor; he took my measure anew every time he saw me, while all the rest went on with their old measurements and expected them to fit me."

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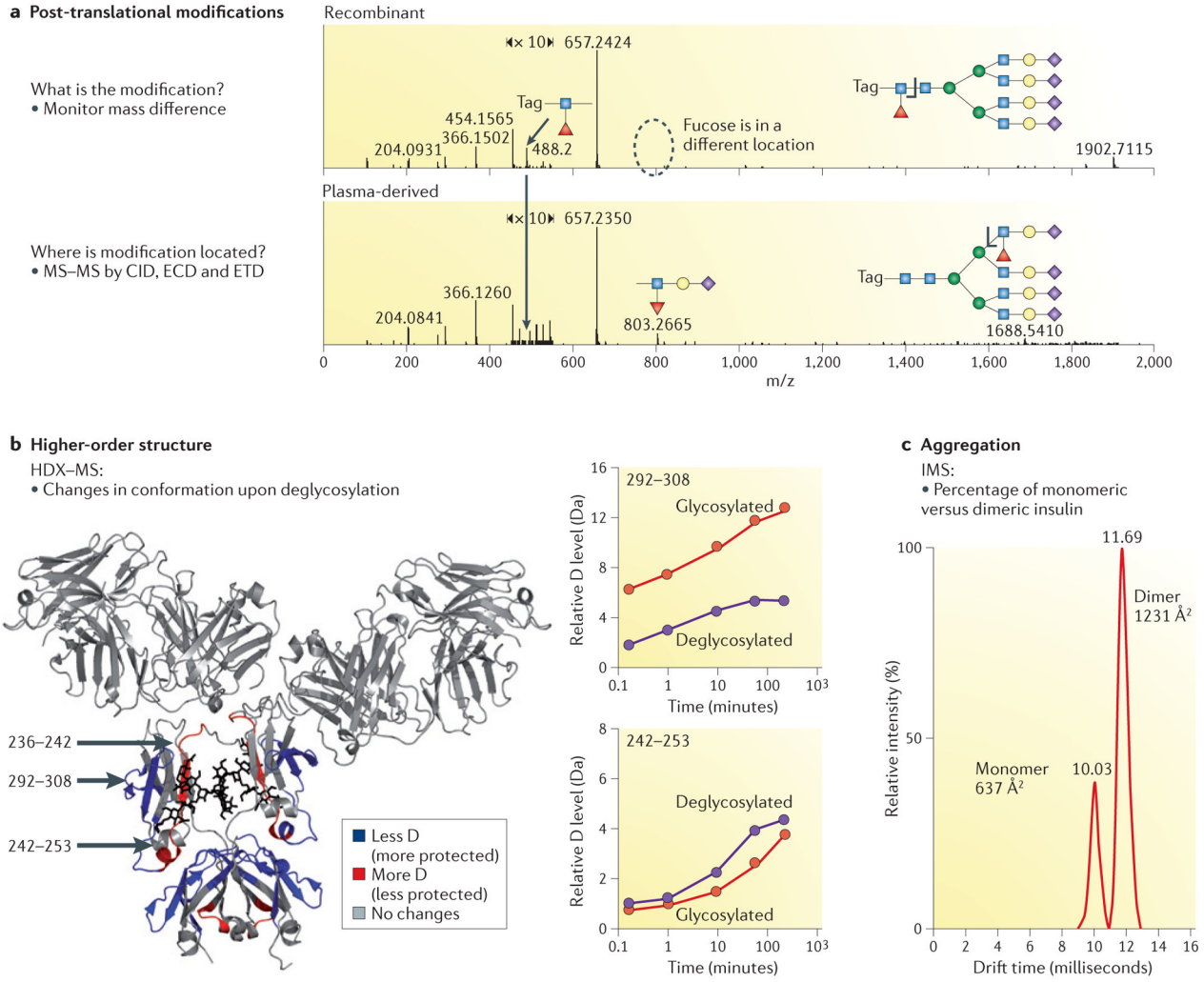

Figure 1. Uses of analytical tools in the characterization of biopharmaceuticals

a | Post-translational modifications are observed as mass increases in peptides, and the location of the modifications can be deciphered by monitoring smaller fragments through tandem mass spectrometry (MS–MS) experiments. In this example, the addition of fucose is monitored in a specific peptide, and its location in the whole protein is known based on its mass and fragmentation pattern. The plasma-derived version of the protein (bottom spectra) contains the fucose but the recombinant form (top spectra) does not. **b** | Higher-order structures can be studied by hydrogen deuterium exchange MS (HDX–MS). In this example, the changes in protein conformation and dynamics in a monoclonal antibody were probed for fully glycosylated immunoglobulin G (IgG) versus deglycosylated IgG. Removal of the glycan affects hydrogen deuterium exchange and therefore conformation in the blue and red regions. **c** | Aggregation can be monitored by ion mobility spectrometry (IMS), in which different species have different drift times in the ion mobility separation. Here, dimeric insulin has a collisional cross-section and therefore a longer drift time than monomeric insulin. This figure is based on REFS 36,77,144. CID, collision-induced dissociation; ECD, electron capture dissociation; ETD, electron transfer dissociation; m/z, mass-to-charge ratio.