Biologics: Targets and Therapy Dovepress

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Biophysical Analysis of Therapeutic Antibodies in the Early Development Pipeline

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Abstract: The successful progression of therapeutic antibodies and other biologics from the laboratory to the clinic depends on their possession of "drug-like" biophysical properties. The techniques and the resultant biophysical and biochemical parameters used to characterize their ease of manufacture can be broadly defined as developability. Focusing on antibodies, this review firstly discusses established and emerging biophysical techniques used to probe the early-stage developability of biologics, aimed towards those new to the field. Secondly, we describe the inter-relationships and redundancies amongst developability assays and how in silico methods aid the efficient deployment of developability to bring a new generation of cost-effective therapeutic proteins from bench to bedside more quickly and sustainably.

Keywords: developability, biophysics, protein, antibody, analytical techniques

Introduction: The "Biologics Era" and the Drug Discovery Pipeline

Therapeutic proteins, or biologics, interact with their targets with a different mechanism of action and often greater specificity than their small-molecule counterparts, giving rise to fewer side-effects.[1] These features have thus made the development of proteins as therapies desirable over the past few decades. The advent of recombinant DNA technology in the 1970's and 1980's brought the biopharmaceutical industry into its modern era.[2–5] Prior to this, therapeutic peptides and proteins derived from animal[6] or donor sources (eg insulin[7] and blood factors[8]) were used to treat patients deficient in these vital macromolecules.[5] A further vital breakthrough was the development of hybridoma technology by Köhler and Milstein,[9] and using this approach, the first monoclonal antibody (mAb) therapy (Orthoclone®) was approved by the Food and Drug Administration (FDA) in 1986.[10] Since then, many technological breakthroughs have been made,[11] including a variety of display methods, (such as phage-[4] and ribosome display[12]) that now allow the routine and efficient identification of highly avid mAbs.[13,14] Additionally, mAbs have additional modes of action (effector function) afforded to them through their crystallizable fraction (Fc) region; the isotype of the antibody can modulate its interaction with different receptors, eg, neonatal receptor allows for "recycling" of the molecule from the serum giving a long half-life[15] or interaction with Fcγ receptor IIIa to trigger antibody-dependent cellular cytotoxicity.[15,16] By contrast, peptide therapeutic agents lack such diverse modes of action nor a means of extending their half-life; unnatural amino acids or chemical modifications are necessary to improve this. For example, the blockbuster type-2 diabetes and anti-obesity drug, Ozempic® has two amino acid substitutions/modifications (Ala8 to α-aminoisobutyric acid and Lys34 to Arg) in addition to conjugating a C18 fatty acid via a hydrophilic linker to the ε-amino group of Lys26 in the semaglutide peptide. This increases its half-life by several days compared to the wild-type peptide.[17,18]

High target affinity, however, is only one feature that is required for a successful mAb therapeutic to transition from "bench to bedside" (Figure 1).[19] These additional properties, identified over the last forty years include specificity, potency and manufacturability, and are probed using a "toolkit" of different techniques.[20] The process of identifying whether any initial "hit molecules" possess the critical quality attributes required for the economic manufacture of an

Received: 12 October 2024
Accepted: 10 December 2024
Published: 21 December 2024

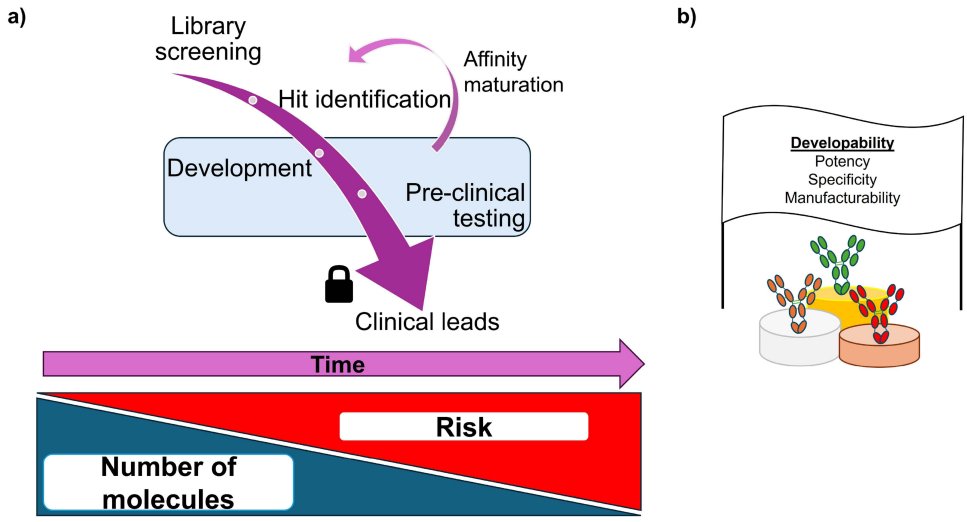

Figure 1 Overview of the drug development pipeline. (**a**) Screening libraries of molecules to identify hits against the target(s) of interest will whittle the number of molecules down from several thousand to tens of molecules. An array of developability assays will be employed to interrogate these hits, with the most successful undergoing further tests to ascertain if they will progress to the clinic. These clinical leads have their sequence locked at this point, with late-stage formulation deciding the dosage and delivery method. Low quantities of material are produced (tens of milligrams from tens–hundreds of mL of cell culture) for early development, increasing to gram and kilogram scale (from thousands of liters of cells) at the clinical end. The cost of clinical trials (both financially and in terms of patient benefit) means that increased risk is attached to molecules that reach the clinic, thus developability assays and the data they generate are so important. The blue region represents the scope of this review. (**b**) Ultimately, one aims to use developability assays to find potent molecules, with no off-target interactions that can be made easily, at scale and administered safely into patients.

efficacious and safe medicine (Figure 1b) is known as "developability".[21–23] The varied physicochemical profile of a molecule needs to be evaluated using multiple different methods, with each method probing (mainly) one specific feature, eg, hydrophobicity Figure 1. Technological advancements in instrumentation, computation and fundamental science have meant that this toolkit is always changing in terms of size and contents.[20]

In this review, we survey the array of biophysical techniques used in early-phase drug discovery to aid the selection of developable biopharmaceuticals. Focusing on antibody-based therapies, commonly used experimental and in silico methods are discussed, prefaced with a simple overview of how they work. New techniques are then highlighted together with the potential of machine learning and artificial intelligence to exploit the ever-increasing quantities of training data, which together may rationalize the developability framework. This is of increasing importance as a wide range of next-generation therapeutics based on mAb scaffolds with more challenging biophysical properties for manufacturing are entering development.[24] This is a challenging yet exciting time, with biophysical characterization fundamental in the development of the next-generation of therapeutic proteins.

Folding and Functionality: From Conformational to Colloidal Stability

Before we look at the methods used to analyze therapeutic proteins, it is important to consider the species one needs to analyze in term of their size, abundance and stability (thermodynamic and kinetic); all of which can be modulated by their physico-chemical environment. We define the native state as the correctly folded three-dimensional structure of protein through which desirable biological function is achieved (Figure 2). Given its functional importance within the context of the biologics sphere, the characterization of this state, excursions from it and the parameters that affect it are a key part of drug developability screening.[25,26] It should be noted that these principles apply to all globular proteins, not just therapeutic mAbs.

Relatively harsh conditions (eg extremes of pH,[27–29] temperature[30–32] or in the presence of chaotropic denaturants such as urea,[33,34] guanidine hydrochloride[34,35] or organic solvents)[36,37] will bring about the global unfolding of a protein (Figure 2). Unfolded proteins can self-associate and aggregate due to exposure of previously buried hydrophobic residues to the solvent. There is a consensus in the literature[25,38,39] that for biopharmaceuticals, most aggregation takes place from

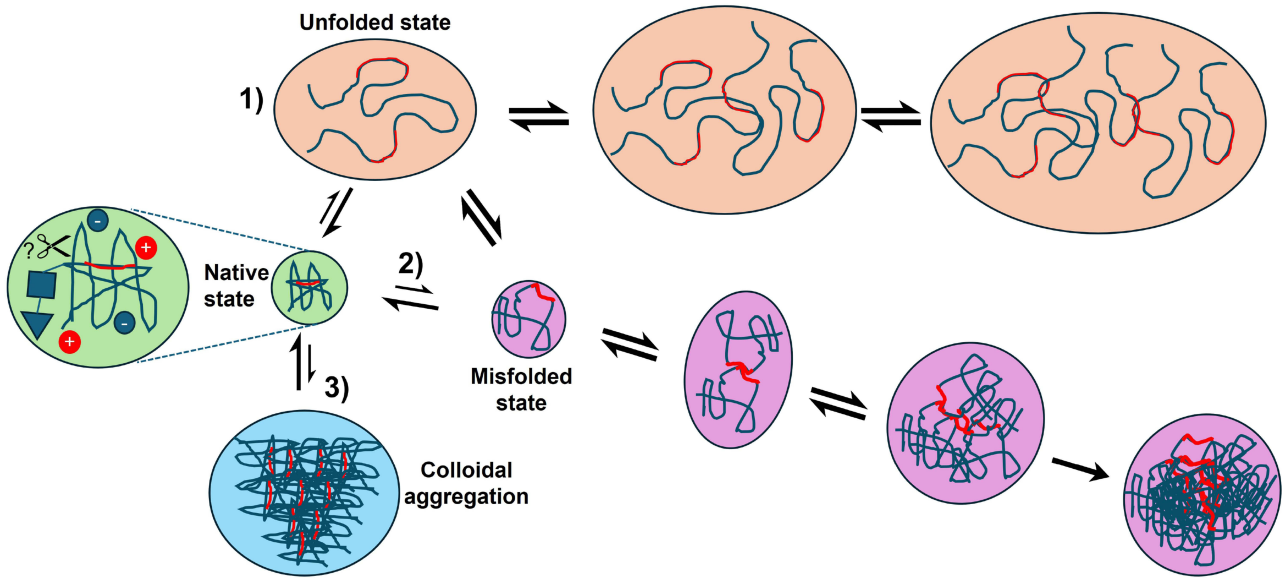

Figure 2 Overview of the aggregation pathways of biopharmaceuticals. Most therapeutic proteins are folded into a defined 3D-structure (green circles). The native protein's surface charge, dynamics, amenability to cleavage by proteases (for example) are all important for it to maintain this structure. Extremes of pH or temperature may bring about the complete unfolding of a protein, leading to aggregation via its exposed aggregation-prone regions (in red) via pathway 1. Along pathway 2 (purple route), partial unfolding of the native state (or misfolding from 1) results in an aggregation-prone misfolded state, with these species able to multimerize and eventually precipitate from solution. Finally, it is increasingly clear that some proteins can reversibly self-associate into crystalline precipitates or a liquid-like phase (colloidal aggregation, blue circle). The length- and timescales of all of the above mean a wide range of assays are necessary to probe the states above and their interconversion.

a misfolded state, brought about by perturbations to the native state by physical, chemical or mechanical means. For this review, we define an aggregate as a proteinaceous entity comprising two or more mis- or un-folded species.[40]

Colloidal stability refers to the dispersity of the sample, ie, a low dispersity sample contains species of mainly one size, eg, monomers, whereas a highly disperse sample would contain monomers and aggregates over a broad range of size/length scales.[41,42] While it is established that under certain conditions, proteins can self-associate into a solid-state crystal lattice (ie native state aggregation),[43] many have shown that proteins can self-associate and undergo liquid–liquid phase separation (Figure 2).[44–46] While crystallization is being explored as an alternative means of purifying proteins at scale,[47] both of these aggregation phenomena are generally deleterious for mAbs during production.

In-Silico Analysis of Therapeutic Protein Sequences: Identifying Risks and Liabilities

A plethora of computational methods have been developed to aid the development of therapeutic proteins (Figure 3).[48–50] Generally, these algorithms use a sliding window of primary sequence to compute an output score describing a certain biophysical property such as net charge or hydrophobicity on a "per residue" basis or averaged over the entire protein.[51] Experimentally relevant conditions can often be fed into the algorithms to tune the output, eg, changing pH or ionic strength, which are known to influence aggregation kinetics by affecting electrostatic interactions between proteins.[25] These algorithms can be applied to either the primary sequence or higher-order protein structures, allowing the structural context of problematic sequences to be taken into account with structures derived either experimentally or from homology models using programs such as MODELLER,[52] and ABodyBuilder.[53] Sequences or regions of protein, which promote aggregation or have low solubility, for example, can thus be identified, aiding the redesign of protein sequences or the selection of more developable candidates. Most algorithms developed to probe this have been trained on intrinsically disordered amyloidogenic peptides, eg, TANGO[54] and AGGRESCAN;[55] however, some of these have been adapted or developed specifically to look at therapeutic proteins, including antibodies.[56–60]

The TANGO algorithm was developed twenty years ago and uses a statistical mechanics approach to predict the β-sheet aggregation propensity of a given protein/peptide sequence.[54] While amyloid aggregates consist of a cross-β architecture,[61,62] thus the excellent prediction of amyloidogenic sequences with this algorithm, non-amyloidogenic aggregates can still contain β-sheet rich aggregates (binding dyes such as Thioflavin T).[63] Antibodies contain multiple

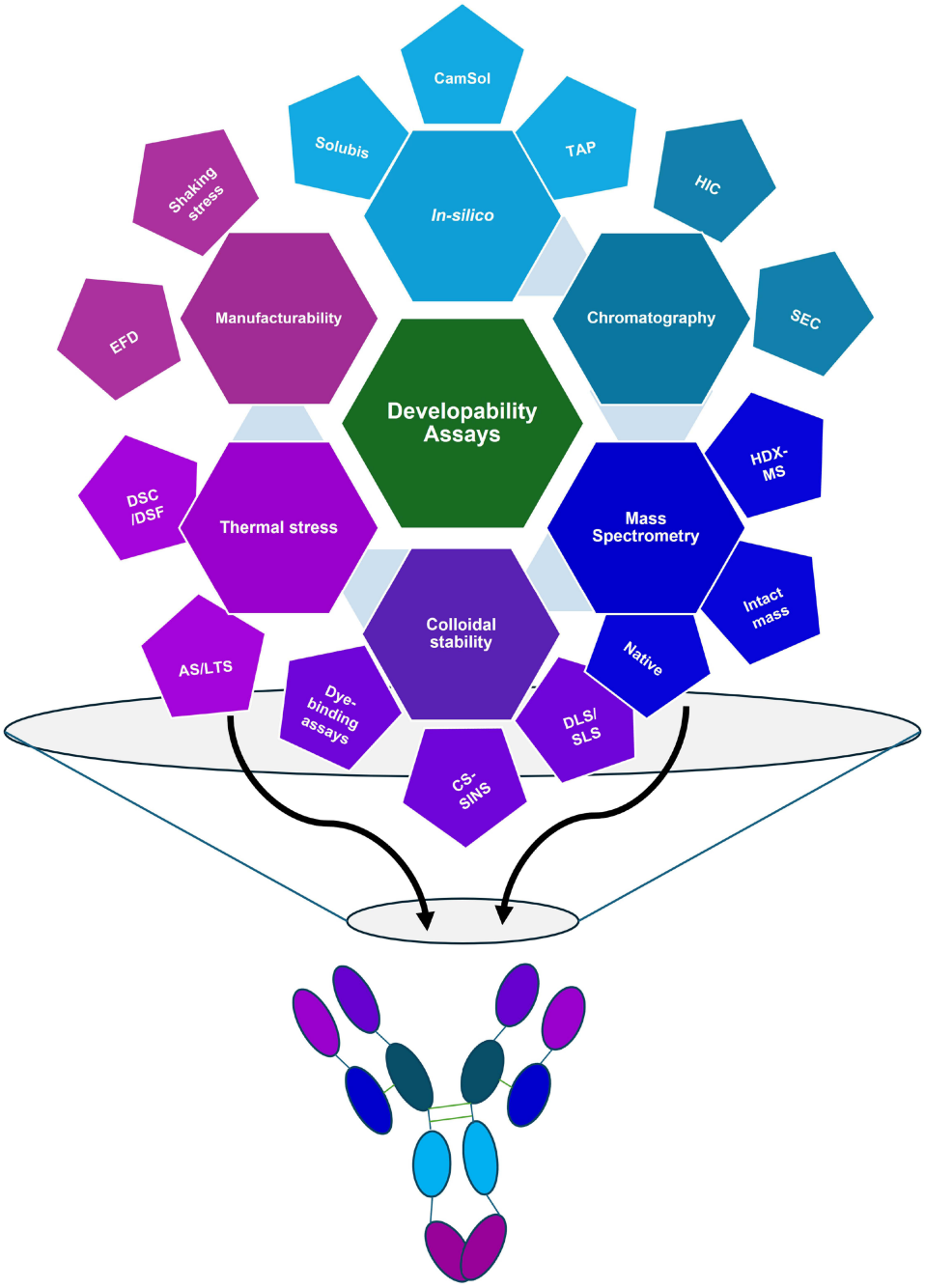

Figure 3 Schematic depicting the "family tree" of developability assays, discussed in this review. The assay groups radiating from the center (going clockwise from the top) are discussed in this review: In-silico assays, Chromatography-based assays, Mass spectrometry-based approaches, Methods to probe colloidal stability, thermal stress assays and finally, assays to probe manufacturability. As developability assays from each branch probe different physicochemical features of a molecule, by "harvesting fruit" from different branches of this tree, one can gain a holistic understanding of a molecule's developability profile, represented by the mAb colored by the branches above.

immunoglobulin domains, comprising two β-sheets cross-linked via a conserved disulfide bond.[64,65] The algorithm can thus predict aggregation for a broad range of input sequences. As shown in Figure 2, aggregation of biopharmaceuticals is thought to mainly take place through the misfolded state,[25] by exposing aggregation-prone regions (APRs) buried in the natively folded core, to the solvent. Here, APRs are defined as a window of five or more amino-acids with a high average TANGO score and while ordinarily buried in the native state, these APRs may become solvent-exposed upon environmental perturbation (eg[38]). The Solubis approach[66,67] calculates both the aggregation propensity (using TANGO) and the contribution of a given APR towards a protein's folding stability ($\Delta G^{\text{contrib}}$) using the FoldX algorithm.[68] Using

this approach, variants with reduced aggregation propensity can then be designed which suppress the TANGO score but do not thermodynamically destabilize the protein. For example, placing a lysine residue, a known "gatekeeper" which disfavors β-sheet formation,[69] into an APR would decrease its TANGO score, but would adversely affect the conformational stability of a protein if the APR in question was found in the hydrophobic core of a protein.

Other algorithms have been developed to identify APRs formed from spatially proximal residues that may be distal in primary sequence. The Spatial Aggregation Propensity (SAP) algorithm has been developed to identify solvent-exposed APRs in antibodies using a molecular dynamics-based approach.[70] Dynamics in the protein, an inherent property of monoclonal antibodies for their function,[71] could expose these APRs to the solvent over time. This algorithm has been used to identify problematic APRs in antibodies, for example, the MEDI1912 protein, whose "WFL" residue patch causes notable developability issues such as self-association[72] and poor manufacturability.[73] This approach was expanded to create a "developability index" which also computes an antibody's net charge,[74] which is increasingly recognized as an important physicochemical parameter to consider for a developable biopharmaceutical.[75–78]

Solubility can be defined as the maximum quantity of a solute that can be dissolved in a given volume of solvent at equilibrium.[79] When proteins aggregate, solvent-solute interactions between water and the protein are disrupted, allowing the APRs to self-associate (to yield a favorable protein–protein interaction).[80] Solubility, while related to aggregation, can be treated slightly differently with respect to its prediction, as charge will greatly affect protein solubility in water (a polar solvent). Hydrophobicity is of course another key parameter related to solubility.[77,81] A notable tool to predict protein solubility is the CamSol algorithm, developed by Sormanni et al.[82] Based on physicochemical properties of amino acids in a protein, a seven-residue sliding window is used to predict the local solubility, which can then be averaged over the length of the sequence. As described above, CamSol scores can also be calculated to account for structural context (ie buried or solvent exposed). Similar to Solubis, rational design of mutants, which favor solubility can be performed with this program,[82] as well as the identification of hotspots which disfavor solubility, such as the WFL example above.[56]

When employing these algorithms for antibody engineering, it is important that any amino-acid substitutions designed to reduce aggregation and or increase solubility do not affect binding affinity to the target,[49,83] which is especially challenging as residues in the complementarity determining regions (CDRs) are the regions usually subjected to mutational scanning during discovery.[84] To minimize this, the Therapeutic Antibody Profiler (TAP) has been developed to interrogate the CDRs for five developability liabilities including charge density and proximity, length and hydrophobicity.[85] An ever-growing database of therapeutic antibodies is used to benchmark amber (warning) and red (danger) thresholds below/above which a given TAP parameter is deemed problematic. Recently, TAP has been expanded to account for differences in both the modelling of antibody structures (aided by machine-learning algorithms (eg ImmuneBuilder)),[86] to distinguish between kappa and lambda light chains in therapeutic mAbs, with the latter trending towards poorer developability outcomes.[87] Interestingly this observation concurs with the observation that lambda light chains are found relatively more commonly in patients with light-chain amyloidosis.[88–90]

Efforts have also been made to predict aberrant post-translational modifications in proteins (eg deamidation) based on known sequence motifs, which are linked to these.[91] Recently, these sequence features have been identified in germline and therapeutic antibodies, based on mining large sequence databases to generate the Liability Antibody Profiler (LAP).[92] Based on their location in the protein (eg solvent exposure) as well as the frequency of these liabilities in germline (ie natural) and therapeutic proteins, a particular liability is ranked in terms of its real risk. For example, identification of a solvent-exposed Met residue which does not exist in the same position in any marketed mAbs may indicate that this is a prime candidate for substitution to minimize the risk of oxidation.[92]

In summary, computation characterization of the physicochemical characteristics of stretches of amino acids in proteins, corrected based on their structural context, has allowed various algorithms to be developed which identify APRs, regions of poor solubility and other developability liabilities in proteins. The prevalence of these tools in the development of therapeutics will continue to increase as the use of artificial intelligence/machine learning models and the availability of large volumes of sequence data becomes more ubiquitous in the 21st century.[93]

Chromatography-Based Methods: The Analytical Workhorses of the Biopharmaceutical Industry

The high-throughput (HTP), robust and versatile nature of high-performance liquid chromatography (HPLC) methods means they are employed routinely in the analysis of biopharmaceuticals.[94] The chemistry of the stationary phase (the column resin) and the mobile phase (the solvent) work in tandem to resolve different analyte(s) within a sample that can be quantified by a variety of (usually) spectroscopic methods. Proteins and peptides can either be analyzed in their native state using aqueous buffers at near neutral pH or in a denatured form by using non-polar resin and polar solvents at low pH in reversed-phase methods[95] with the latter method often prior to analysis by mass spectrometry (MS, see next section). As the characterization of the native state is often the principal aim of developability assessment (see above), this section will focus on normal-phase chromatographic methods.

Size-exclusion chromatography (SEC) has been described as the workhorse method to characterize therapeutic proteins.[96,97] Molecules are fractionated by the resin according to their mass and shape.[98] Detection of the species as they elute from the column, most frequently by in-line UV-Vis absorbance spectroscopy, allows for the separation and simultaneous quantification of aggregates, monomeric protein, and fragments.[97] By measuring samples stored over a period of time, this method is routinely employed to track changes in monomer/fragment/aggregate content in a sample over time at a defined temperature, setting its shelf-life.[99,100] The guidelines from the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) mandate this method for therapeutic proteins, based on its power in this regard.[101,102] However, SEC does have some caveats. Samples are often diluted prior to analysis or dilute during resolution on the column, which may favor the dissociation of some aggregates back to monomer (thus masking the true aggregate content of the original sample).[11,103]

Furthermore, some proteins will interact with the column resin (often silica-based for HP-SEC), leading to longer retention times[72] erroneously suggesting,[98] a much smaller apparent molecular weight. In the absence of changes in mass (verified by MS, for example) longer retention times are usually indicative of "sticky" molecules.[72,104] This property has been shown to correlate with other indicators of poor developability, such as poor growth in the presence of beta-lactam antibiotics (indicative of high aggregation propensity)[105,106] in an *in-vivo* tripartite beta-lactamase assay (TPBLA) of antibody variants.[83] Chromatography column vendors have developed SEC resins with a stand-up monolayer, which mimic the exterior of proteins.[107] For these resins, long retention times (caused by aberrant adsorption to the resin) have been shown to correlate with unfavorable developability parameters, such as colloidal stability as measured using cross-interaction chromatography (CIC), a method, which functionalizes SEC resin with a different IgG sequence to that of the analyte.[108]

As both hydrophobicity and overall charge can dictate the aggregation and solubility of protein. Hydrophobicity can be probed directly using hydrophobic interaction chromatography (HIC), where a high ionic strength mobile phase (eg 2 M ammonium sulfate) is used to "salt" proteins onto a hydrophobic resin. Dilution of the salt in the mobile phase results in the elution of proteins in order of increasing hydrophobicity (thus long retention times correlate with increased molecular hydrophobicity).[104,109] Whether the adsorption process leads to increased exposure of previously buried hydrophobic residues (eg[110–112]) is a potential caveat of the technique.

Resins of both negative or positive charge (for cation- and anion-exchange chromatography, respectively) are used in ion-exchange chromatography (IEX) to separate analytes based on their charge. The pH of the mobile phase can be used to tune adsorption to the resin, exploiting the isoelectric point (pI) of the analyte of interest, with high ionic strength typically used to elute species from the column.[113] While generally employed during bioprocessing in polishing steps to remove nucleic acids or charge variants from the desired drug substance,[114] both cation-[115,116] and to a lesser-extent, anion-exchange chromatography[117] have been used to identify molecules with aberrant charge profiles, often coupled to mass spectrometry[113,116] (see below). So-called "mixed mode" resins have been developed which have characteristics encompassing both hydrophobic and charged (ie polar) character.[118] Such resins have been used to separate different heavy- and light-chain combinations of bispecific antibodies when used in a SEC-resin, with multi-angle static light scattering used to corroborate the molecular weights of the species separated by these.[119]

Whilst not a chromatographic method per se, capillary electrophoresis uses an electric field in a functionalized capillary[120] or microfluidic chip[121,122] to separate molecules based on their charge and mass (ie charge density). By varying the pH in the capillary, the device can be used to perform capillary isoelectric focusing (cIEF). This has been used to look at charge variants of antibodies, with such deviations stemming from aberrant post-translational modifications (PTMs) caused by deamidation, glycosylation and other chemical changes to the higher order structure of the protein.[122–124]

The varied chemistry of chromatography resins, together with the precision of a HPLC autosampler, mean that HPLC-based methods have become routine for the analysis of therapeutic proteins. Aberrant biophysical behavior (namely fragmentation, aggregation, unwanted surface adsorption and charge variation) can be detected and quantified, depending on the combination of stationary and mobile phases employed. As alluded to above, MS is often employed downstream of these methods.

Mass Spectrometry (MS)- The Main MAM in Developability Assessment

Multi-attribute methods (or MAMs) are analytical methods capable of discerning multiple critical quality attributes (such as low aggregation propensity) from one sample, as described under the Quality-by-Design framework.[125,126] MS is one such MAM which has found itself well-placed in the development of therapeutic proteins, due to the rapid advancement and commercialization of these instruments over the past decade,[127] and in the software used to analyze the complex data these instruments generate.[128] In its simplest application (the accurate mass determination of proteins or their peptide derivatives), most MS measurements of therapeutic proteins are prefaced by a HPLC-based separation of the analyte, often by reversed-phase (RP) liquid chromatography. An ion-pairing agent, such as formic acid or trifluoroacetic acid (TFA), is typically added to the mobile phase, making protein analytes more hydrophobic than usual, thus increasing their resolution from an RP column.[95,129] The protein is eluted in a volatile organic solvent (typically acetonitrile), facilitating the transmission of the analyte from solution phase into the gas phase. For an in-depth discussion of different MS configurations, we direct the reader to more specialist reviews.[130,131]

Measured masses, obtained with a high degree of precision using MS (typically ± 0.01% of the protein's molecular weight) that are different to the calculated mass of the primary sequence can indicate proteolysis of the sample or incorrect disulfide bonding.[132] To look for residue-level evidence of PTMs, a "bottom-up" approach is often employed, whereby the protein will first be enzymatically digested by a protease (typically trypsin or a mixture of other proteases); if disulfide bonds are present, the sample is reduced and alkylated using standard protocols,[133] with salts then removed prior to the LC-MS experiment.[134] It is known that certain PTMs can adversely affect the chemical stability of a protein, namely: deamidation,[135] incorrect disulfide bonding[132,136] or glycosylation[137,138] and oxidation.[139–141] The primary sequence of a protein, particularly if this is an mAb, can be analyzed using algorithms to identify motifs, which are liable to these aberrant PTMs. For example, it is known that the dipeptide motifs of Asn followed by either Gly or Ser are liable to undergo deamidation at the Asn residue,[135,142] decreasing the chemical and conformational stability of the mAb. These can be readily engineered out of the sequence (identified using the LAP algorithm described above)[92] if they lie outside of functional regions of the mAb, eg, the paratope.

Glycosylation is another key attribute of antibodies, as N-linked glycosylation of a conserved Asn residue in the CH2 domain of an IgG is important for both the thermal stability of an antibody[137,138,143] and Fc-receptor binding.[144] The so-called effector functions of an antibody can dictate half-life in vivo (due to binding to the neo-natal Fc receptor) or aid killing of target cells through, eg, antibody-dependent cellular cytotoxicity.[15] Cell line engineering,[145] as well as modifications to residues in the hinge,[146,147] can affect the type of glycans formed in the endoplasmic reticulum (ER) during expression. mAbs can be deglycosylated to analyze the intact mass (of both mAb and glycan, the latter particularly important for the detection of different glycoforms) using enzymes such as PNGase F,[148] using the HPLC to aid their separation.

Due to the development of commercially available automated systems,[149] Hydrogen-Deuterium exchange (HDX) coupled to MS is being increasingly used to identify particularly dynamic regions in therapeutic proteins.[150] Typically, experiments are performed where the (protiated) protein of interest is incubated in a D_2O-based buffer. Under these conditions (a vast excess of deuterons versus protons) backbone amide protons exchange with deuterons,[151] the rate of

which being dependent on pH, temperature, solvent accessibility and amino acid side chain.[152] After incubation for variable time periods, exchange is quenched (through low pH and cold temperature), and the protein digested (typically with immobilized pepsin, which is most active at pH 2.5), then performing LC-MS. The peptide ions are often fragmented in the gas-phase to facilitate their sequencing (see[134] for more detail). One can then piece together the peptide information and map this back onto the 3D structure (or model) of the protein, to identify dynamic regions from protected regions. This has been done to identify regions of proteins/amino acid substitutions which modulate aggregation.[150,153–155]

The structural mass spectrometry toolkit allows one to probe the HOS of a protein, with a growing body of literature linking these data to favorable/aberrant developability parameters.[156–159] By introducing proteins (or other analytes) into the gas phase under much gentler conditions, using volatile additives such as ammonium acetate, native mass spectrometry proteins and their higher-order species can analyzed in conditions that maintain tertiary and quaternary structure.[160,161] A more novel use of MS for protein developability has recently been the use of collision-induced unfolding to probe the gas-phase stability of different therapeutic proteins.[162,163] This involves incrementally increasing the energy of ions in the ion trap of mass spectrometer, then measuring their collisional cross-sectional (CCS) area using an ion-mobility cell. This acts like a "gas-phase chromatography column", with compact ions interacting less with the buffer gas in the ion mobility cell than more expanded ones, thus the former arrive at the detector first.[164,165] Increased collision energy thus results in the unfolding of a protein in the gas phase, detected by a later arrival time. This has been shown to be able to identify different glycoforms based on their gas-phase stability.[166] Another approach to "gently" introduce proteins into the gas phase is to use capillary electrophoresis, as described above. This can be applied to both intact molecules (ie in a "top-down" fashion), as well as on peptides, in a bottom-up fashion[167] analogous to the LC-MS /MS approach above.

From the perspective of early-stage developability assessment, while confirmation of the correct intact mass of a protein is routinely employed for hits, more in-depth analysis would only typically be performed as molecules progress through the development pipeline, due to the increased amount of analytical effort (in terms of time) required to perform these latter experiments. We envisage this becoming more routine over time, as suggested by the increasing amount of literature in this area (with over 200 papers a year published during this decade).[131]

Colloidal Stability Assessment: Probing Self- and Cross-Interactions in Therapeutic Protein Samples

The word "colloid" stems from the Greek, meaning "glue-like". Protein solutions can be thought of as colloids based on their properties.[168,169] In Figure 2, self-association between monomers, through native state aggregation, or non-native aggregation (leading to the formation of oligomers and eventually precipitates) pertains to the colloidal stability of a therapeutic protein solution. In addition to self-interaction, therapeutic proteins will encounter other biological macromolecules throughout their lifetime (expression, purification and after administration), where cross-interaction with other antibodies, antigens or other off-target biomolecules can lead to undesired manufacturing and therapeutic outcomes (see Aussewöger et al for more detail).[170] A variety of methods have been developed to probe colloidal stability in its broadest sense.

Dynamic light scattering (DLS), which correlates the time-dependent fluctuations in the intensity of light scattered by solutes within a solution, is commonly employed to characterize colloidal stability.[171] This allows one to infer the diffusion coefficient of solutes and by extension, their hydrodynamic radii (R_H).[42] Further analysis (using the regularization algorithm for example)[172] allows the size-distribution of species within the sample to be determined. As the intensity of scattered light is proportional to R_H^6 aggregates will scatter far more light than the native monomer.[171] This makes DLS a particularly sensitive means of detecting aggregation within a sample but conversely makes precise quantification of the populations within polydisperse solutions extremely challenging.[171,173] Nanoparticle tracking analysis (which visualizes and counts aggregates larger than 50 nm in diameter),[173,174] mass photometry (an imaging-based method where the scattering footprint of a protein landing on the imaging surface is proportional to its mass)[175,176] and microfluidic-based technologies (reviewed by Otzen et al recently[177]) can be used to circumvent these issues.

DLS can now be performed on multiple samples in a plate-reader format.[178] By measuring the DLS of protein samples across a concentration range (typically from sub-milligram/mL to 5 mg/mL), the diffusion coefficient can be determined and plotted as a function of concentration. The intercept of a straight-line graph fitted to these data (which will have either a negative (attractive), flat or positive (repulsive) gradient, yields the diffusion coefficient at infinite dilution for the sample. The gradient of the straight-line graph relates to the diffusion interaction parameter (*kD*, units mL/g).[179] This parameter can give an indication of whether or not proteins interact (negative *kD*) or repel (positive *kD*) one another as a function of concentration, which is useful when screening molecules[179] or different formulations.[72] Kingsbury et al's determination of the *kD* values for a large panel of mAbs and correlation of these against 22 other properties showed that repulsive behavior between the molecules correlated with favorable pharmacokinetic properties, highlighting the importance of *kD* as a developability parameter.[179] It should be noted that the sign of the *kD* value is a rule of thumb, as recently the opposite trend has been tied to unfavorable viscosity at elevated concentration.[180]

In contrast to DLS, static light scattering (SLS) measures the time-independent scattering of light by solutes in a sample.[42,181] The intensity of the scattering is dependent on the concentration and mass of the sample.[181] Using more sophisticated equipment, one can measure the SLS of protein solutions as a function of concentration in the same fashion as above. The second osmotic virial coefficient (commonly called B22 or A22) describes attractive (positive values) or repulsive interactions (negative values) between proteins as a function of concentration.[182,183] It can be seen therefore that *kD* and B22 values are generally inversely correlated;[182] (but note[180] is an important exception). Both parameters are important as attractive interactions as a function of concentration are likely to impact the final drug product, which is increasingly formulated at concentrations exceeding 100 mg/mL if administered by sub-cutaneous injection via a pre-filled syringe.[184–186]

In addition to these sophisticated measurements, simple turbidity assays (such as the optical density of a protein sample at 350 nm) can be performed but is very sensitive to the size range and distribution of aggregates within the sample.[187] The sizing and counting of subvisible and visible particles (spanning 10's–1000's microns in size) is critical,[188] as aggregates in this range are linked to adverse immunogenic effects in vivo.[189,190] Light obscuration and microflow imaging[191,192] have traditionally been used to achieve this aim, with machine learning approaches being developed to aid discrimination of proteinaceous particles from other adventitious contaminants.[193] More recently, background membrane imaging (which uses image processing to improve signal to noise) has been developed to look for these particles, using sub-mL volumes for analysis.[194]

Molecular crowding (see[195] for physical basis) has been a commonly employed method to indirectly probe the colloidal stability of therapeutic proteins. Polyethylene glycol (PEG) has been used as a crowding agent to force proteins closer to one another (akin to their environment under high concentrations).[196] By determining the concentration of protein remaining in solution (using a constant initial protein concentration) over a range of PEG concentrations, one can determine the colloidal stability of the protein,[56,72] including the inference of its "concentratability" (ie a protein's theoretical solubility limit).[197]

More sophisticated HTP methods have been developed to monitor self-association of proteins. Affinity-capture self-association nanoparticle spectroscopy (AC-SINS) was developed by the Tessier group, conjugating polyclonal human IgG-targeting antibodies onto gold nanoparticles (AuNPs).[198,199] If a target mAb exhibits low dispersity (ie was monomeric), the AuNPs remain disperse in solution. Conversely, self-association of the target mAbs results in the agglomeration of the AuNPs, with the plasmon wavelength (absorbance) of the particles red-shifting as a result. This has been used to screen panels of antibody sequences,[104,199] with trends being identified between net-positively charged CDRs and self-association by AC-SINS.[200] Modification to the AuNP preparation protocol increases the diversity of formulation buffers compatible with the method.[201,202]

Cross-interaction (ie off-target binding to bio-molecules other than the epitope) is an important developability parameter as increased retention times on this resin have been linked to poor solubility at elevated protein concentrations.[108] Methods have consequently been developed which conjugate or adsorb other proteins, including antibodies {Clonal self-interaction Biolayer Interferometry (CSI-BLI)[203] and Cross-interaction chromatography[108,204]} and either specific antigens {Baculovirus particle (BVP) enzyme-linked immunosorbent assay (ELISA),[205] or a broad range of antigens (Poly-specificity reagent (PSR))}[206] to different stationary phases (chips, chromatography resin and

well-plates). Test proteins are then incubated with these functionalized species, with interaction yielding longer retention times/increased ELISA signals with rapid clearance of drugs in animal models when BVP scores are high.[109] The reader is directed to Jain et al,[104] Ausserwöger et al[170] and Norden et al[207] for more details of these methods.

In summary, the examination of the colloidal stability of a therapeutic protein is important with respect to its pharmacology and eventual formulation as a medicine. Aggregates have also been shown to cause illicit immunological effects in vivo, including neutralization of drugs (Anti-drug antibodies)[208] or anaphylaxis. Thus, the characterization and mitigation of aggregation and off-target interaction is pivotal in early-phase drug development.

Thermal Stress: Turning Up the Heat on Biopharmaceuticals

It is well-known that heating proteins can trigger their unfolding and aggregtion. From the perspective of biopharmaceutical development, the final drug product is often formulated to be kinetically stable at refrigerated temperatures for typically two years or more.[101,186,209] It would be impractical to subject all early-stage candidates to a real-time stability screen based on both the long timescales and shallow amplitude of the degradation kinetics under real-time storage conditions.[99,100,210–212] Instead, samples are often incubated at elevated temperatures to increase their rate of degradation. Typical "accelerated stability" (AS) conditions for early-phase biologics are incubation at 40°C for 2–4 weeks.[210,213–215] The regulatory agencies mandate such testing as part of the development of new drugs.[216]

Can one use the rates of degradation at elevated temperatures to infer what happens at lower (refrigerator) temperature? Classically, the temperature dependence of reaction rate follows Arrhenius kinetics, whereby the rate of a reaction increases as a function of temperature (a 10 K increase in temperature typically doubles the observed reaction rate). Recently, several Arrhenius-based kinetic models have been developed which can exploit protein-degradation kinetics at elevated temperatures (and short time frames) and use the data to directly predict the long-term storage stability of molecules.[99,100,212,217,218] Others in the field, however, have shown that not all proteins obey this kinetic regime,[210,211,219,220] meaning AS serves as a "rule of thumb" test to identify particularly thermally labile molecules or formulations from a panel.[104,209]

Reasons for deviation from an Arrhenius regime may depend on the distinct aggregation mechanism of the protein in question. AS conditions are typically below the apparent melting temperature ($T_{m,app}$) of a protein (the temperature at which 50% of the molecules are unfolded). $T_{m,app}$ can be deduced in a HTP fashion using differential scanning fluorimetry (DSF). If a protein contains a Trp residue (conveniently, Ab-based therapies contain several Trp residues as they are conserved as part of the immunoglobulin fold),[221] then the intrinsic fluorescence can be followed as a function of temperature. Protein unfolding will result in a red-shift in the fluorescence emission maximum of the Trp residues. Plotting the ratio of the emission at 350 nm (Trp exposed suggestive of an unfolded protein) versus 330 nm (Trp buried and suggestive of a folded protein) allows a sigmoidal curve to be fitted to the data and the $T_{m,app}$ extracted from the mid-point.[222–224] Furthermore, the inflection point of this curve represents T_{onset}. As well as setting the upper limit for an AS study (typically 10 K below the $T_{m,app}$ to prevent aggregation taking place predominantly from the unfolded state, not the misfolded state), this method can identify thermally labile molecules and formulations.[213] Some instruments which monitor intrinsic fluorescence can also use SLS simultaneously to infer the apparent temperature of aggregation (T_{agg}) based on an increase in scattering intensity as a function of temperature.[224,225] Such instruments fit into the MAM framework by allowing one to obtain multiple developability parameters from one sample, maximizing characterization from the smaller quantities of material available in early drug development.

The "gold standard" method for obtaining $T_{m,app}$ is differential scanning calorimetry (DSC). This is because cooperativity of unfolding observed by DSF can often mean that the unfolding transitions of different domains in the protein are poorly resolved.[226] DSC involves heating up a sample and measuring the difference in temperature between the reference cell and sample cell upon unfolding of different domains in the protein (ie release of heat, due to bond breaking, being exothermic). The transition midpoint yields the $T_{m,app}$, whereas the peak area would yield change in heat capacity.[227] The sensitivity of the method means multiple transitions can be observed for Ab domains.[227] Blech et al have recently shown that multiple thermodynamic parameters can be obtained from DSC measurements (eg enthalpy change between folded and unfolded states), which can then be linked to the kinetic aggregation mechanism for a protein in question.[228] However, larger quantities of material (>100 μL at protein concentrations ~1 mg/mL, compared to tens of

microliters at a similar concentration for DSF) are needed to perform these measurements. DSF is thus likely to be performed in the first instance when quantities of material are lower, albeit with the caveats mentioned above.

Assessing Manufacturability: Shaking Up the Developability Pipeline

Of the various stresses biologics experience as part of their manufacturing, hydrodynamic forces (namely shear and extensional flows, as well as interaction with both solid-liquid and air-liquid interfaces) are ubiquitous.[229–231] Such mechanical forces are thought to be able to initiate protein aggregation, mainly through the adsorption of proteins to interfaces. Subsequent partial unfolding and aggregation can take place on the surface, with flow then able to dislodge these species into bulk solution to regenerating these interfaces.[110,231,232] The bulk flow may also induce formation of the aggregation-prone state,[233–236] though this is controversial.[231,237.] Although poorly defined, orbital shaking studies appear to generate many of the hydrodynamic stresses described above and have been used to identify aggregation-prone molecules,[187,238] as well as the type and concentration of surfactant excipients, which protect against these interfacial stresses.[239,240]

However, the mechanistic drivers of aggregation in this mixed type of stress can be difficult to define. Devices have been developed to subject proteins to defined shear flows, often in the presence of a solid–liquid interface of known chemistry,[241–243] with this latter factor playing the dominant role in any subsequent aggregation. We have developed a small-volume extensional flow device (EFD), which subjects proteins to a predominantly elongational flow in the presence of solid (glass)-liquid interfaces.[73,187,236,244] Others have built similar devices,[232,237,245] including a recent example that can subject later-stage formulations to realistic hydrodynamic forces encountered during syringe actions.[246] Though this latter application falls slightly outside the scope of this review, one could envisage the utilization of these devices for the rapid small-scale assessment of proteins to manufacturing stresses, something which appears to be a unique developability feature not described by the other assays mentioned in this review.[244]

How Do We Make Sense of Developability Data?

The many critical quality attributes required by a candidate therapeutic has driven the deployment of a wide array of methods to assess developability, but how these can be integrated is unclear. For example, if multiple methods are available to measure the same developability attribute (eg HIC and SMAC chromatography measuring molecular "stickiness"), then will one method provide adequate insight? If these assays predict conflicting developability outcomes, which do you trust? Once the data have been generated, how can one integrate the outputs to make an informed holistic decision on candidate selection?

Firstly, it is important to understand how assays relate to one another. Jain et al used Spearman's rank correlation to look at the pairwise interaction between the behavior of 137 clinically relevant mAb sequences in 12 different developability assays.[104] Hierarchical clustering was used to group the assays based on the responses of the mAbs; for example, HIC and SMAC chromatography yielded similar results.[104] Both we[244] and others[228,247–251] have subsequently used similar approaches (sometimes using Pearson's correlation instead of Spearman) to understand how novel assays/analyses compare to the established methods discussed herein, as well as infer (numerically) relationships between developability assays. There is an argument that applying multiple methods that probe the same biophysical property of a molecule is not the best use of resources, as one ends up with degenerate data. Using one technique from each branch of the "family tree" of developability assays (outlined in Figure 3) may be a better approach.

Secondly, statistical (and in a more simplistic sense, anecdotal) evidence can aid the decision to remove a molecule from a development campaign based on its behavior in a given developability assay. While liabilities (such as primary sequence motifs, which could lead to poor chemical stability[252] or aggregation propensity[60]) can be engineered out in early-stage development, or by the addition of surfactant during formulation[253,254] this becomes increasingly difficult the further the molecule progresses through the clinic. Acceptable thresholds are often placed on particular developability assays either from the regulator (eg particle levels[255]), scientific reasoning (based on long-term stability data, for example), or other more arbitrary cut-offs.[99,100] This latter approach would allow one to discard (for example) the worst molecules in a panel if they score a "red flag" in a particular assay.[104] This becomes less clear cut if a molecule performs poorly in one DA but well in other unrelated assays.

Machine learning/artificial intelligence approaches have gained much traction over the past five years in the biophysical analysis of proteins.[93,202,256,257] For example, Makowski et al used CS (charge stabilised)-SINS and PSR to screen the self-association and polyspecificity, respectively, of a panel of 80 clinically-relevant antibody sequences. A machine-learning model was trained on these data (plus others) to effectively predict which mutations would ameliorate aberrant behavior in these assays, which was validated experimentally.[202] Most recently, clinical and natural sequence databases have been mined to compute different sequence and structural liabilities across antibodies.[92,257] As the biopharmaceutical industry embraces Industry 4.0, the leveraging of large datasets in this fashion will only aid the identification of the rules of developability for both current and next-generation molecules.

The biopharmaceutical pipeline has seen an increase in diversity in terms of both the pharmacological targets and the molecular architecture of the molecules used to interact with these.[24] The number of modalities is only set to increase over the next few years.[24] How the relatively established rules used to develop mAbs applies to these modalities in the future, remains to be seen, though efforts to this aim have begun.[251] Some rules could be the same (for example, a molecule obeying Arrhenius kinetics will allow prediction of long-term stability).[100] Others could change in light of allosteric effects, or due to the availability of a richer and larger dataset as technical challenges in analyzing these modalities are overcome . The field will inevitably rise to the challenge to bring about the successful development of the next-generation of biopharmaceutical proteins.

In conclusion, therapeutic proteins, in particular, monoclonal antibodies, have emerged as powerful medicines over the past forty years due to their potency, specificity and multiple modes of action. However, the multiple mechanisms by which they can degrade means an array of "developability" assays have been developed to identify defective molecules and select only those with drug-like properties. These analyses probe the varied molecular features of these complex molecules. The integration of computational and statistical workflows with experimental methods, especially as the field continues to grow, will aid the development of the therapeutic proteins of the future.

Funding

All the authors acknowledge funding from the EPSRC (EP/Z533063/1). NK is supported by a Royal Academy of Engineering Chair. SER is also funded by a Royal Society Professorial Fellowship (RSRP\R1\211057).

Disclosure

The authors declare no competing financial interests arising from this work.

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