Chapter 2
Molecule and Manufacturability Assessment Leading to Robust Commercial Formulation for Therapeutic Proteins

Ranjini Ramachander and Nitin Rathore

Abstract The transfer of lead molecules from discovery into process development at a relatively fast pace requires a process of candidate selection that assesses if a candidate is not only active and safe but also "manufacturable." Formulation and process stability of potential candidates help narrow down lead candidates at an early stage, prior to large-scale manufacturing, by a process of rank-ordering properties generated from process and long-term stability studies. Such an assessment of the molecules' manufacturability is especially useful when binding affinity and bioactivity are comparable among the various candidates under question. This chapter reviews several case studies that explore the utility of early-stage molecule or manufacturability assessments in moving forward therapeutic candidate/s by finely balancing potency and pharmacokinetics with the manufacturing capability of the candidate/s under question.

2.1 Introduction

Molecule and manufacturability assessments are key components of commercial formulation development. Molecule and formulation selection based on manufacturability assessment is critical to designing robust drug product formulation. Molecule assessment involves work that includes assessing sequence attributes for product quality, robust cell line expression, desirable purification properties that include lesser propensity to aggregate under process conditions, and the evaluation of physical and covalent stability of biotherapeutics under formulation and storage

R. Ramachander (✉)
Product Attribute Sciences, Amgen, Inc., Thousand Oaks, CA, USA
e-mail: ranjini.ramachander@gmail.com

N. Rathore
Drug Product Technology, Amgen, Inc., Thousand Oaks, CA, USA

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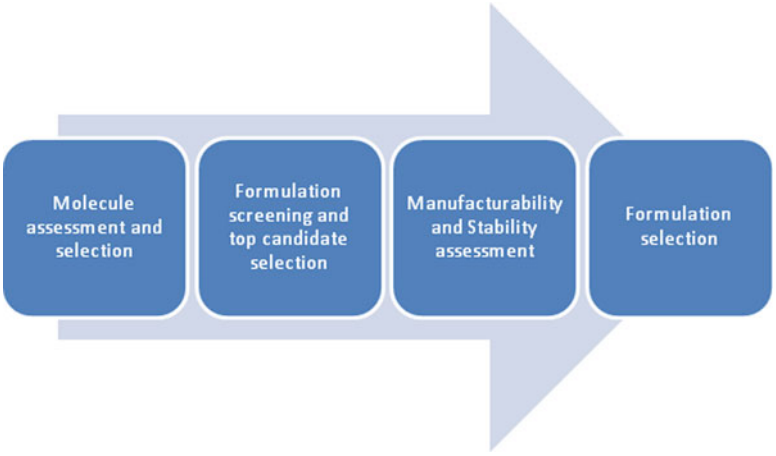


Fig. 2.1 Steps in molecule and manufacturability assessment process leading to selection of robust formulation

conditions (Narhi et al. 2010). These activities typically commence on a set of candidates that are initially chosen or narrowed down based on favorable target binding or bioactivity and pharmacokinetic considerations. Upon assessment and mitigation of potential unfavorable sequences that may result in product heterogeneity, optimal cell expression, and protein purification yield, the molecules are subject to several stability studies. These studies are not only designed to predict stability in the long term but oftentimes used to also define the formulation conditions to store the therapeutics in. The top formulation candidates can then be subjected to stress conditions representative of the manufacturing process to select the final formulation. Small-scale studies are often employed to conduct such manufacturability assessments. This kind of early evaluation is the holy grail of quality by design (QbD) where prior knowledge of the evaluated therapeutic is used to design more robust molecules with the desired properties and characteristics (Rathore and Winkle 2009).

Figure 2.1 provides an outline of the steps leading to robust formulation selection. This chapter will discuss the molecule assessment approach in detail. Case studies related to manufacturability assessments are also included.

2.2 Molecule Assessment

2.2.1 Protein Sequence Analysis and Assessment of Product Quality

In addition to maintaining product homogeneity for the therapeutic of interest, accurate determination of molecular weights and modifications is also highly desired. Uncommon framework sequences and non-germ line mutations that affect expression

are identified, and appropriate engineering is carried out on these residues. Sequences that show a greater propensity for chemical or covalent modifications are also mutated to less labile amino acids especially if they decrease stability or result in loss of activity. Further, residues that have a greater propensity to increase deamidation/oxidation are also identified and mutated if needed from a product quality standpoint or due to their possible detrimental effect on activity. Specific examples will be discussed under chemical stability in the case studies below.

2.2.2 Assessment of Physical Stability

Upon the completion of sequence analysis and engineering, candidates are moved forward based on desirable upstream expression and favorable downstream purification properties such as stability to common process conditions, including low pH for viral clearance for mammalian-derived proteins, exposure to denaturing buffers during chromatography steps, and resistance to formation of high molecular weight species (HMWS). The purified candidates are assessed for both physical and chemical stability. Depending on the molecular modality of the protein, physical and chemical stability assessment may also involve deducing the appropriate storage formulation buffer after purification in addition to understanding relative stabilities in different formulations under accelerated conditions.

2.2.2.1 Recommendation of Initial Formulation for a Single Candidate: Assessment of Stability at Different pH Conditions

Short-term and long-term formulation stability of the therapeutic or candidates is assessed by subjecting them to accelerated temperature stress in appropriate formulation conditions over time. Several assessments including melting temperature determination to predict long-term storage stability, presence of HMWS and clipped species, and other degradation products can be carried out. The candidates and formulations are then rank ordered based on predetermined stability criteria.

A formulation stability assessment was carried out on Fc-fusion protein Y in several potential formulations over a wide pH range due to lack of prior knowledge of stability at different pH conditions. Based on the stability results at 25 °C over a month, an appropriate storage formulation was recommended for further stability studies.

Figure 2.2 shows a comparison of the percent increase in HMWS generated under different buffer pH conditions at accelerated temperature for Fc-fusion protein Y.

It was seen that the greatest increase in HMWS was in the pH 4.8 buffer over a month long period, followed by pH 7.4 buffer. This Fc-fusion protein showed the greatest stability in the pH 8 buffer which was thus recommended as the ideal buffer to store the protein after purification.

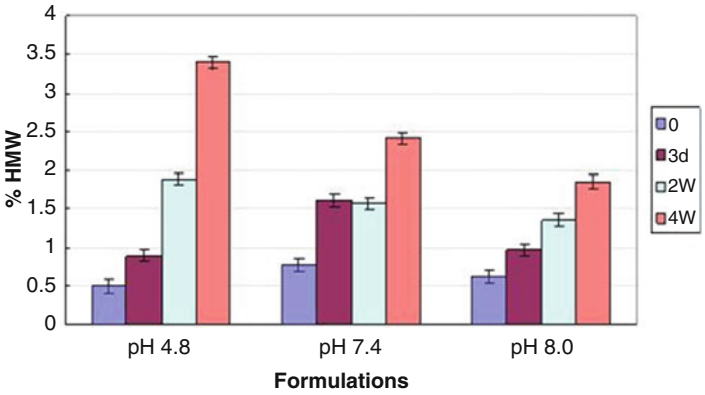


Fig. 2.2 Percent high molecular weight species (HMWS) formation over 4 weeks at different pH conditions at 25 °C for Fc-fusion protein Y. At each pH condition, the Fc-protein was assessed up to 4 weeks

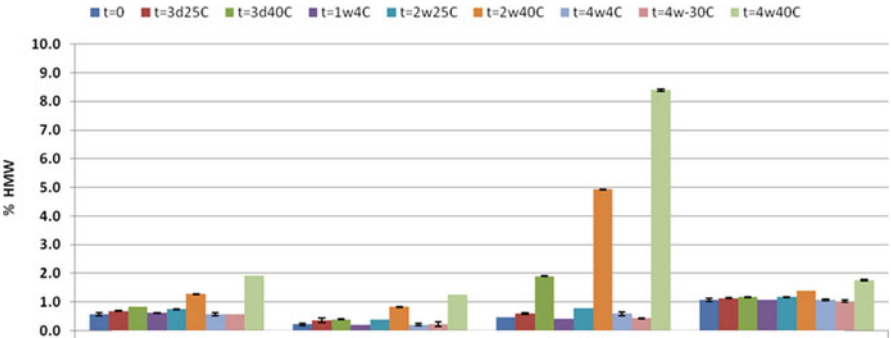
2.2.2.2 Stability of Several Candidate Molecules at a Single pH

Assessments are oftentimes also carried out across several candidates, especially antibodies or molecules with established stability in a standard storage buffer. These assessments rely on the fact that protein therapeutics generally have established storage stability in buffers and stability assessments for these buffers are made by accelerated temperature stress. Accelerated temperature stability experiments are carried out at 40 °C for more stable therapeutics to predict impact of long-term stability.

Figure 2.3 shows a comparison of the stability of different monoclonal antibody candidates at accelerated temperatures of 25 and 40 °C. Of all four mAb candidates evaluated, candidate 3 showed the highest HMW formation under the conditions tested, followed by candidate 4. In other experiments, candidate 1 also showed significant aggregation during low pH purification on a protein A column indicating process instability which was also reflected in a short-term stability study of the purified protein (data not shown). Candidate 2 was therefore selected as the lead for further development.

2.2.2.3 Stability to Physiological pH Conditions

The effect of the transition from formulation pH to physiological pH conditions is monitored by assessing the change in pH of the therapeutic going from actual formulation pH to that for phosphate buffer saline (PBS) (pH 7.4). The effect of this transition on precipitation or the formation of oligomers is monitored using a variety of tools including visual assessment, size-exclusion chromatography (SEC), and dynamic light scattering (DLS). The change is monitored in PBS at 37 °C over a



	Candidate 1	Candidate 2	Candidate 3	Candidate 4
t=0	0.6	0.2	0.5	1.1
t=3d25C	0.7	0.4	0.6	1.1
t=3d40C	0.8	0.4	1.9	1.2
t=1w4C	0.6	0.2	0.4	1.1
t=2w25C	0.7	0.4	0.8	1.2
t=2w40C	1.3	0.8	4.9	1.4
t=4w4C	0.6	0.2	0.6	1.1
t=4w-30C	0.6	0.2	0.4	1.0
t=4w40C	1.9	1.3	8.4	1.8

Fig. 2.3 Four monoclonal antibody (mAb) candidates were evaluated for their stability towards aggregation as a function of temperature and time. mAb candidate 3 shows a greater rate of HMWS formation of about 8 % compared to the other candidates over the same period of time at 40 °C. Candidate 2 showed the least amount of HMWS followed by 1 and 4

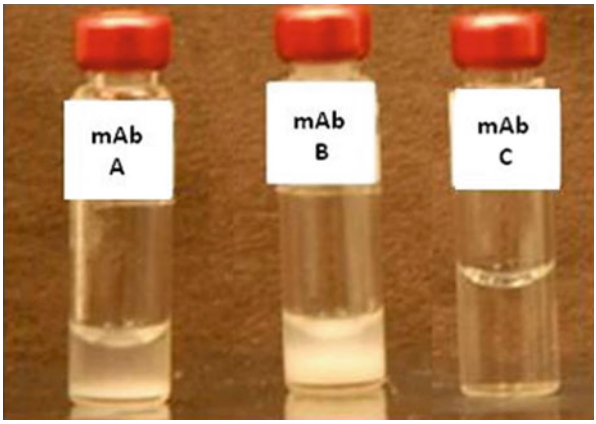


Fig. 2.4 Monoclonal antibodies A and B show visible precipitation upon transition from formulation pH of 5 to PBS at pH 7.4; mAb C remains clear even after 24 h

period of several hours (up to 24 h). Figure 2.4 shows the effect of the pH change on three antibody therapeutics, mAbs A, B, and C. It was seen that this pH change caused precipitation in two antibodies (A, B), while no visual change was observed for mAb C. Interestingly, SEC did not show significant change in smaller oligomers for any of these three antibodies (data not shown).

2.2.2.4 Stability to Low pH Conditions

Relative stability of therapeutic candidates is also assessed based on their ability to withstand purification process conditions. Low pH stability of proteins, particularly monoclonal antibodies, is determined by analysis of HMWS formation during elution from the protein A column and viral inactivation steps. This assessment is performed both during real-time purification and under accelerated conditions that simulate process excursions. Samples are checked for turbidity, and SEC is carried out to look for the presence of oligomers and other HMWS. For molecules that show propensity to aggregate under process conditions, structural integrity of these therapeutics is determined using biophysical techniques such as Fourier transform infrared (FT-IR) spectroscopy, circular dichroism (CD) spectroscopy, fluorescence spectroscopy, and differential scanning calorimetry (DSC). The reversibility of these changes is also determined. Criteria are set to eliminate candidates based on the amount of HMW species or aggregates obtained at the various process steps (data not shown). Only candidates that display robustness towards these conditions are moved forward through the pipeline (Ramachander 2008; Jiang et al. 2008).

2.2.3 Solubility and Viscosity

Subcutaneous administration of high-concentration antibody therapeutics is desirable in order to minimize the volume injected in a single delivery. This often can result in significant challenges achieving the right viscosity for administration, targeted solubility at concentrations as high as 70–150 mg/mL, and overall stability under high-concentration formulation conditions. As part of screening therapeutic candidates during molecule assessment, solubility and viscosity are determined at a series of concentrations up to about 150 mg/mL. Molecules exhibiting very high viscosities are flagged and given a lower rank compared to other candidates with lower viscosities (Yadav et al. 2011). Shear-independent viscosities of candidates are initially screened using a plate-based DLS method followed by a shear rate-dependent viscosity method (typically a cone and plate method).

Figure 2.5 shows a plot of viscosities of a number of monoclonal antibodies as a function of concentration. mAbs A, B, and C show significantly high viscosity compared to the others and were flagged to indicate the possibility of greater challenges during manufacturing and delivery.

2.2.4 Stability to Agitation-Induced Particulation

Particulation or aggregation due to agitation can be encountered during fill-finish operations or during transportation (Kiese et al. 2008). Potential candidates are screened for their propensity to particulate under agitation by subjecting them to

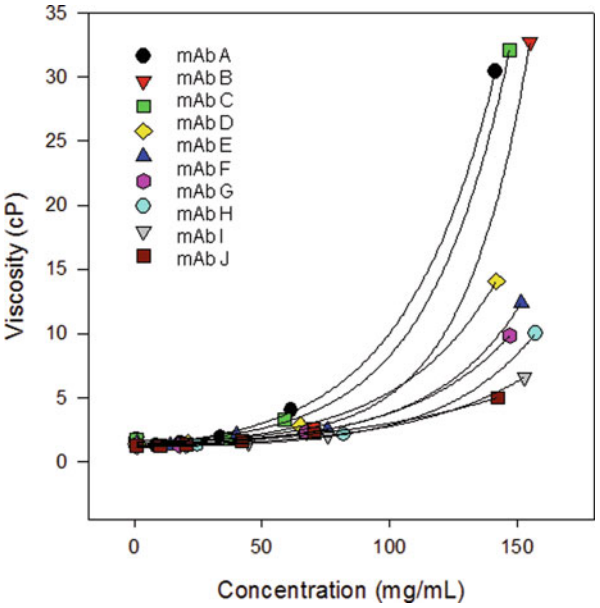


Fig. 2.5 Viscosity as a function of protein concentration was determined for several monoclonal antibodies, mAbs A–J

shaking stress on a tabletop orbital shaker at high speed over a significant period of time (up to 3 days). Samples are then observed for visual precipitation and tested by SEC for observing oligomers formation and DLS to look for a qualitative size distribution.

Figure 2.6 shows the precipitation of mAbs B and C under agitation stress, whereas mAb A remains clear even after 3 days. SEC of the precipitated mAbs also showed a greater increase in HMWS, and DLS showed a significant increase in light scattering corresponding to the larger species present in the sample (data not shown). Molecules with greater propensity to aggregate upon agitation are flagged for potential concerns during formulation development, and other candidates with desirable manufacturing properties are recommended.

2.2.5 Chemical Stability

Chemical degradations of monoclonal antibodies such as deamidation, isomerization, methionine and tryptophan oxidation, and succinimide formation in the complementarity determining regions (CDRs) regions may possibly lead to decreased potency of antibody therapeutics. Several of these "labile" sites may be modified or as part of the molecule assessment or the therapeutic candidates can be assessed for chemical modifications in the appropriate formulation buffer/s by subjecting them to accelerated conditions under which modifications such as deamidation, oxidation, or isomerization can be detected. While considering the various covalent

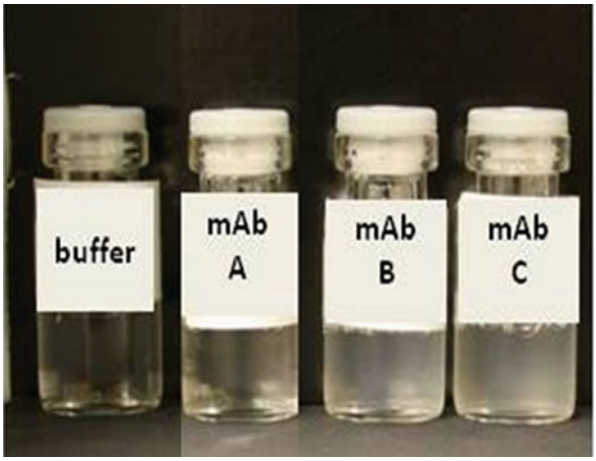


Fig. 2.6 Monoclonal antibody (mAb) A shows no precipitation while mAbs B and C show visible precipitation upon agitation-induced particulation

modification pathways, it appears that asparagine deamidation and aspartate isomerization in the CDRs are significant routes of degradation. One option that is commonly adopted is to substitute the asparagine of interest into another amino acid while still preserving antigen binding and not introducing any new routes of degradation.

An illustrative example is the study of panitumumab (antiepithelial growth factor receptor (EGFr) immunoglobulin 2 (IgG2) antibody) by Rehder et al. (2008), which contains an aspartate at position 92. Upon long-term storage, this aspartate was found to isomerize to iso-aspartate, which was captured as a separate peak in a reduced/alkylated reverse-phase assay that these authors developed, in conjunction with ion-trap mass spectrometry. The isomerization phenomenon increased with higher temperature and lower pH. These authors also developed an in vitro size-exclusion-based antigen-binding assay, which showed that a single molecule was able to bind to two EGF receptors, indicating that each antibody arm participated in binding one EGF receptor. Their data indicated that the isomerization event deactivated the binding reaction. Thus, the intact antibody was able to bind two EGF receptors, while panitumumab with one arm isomerized only bind one EGF receptor, and panitumumab with both arms containing iso-aspartate did not bind to EGF at all.

In another study, Nakano et al. (2010) detected an asparagine isomerization event in an anti-glypican 3 IgG1 antibody. This asparagine was found to deamidate significantly due to being present in an asparagine-glycine sequence. Since deamidation is highest in asparagine-glycine and asparagine-serine sequences, it was decided to mutate the glycine, and not the asparagine, to mitigate the problem. In this case, the authors mutated the glycine to an arginine which substantially reduced deamidation rates while still preserving the antigen-binding capability due to the continued presence of the asparagine residue in the mutated antibody as well.

It is thus recommended that antibodies with the instabilities mentioned above should be given lower priority as a clinical candidate. One option to mitigate such issues is by sequence engineering and applying structural analysis to generate

antibodies that do not contain residues which are prone to modification. In such cases, a backup antibody candidate could be designed that avoids or minimizes such tendencies. This area is still new and upcoming, and the effects of specific chemical modifications on potency, aggregation propensity, and immunogenicity are being intensely evaluated in the industry.

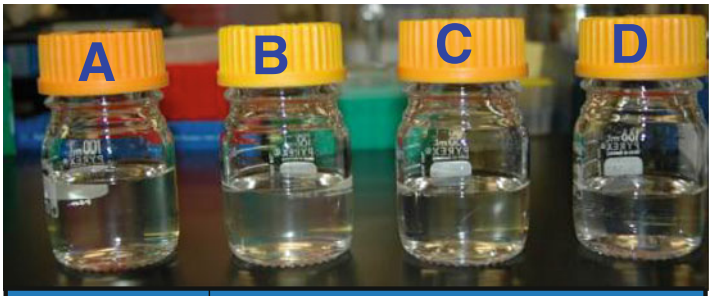
2.3 Manufacturability Assessment

Once the optimal molecule is selected, formulation screening experiments are conducted to identify the buffering conditions, ionic strength, and product concentration suitable for product stability in the desired primary container. Design of experiments (DOE) approach can be used for defining and characterization of formulation design space including identification of critical factors impacting product stability and the interactions among them. A series of forced degradation studies and DOE can be used to demonstrate the robustness of protein formulation under long-term storage (Grillo 2010).

Manufacturability assessment provides a mechanism to rank top formulation candidates for their robustness against stresses associated with manufacturing, storage, transportation, and end use. Bench-scale studies can be conducted to more closely simulate the stresses that are associated with the manufacturing process and are known to impact product stability. This requires identifying the key process parameters suspected to impact product quality and simulating them on a small scale. The following sections present some examples of such lab scale assessments.

2.3.1 Freeze–Thaw

Freezing of drug substance for long-term storage is a standard practice widely used in the industry for the obvious advantage it present in terms of product stability and manufacturing flexibility. However, freeze and thaw steps themselves could bring stresses that could potentially impact product quality. Chapter 7 discusses these stresses in details. Freezing of drug substance in large volume containers could result in cryoconcentration due to the exclusion of solutes from the ice-water interface (Singh et al. 2009; Lashmar et al. 2007). Such concentration gradients can be further amplified during the thaw step due to the migration of ice to the top of the container. For example, static freeze–thaw in a 10 L container showed protein concentration can increase by more than 50 % at the bottom of the container. At the same time protein and excipient concentration at the top of the container are reduced to half of the starting composition. Product destabilization may occur due to extended exposure of protein molecules to an environment significantly different from the recommended formulation conditions. The extent of gradient formation and the resulting impact on product quality attributes could be scale specific. Stability studies conducted at very small scale (e.g., vials) may not capture the full



Formulation Candidate	HIAC count (cumulative/mL)				
	2 µm	5 µm	10 µm	25 µm	50 µm
A	57	17	2	0	0
B	15588	3208	68	7	0
C	37	12	5	0	0
D	47	25	13	3	0

Fig. 2.7 Subvisible particle data and visual observations for post-freeze–thaw samples collected for four different formulation candidates. Formulation B exhibited higher turbidity as well as elevated particle count

commercial scale freeze–thaw 10 L carboys. It is therefore important to assess the stability in more realistic conditions. However, large amount of bulk is usually not readily available during the early stage of product development. An intermediate scale using a container made of representative material offers a reasonable mechanism to assess product sensitivity to freeze–thaw stresses and rank the top formulation candidates. Figure 2.7 shows the outcome of such an evaluation where freeze–thaw stability of four formulation candidates was evaluated at a scale of one liter. Unlike the freeze–thaw in vials where no impact was observed, results show that one of the formulation candidates experienced significant destabilization resulting in protein aggregation and formation of subvisible particulates. For controlled rate technologies such as Celsius Pak systems and CryoVessels, appropriate scaled down models are available that ensures heat transfer path length and freeze–thaw durations are consistent between small and large scale (Lashmar et al. 2007; Kolhe et al. 2009; Jameel et al. 2010). This provides the flexibility to conduct product quality assessments at lab scale using very limited material.

2.3.2 Filtration

After the bulk drug substance has been formulated, it goes through filtration step(s) that assures sterility of the filled drug product. Design of a robust fill-finish process requires understanding of the filterability of drug substance as well as the impact of

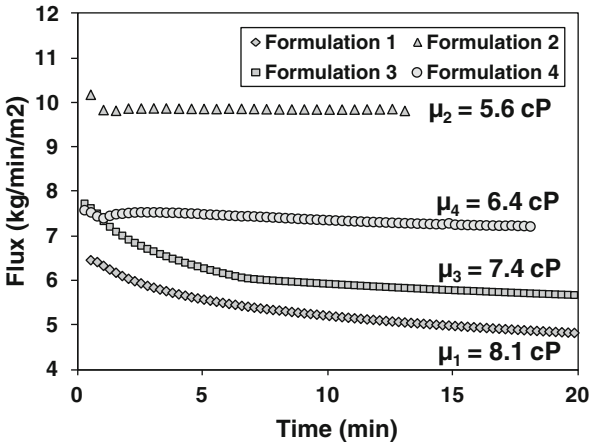


Fig. 2.8 Filtration flux as function of filtration time for four different formulations for a monoclonal antibody. The starting flux decreases with increase in viscosity; the decay in flux is specific to formulation candidate and directly impacts filtration time

filtration on drug product quality attributes. Such filterability evaluations can be conducted on small scale using filters of representative geometry and membrane but with smaller filtration area in order to lower the material demand. Various product attributes including viscosity, subvisible, and visible particle load impact the filtration process performance. Early during formulation development, bench-scale experiments can provide relative comparison for different formulation candidates. Filtration laws and mathematical models such as V_{max} methodology (Badmington et al. 1995) can also be used to estimate filter capacity and perform process scale-up for commercial scale.

Figure 2.8 shows filtration flux data for a monoclonal antibody in four different formulation buffers. The buffer excipients govern the viscosity of the final formulation as well as the particle load. The starting filtration flux is inversely related to solution viscosity, with less viscous solutions having higher starting flux. The particle load present in the protein solutions can also potentially plug the filter pores and result in flux decay during the course of filtration. Formulations 1 and 3 show significant flux decay in comparison to formulations 2 and 4, suggesting elevated levels of particle loads. Filtration models can be utilized to estimate the filter capacity for these candidates and rank them based on the expected process performance requirements (batch size, filtration time) for the large-scale process. In addition to filter capacity assessments, small-scale filtration experiments also help to assess impact of multiple filtration steps on product quality and to estimate any potential losses in protein or excipient concentrations due to membrane binding (Rathore and Rajan 2008).

Sections 3.1 and 3.2 provide two examples of commonly used small-scale manufacturability assessments and their application to ranking of formulation candidates. Similar studies can be designed to simulate other manufacturing stresses. Bench-scale filler assessments provide a mechanism to assess impact of fill operation on product quality. Recirculation of monoclonal antibody solution through a piston pump has been shown to contribute to higher turbidity as well as increase in subvisible particles (Cromwell et al. 2006; Nayak et al. 2011). Simulated transportation

studies that expose product to a range of temperature and pressure variations can be used to assess risk of product quality impact during actual shipments. Photosensitivity studies can be designed to mimic the exposure to light conditions that are representative or worse than the levels experienced in the manufacturing site and during end use.

2.4 Summary

Early-stage candidate screening during molecule assessment is one of the first steps in understanding the feasibility of manufacturing a therapeutic with the desired attributes. Establishing in vitro and in vivo potency and favorable pharmacodynamic attributes is key to every therapeutic program followed by high cell expression and a purification process that results in folded protein with minimal aggregates. Subsequently, molecules are evaluated for both short- and long-term stability that includes both storage and transportation stresses in addition to the effect of chemical modifications on stability, potency, and immunogenicity. Small-scale studies also provide mechanism to assess product stability against stresses associated with manufacturing, shipment, and storage. Candidates are rank ordered, eliminated, or moved forward based on these assessments for further scale-up and downstream processing. The predictability of these small-scale assessments during scale-up is currently being established. Most stability parameters at the early-stage assessment have been used to develop or streamline downstream formulation and scale-up processes. The desire to move a therapeutic with favorable potency and pharmacokinetics over the difficulties of processing the molecule with significant aggregation will have to be balanced by assessment of risks such as immunogenicity in administering such a therapeutic to a patient versus superior bioactivity.

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