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REPORT

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Developability studies before initiation of process development

Improving manufacturability of monoclonal antibodies

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Monoclonal antibodies constitute a robust class of therapeutic proteins. Their stability, resistance to stress conditions and high solubility have allowed the successful development and commercialization of over 40 antibody-based drugs. Although mAbs enjoy a relatively high probability of success compared with other therapeutic proteins, examples of projects that are suspended due to the instability of the molecule are not uncommon. Developability assessment studies have therefore been devised to identify early during process development problems associated with stability, solubility that is insufficient to meet expected dosing or sensitivity to stress. This set of experiments includes short-term stability studies at 2–8°C, 25°C and 40°C, freeze-thaw studies, limited forced degradation studies and determination of the viscosity of high concentration samples. We present here three case studies reflecting three typical outcomes: (1) no major or unexpected degradation is found and the study results are used to inform early identification of degradation pathways and potential critical quality attributes within the Quality by Design framework defined by US Food and Drug Administration guidance documents; (2) identification of specific degradation pathway(s) that do not affect potency of the molecule, with subsequent definition of proper process control and formulation strategies; and (3) identification of degradation that affects potency, resulting in program termination and reallocation of resources.

Introduction

Development of a therapeutic protein is a long and costly process that can take over a decade from discovery to commercialization. Although therapeutic biologics generally have a higher probability of success than their small molecule counterparts,[1] the rate of attrition remains substantial. While decisions to terminate projects can be based on the competitive landscape and commercial opportunities, projects are also terminated for technical reasons, including unsuitable safety profile, lack of efficacy in human, instability of the molecule or formulation, poor expression and purification issues. To reduce the risk associated with a given project, the sooner these technical challenges are identified, the sooner appropriate control strategies can be put in place. When irreducible challenges are identified, the project can be terminated and resources diverted to the development of a more promising molecule. Derisking process development early enough is critical to success because challenges not identified early enough may lead to expensive and time-consuming remediation steps later in the development of a given program. Determination of the safety profile is usually performed during preclinical studies and Phase 1 clinical studies in human, which is well into the development path. Likewise, the true assessment of a molecule efficacy is obtained during Phase 2/3 clinical studies.

In contrast with safety and efficacy assessments, a number of technical hurdles can be evaluated early during development programs. To identify technical challenges, we devised a comprehensive set of developability experiments and applied the strategy to a number of monoclonal antibodies (mAbs) currently in our pipeline. This strategy includes a series of in silico and experimental approaches that we grouped under the *Developability Assessment* term. In silico work includes structure sequence analysis based on primary sequence alignment and molecular model; prediction of possible degradation pathways is based on prior experience and on published literature. This constitutes the first step toward establishing potential critical attributes within the Quality by Design (QbD) paradigm encouraged by the US Food and Drug Administration.[2] The experimental part of the developability assessment includes short-term stability studies at various temperatures, freeze-thaw studies and limited forced degradation studies. This series of studies determine the biochemical (e.g., sensitivity to oxidation and deamidation) and biophysical

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Table 1. Developability stability schedule

Conditions	Initial	t = 0.5 mo	t = 1 mo	t = 2 mo	t = 3 mo	t = 6 mo
−80°C	X	/	/	/	/	X
2–8°C		X	X	X	X	X
25°C		X	X	X	X	X
40°C		X	X	X	X	X

/ No samples staged at these conditions/timepoints. X Samples staged and analyzed at these conditions/timepoints. Details of the assays used during stability are reported in **Table S1**.

(e.g., unfolding and formation of large molecular weight species) stability profiles of the molecule of interest. Determination of maximum solubility and associated viscosity are also essential for molecules destined to be formulated at high concentrations for subcutaneous injections. Developability studies offer the first opportunity to assess the technical viability of a project at the industrial scale and function as a bridge between discovery and process development activities. We present in this report three case studies and include data generated during developability assessment of antibody molecules.

Results

General strategy for developability studies. Developability studies are considered a derisking activity designed to provide understanding of the colloidal properties and prevalent degradation pathways of a molecule early in the development process, thereby allowing the analytical strategy to be tailored to the molecule of interest. To be relevant, developability studies should be performed on material produced with a stable cell line of the same host as the one that will be used later for process development; when possible, use of the final stable clone is preferable.

The most widespread host for expression of mAbs is the Chinese hamster ovary (CHO) cell line; however, a developability strategy can be applied to material produced by any expression system. The recombinant protein is produced via an upstream early cell culture process and purified by a downstream, 2–3 columns standard process. It is important to note that, at this stage of development, the formulation has not been optimized and the protein matrix might be suboptimal. Keeping these limitations in mind, developability studies are aimed at detecting gross changes in the protein that could hinder the stability or potency of the molecule.

Sequence alignment and molecular modeling. Developability assessment starts with analysis of the sequence of the protein entering the development phase. Some of this work has likely been done in the discovery space. For example, potentially problematic residues such as methionine, asparagine or aspartic acid residues localized in exposed region of the mAb, including the complementarity-determining regions (CDRs), may have been removed. Relevant knowledge and experience generated during the discovery phase, such as whether the molecule was generated through phage display or hybridoma cells, should be taken into account. The first examination of the sequence during developability assessment is therefore aimed at identifying potential hot spots for degradation that remain in the sequence. Sequence alignment can be performed by any software available on the market. To complete the analysis of the primary sequence, building a molecular model may help to visualize residues exposed to the solvent, which are susceptible to degradation. The software we currently use for this exercise is MOE (CCG, Montreal, Canada).

Short-term research stability studies and freeze/thaw cycles. To gain a first impression about how the molecule will degrade, short-term stability studies are performed. Material is staged at −80°C, 2–8°C, 25°C and 40°C for up to 6 mo (**Table 1**). Samples are removed at different time points and analyzed by size exclusion (SEC) HPLC to detect the formation of high molecular weight species and by ion exchange (IEX) HPLC to measure the effect of stress on distribution of charges at the surface of the molecule due to the deamidation and isomerization of asparagine and aspartic acid residues. Samples are also analyzed by reducing and non-reducing sodium dodecyl sulfate capillary electrophoresis (CE-SDS) to detect proteolytic cleavage of the heavy or light chains, peptide mapping to quantify the formation of post-translational modifications formed during stress and a potency test, which is most commonly in a binding ELISA format in the very early stage of the program development.

Propensity of the recombinant protein of interest to form aggregates is gauged during freeze/thaw cycles. The protein is subjected to five cycles. Aliquots are analyzed by SEC-HPLC, SEC-multi-angle laser light scattering and dynamic light scattering at each of the five cycles.

Limited stress conditions. To complete the initial assessment of the stability of the molecule, a series of stress conditions are applied (**Table 2**). These conditions include standard stresses suggested by regulatory agencies, such as exposure to low and high pHs, light and oxidative reagents.[3] More emphasis may be put on some of these stress conditions if a potential degradation pathway has been identified by molecular modeling or during early experience with the material. The stresses are likely to trigger deamidation, isomerization, aggregation and oxidation of amino acid side chains. These degradation pathways are the most commonly described for mAbs.[4] A direct measurement of the effect of the degradation on potency is also taken.

Determination of viscosity and ability to concentrate formulated materials (surrogate for solubility studies). High concentrations of protein are usually required when mAb therapeutics are administered via subcutaneous injection. To verify whether the drug candidate can achieve a target concentration, the protein is concentrated to 100–200 mg/mL in standard buffers. Viscosity of the high concentration solutions is measured to evaluate purification and delivery device strategies.

mAb A case study: No major or unexpected degradation. The first case study concerns an IgG1 antibody, termed mAb A. Sequence structure analysis of the CDRs did not reveal any particularly exposed asparagine or aspartic residues in an amino acid context prone to deamidation or isomerization (i.e., located directly upstream from a glycine, serine or proline residues).[7] CDRs did not contain exposed methionine or unpaired cysteine residues. The short-term stability of early representative

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Table 2. Typical limited forced degradation conditions

Condition	Step	Duration	Tests
Low pH	Adjust pH with HCl to 3.0	48 hr	UV, SEC, IEX, peptide mapping, potency
High pH	Adjust pH with NaOH to 9.0	48 hr	UV, SEC, IEX, peptide mapping, potency
Light stress	1X ICH (UV, Visible)	N/A	UV, SEC, IEX, peptide mapping, potency
Methionine oxidation	0.05% tBHP	2–5 h	UV, SEC, IEX, peptide mapping, potency
pH jump	Coordinate with purification area	N/A	Measure A280, A320 and DLS before and after pH adjustment to pH 5.5

tBHP, tert-Butyl hydroperoxide a reagent.[5] ICH, International Conference on Harmonisation; details on guidance for Light stress can be found in ICH guideline Q1B.[6] Details of the assays used during forced degradation are reported in **Table S1**.

material was evaluated for up to three months at 2–8°C, 25°C and 40°C via a battery of assays. IEX-HPLC profiles after one and three months are shown in **Figure 1A and 1B**. Decrease of relative peak area of the main and basic species (eluting after the main) and increase of the relative peak area of the acidic variants (eluting before the main) was noted for samples stored at 40°C. A decrease of basic variants is typical for mAbs; temperature accelerates the cyclisation of N-terminus glutamine into pyroglutamic acid, a post-translational modification common to many therapeutic and endogenous human mAbs.[8] Likewise, an increase of acidic variants upon exposure to elevated temperature is a common occurrence that may reflect deamidation of asparagine residue, isomerization of aspartic residues into isoaspartic or structural changes affecting the overall surface charge distribution of the molecule.[8] In the present case, deamidation was only detected at conserved position 385 within the Fc portion of the mAb and did not exceed 10% (**Table 3**). Limited forced degradation studies at low and high pH (3.0 and 9.0) after 48 h at 40°C, resulted in pH dependant degradation routes: exposure to high pH resulted in an increase of acidic variant (and deamidation of position 385), while low pH resulted in the formation of basic species not identified by peptide mapping (**Fig. 1C**, **Table 3**). Exposure to other forms of stress was performed and did not result in unexpected alterations of the primary, secondary or tertiary structure of the molecule. Exposure to UV/visible light following ICH guidelines, for instance, resulted in the formation of a basic species reflecting oxidation of conserved Fc methionines 253 and 429 (**Fig. 1D**, **Table 3**). Oxidation of the conserved Fc methionine residues under these conditions was expected and documented in the literature.[4] While developability studies of mAb A did not identify major chemistry, manufacturing and control (CMC) challenges, the outcome of these studies provided information about primary degradation pathways and potential critical quality attributes (CQA) for the molecule.

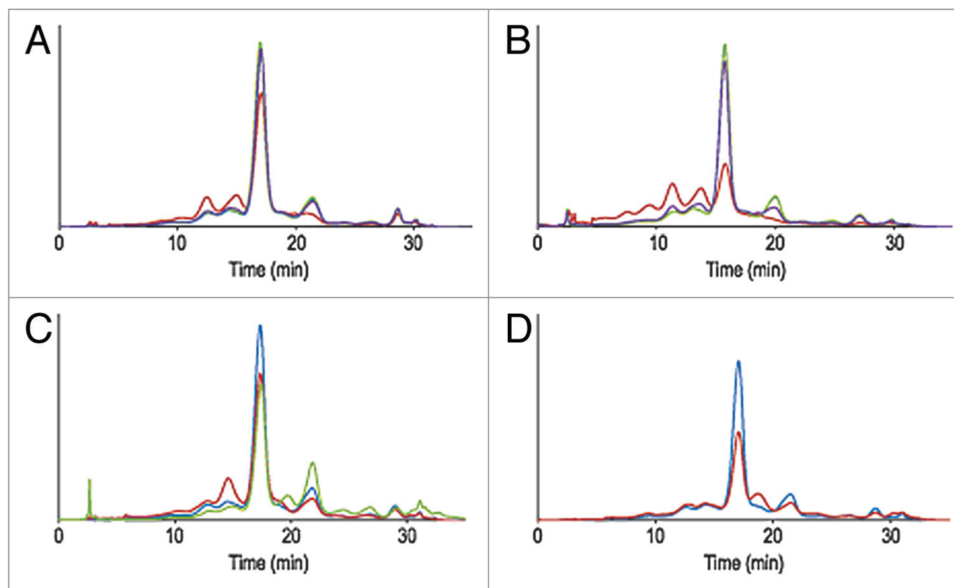

Figure 1. Overlay of IEX-HPLC chromatograms of mAb A samples stored for one month (**A**) and three months (**B**) at −80°C, 2–8°C, 25°C and 40°C. Overlay of IEX-HPLC chromatograms of mAb A samples challenged with high and low pHs for 48 h at 40°C (**C**) and light induced (0.2xICH) stress (**D**).

Table 3. Relative amounts of PTMs in mAb A by LC-MS peptide mapping

Storage Temperature	Met 253 ox	Met 429 ox	N385D
Initial	7%	4%	4%
1 mo time point			
5°C	8%	4%	5%
25°C	8%	4%	5%
40°C	9%	4%	6%
3 mo time point			
5°C	8%	4%	8%
25°C	7%	5%	8%
40°C	21%	4%	8%
Limited forced degradation			
pH 3.0/40°C	8%	4%	14%
pH 9.0/40°C	8%	4%	7%
Photostress	22%	17%	5%
Dark Ctrl	7%	4%	6%

Met253ox and Met429 ox refer to methionine oxidation position 253 and 429, respectively. N385D refers to relative amount of aspartic acid at position 385. Photostress conditions were 0.2x ICH, Dark Ctrl was the

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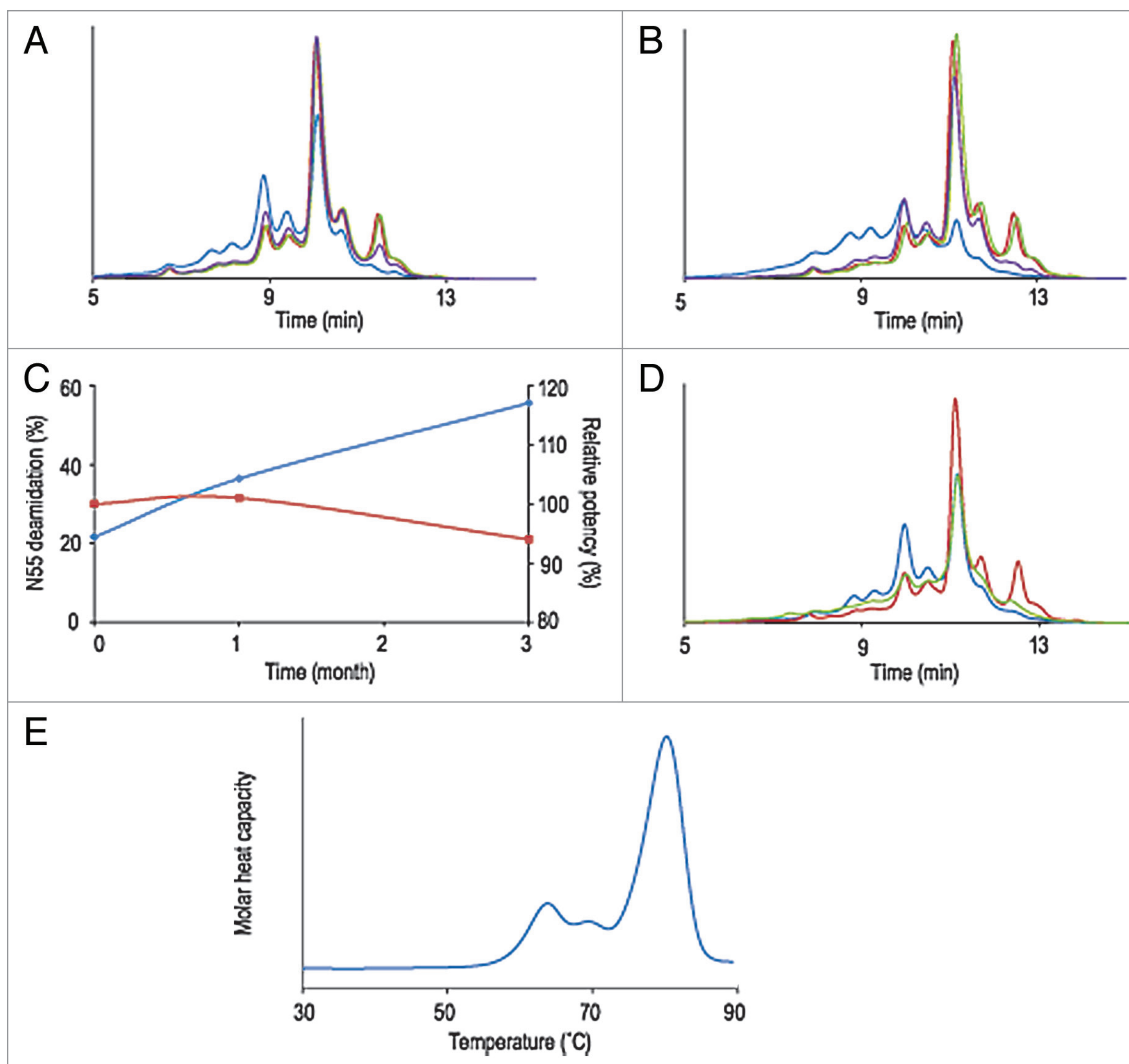

Figure 2. Overlay of IEX-HPLC chromatograms of mAb B samples stored for one month (**A**) and three months (**B**) at −80°C, 2–8°C, 25°C and 40°C. Evolution during stability studies of relative amount of deamidation at HCAsn55 (N55, blue trace) and mAb B potency (red trace) (**C**). Overlay of IEX-HPLC chromatograms of mAb B samples stored for one month at 40°C at pH 4.5 and pH 6.0 (**D**). Thermal unfolding profile ofsolution of mAb B (**E**).

mAb B case study: Identification of degradation pathways informs appropriate control strategy. The second example of developability studies involves a S228P hinge-modified IgG4 molecule, termed mAb B. Scanning of the primary sequence for exposed asparagine, aspartic acid, tyrosine, tryptophan and methionine residues did not predict the presence of particularly sensitive hot spots. Results from the short-term stability studies, however, show a collapsed IEX-HPLC profile after three months at 40°C and a significant increase of acidic variants at 25°C and essentially no changes when stored at 2–8°C or frozen (**Fig. 2A and B**). No increase of aggregates or fragments resulting from chemical proteolysis was detected by SEC-HPLC (data not shown). Peptide mapping and LC-MS analysis indicated that deamidation of heavy chain Asn55 exceeded 50% and thus was largely responsible for the instability of the molecule (**Table 4**). Other expected post-translational modifications, such as oxidation of the conserved Fc methionine residues were also detected, albeit to low levels. Testing of relative potency by binding ELISA showed that, in spite of the chemical degradation, mAb B retained its complete ability to bind its target antigen (**Table 4**, **Fig. 2C**). Lowering the pH of the mAb B formulation from 6.0 to 4.8 reduced greatly the amount of deamidation after one month at 40°C (**Fig. 2D**). For this particular molecule, deamidation at Asn55 was recognized as a potential CQA. A proper control strategy involving monitoring deamidation during upstream and down stream process development and manufacturing was put in place. Efforts toward defining an adequate formulation and storage conditions of the drug substance were made to ensure protein stability and the longest possible product shelf-life. mAb B's desirable commercial drug product form is a highly concentrated solution for subcutaneous injection. Part of the developability exercise is to verify that the protein can remain in solution at concentrations above 100 mg/

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