

Antibody stability: A key to performance - Analysis, influences and improvement

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Antibody stability: A key to performance - Analysis, influences and improvement

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ARTICLE INFO

Article history:

Received 3 March 2020

Received in revised form

28 August 2020

Accepted 28 August 2020

Available online 3 September 2020

Keywords:

Antibody stability

Protein stability measurement

Protein expression

Antibody engineering

Formulation

ABSTRACT

An antibody's stability greatly influences its performance (i.e. its specificity and affinity). Thus, stability is a major issue for researchers and manufacturers, especially with the increasing use of antibodies in therapeutics, diagnostics and rapid analytical platforms. Here we review antibody stability under five headings: (i) measurement techniques; (ii) stability issues in expression and production (expression, proteolysis, aggregation); (iii) effects of antibody format and engineering on stability and (iv) formulation, drying and storage conditions. We consider more than 100 sources, including patents, and conclude with (v) recommendations to promote antibody stability.

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Abbreviations

CD	circular dichroism
CDR	complementarity-determining regions
CE	capillary electrophoresis
C _{H1,2,3}	constant domain one, two, three, of the antibody heavy chain
C _L	constant region of antibody light chain
COOH	carboxylic acid group;
DLS	dynamic light scattering
DSC	differential scanning calorimetry
DSF	differential scanning fluorimetry
EGFR	epidermal growth factor receptor
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding fragment
Fc region	fragment crystallizable region
H/D-MS	hydrogen/deuterium exchange mass spectroscopy
HER	human epidermal growth factor receptor
IC ₅₀	ligand concentration leading to 50% inhibition of binding
Ig	immunoglobulin
LC-MALDI-TOF/TOF	liquid chromatography-matrix assisted laser desorption ionization-time of flight/time of flight

mAb	monoclonal antibody
NH ₂	amino group;
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline;
PEG	polyethylene glycol
RP-LC-MS	reverse-phase liquid chromatography-mass spectroscopy
scAb	single-chain antibody
scFv	single-chain variable fragment
sdAb	single domain antibody
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography
SPR	surface plasmon resonance
S–S	disulfide bond
TBS	tris-buffered saline;
T _m	“melting temperature” where a protein is 50% unfolded
V _H	variable region of antibody heavy chain
V _{HH}	camelid variable heavy-chain antibody fragment
V _L	variable region of antibody light chain
V _{NAR}	variable new antigen receptor
WB	western blot

1. Introduction

Antibodies with high specificity and affinity are very widely used in diagnostics and therapeutics. The most common forms used are polyclonal and monoclonal antibodies (mAbs) and various antibody fragments (e.g. single-chain variable fragment (scFv), fragment antigen-binding (Fab) fragment and single-chain antibody (scAb); Fig. 1). Here we focus on both monoclonal and genetically derived antibodies as these are the best defined and

most widely utilised. They are used in many diagnostic applications, to treat various cancers (e.g. head and neck, colorectal, breast, colorectal and ovarian cancers, leukemia, and multiple myeloma), and also in arthritis, asthma and psoriasis. By May 2018, the US FDA had approved over 80 therapeutic monoclonal and genetically derived antibodies [1].

Instability is reported as a highly significant problem in all aspects of antibody generation and utilisation and production conditions must be tailored to ensure antibody stability [2]. Antibody

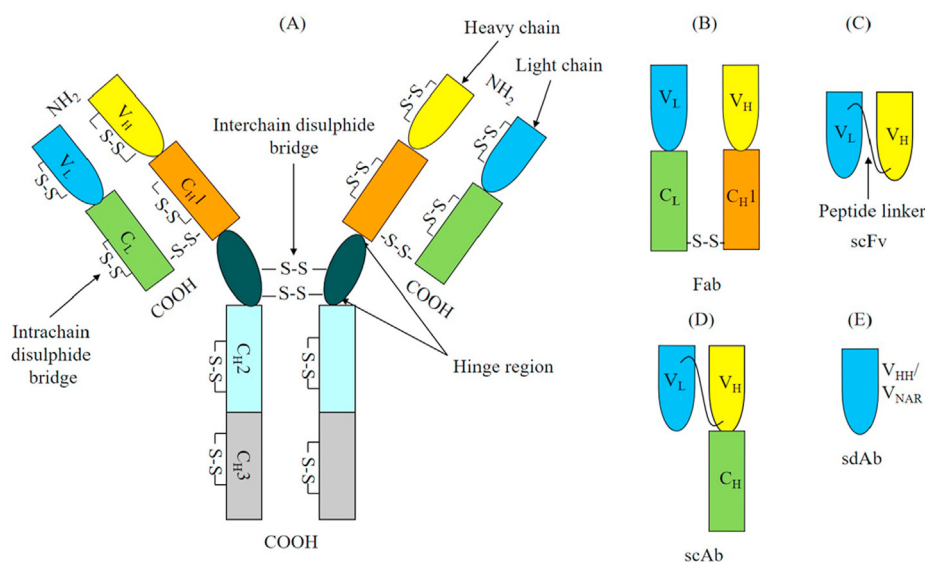


Fig. 1. Illustration of mAb, Fab, scFv, scAb and sdAb. (A) The basic IgG mAb consists of two light chains and two heavy chains. (B) Fab consists of the full antibody light chain, connected to a second antibody domain comprising V_H and C_{H1} regions by disulfide bonding. (C) scFv consists of variable regions of antibody V_L and V_H, which are linked by a short peptide chain. (D) scAb consists of a scFv region fused to a constant domain. (E) sdAb consists of a single monomeric variable antibody domain. NH₂ = amino group; COOH = carboxylic acid group; V_H = variable region of antibody heavy chain; V_L = variable region of antibody light chain; C_L = constant region of antibody light chain; C_{H1,2,3} = constant domain one, two, three, of the antibody heavy chain respectively; S–S = disulfide bond.

instability can lead to reduced production yields, lost or impaired efficacy, harmful immune reactions, patient-associated complications and limited or lost function when working in extreme conditions or during long-term storage (which is crucial for many antibody-based test formats and sensors) [3–5]. Because stability affects the efficacy of increasingly important antibody-based diagnostics and therapeutics, the study, analysis and promotion/enhancement of antibody stability during expression, production, storage (shelf-life) and application, are pressing issues. Here we review antibody stability under five headings: (i) measurement techniques; (ii) stability issues in expression and production (expression, proteolysis, aggregation, yields); (iii) effects of antibody format and engineering on stability; and (iv) formulation, drying and storage conditions. We consider more than 100 sources, including patents, and conclude with (v) recommendations to promote antibody stability.

2. Monitoring antibody performance and stability

A variety of assays is required to analyse and monitor antibody properties and stability. Test parameters include specificity, binding affinity, purity, aggregation, degradation and thermal stability. Although a detailed consideration of methods is beyond the scope of this short article, some of the main techniques are summarized and depicted in Table 1 and Fig. 2, respectively. ELISA, Western blotting and affinity analysis, using approaches such as SPR/Biacore, can be utilised to indicate the retention of biological function (antigen binding, specificity, affinity) during conditions encountered during production/isolation and following storage. In addition, circular dichroism (CD), nuclear magnetic resonance (NMR) and mass spectroscopy have been used to probe antibody stability.

CD is applicable as a high-throughput screening system for stability measurement of antibody derivatives. For example, for various formulations of a V_{HH} antibody, CD spectral changes (corresponding to structural alterations) correlated very well with the effects of denaturation temperatures [21]. Similarly, Dahab and El-Hag [22] reported the use of CD, together with simultaneous absorbance and turbidity measurements, to study the solution stability of an IgG2A mAb. Their approach can also aid in distinguishing between unfolded, refolded, aggregated and flocculated states.

NMR can be used for antibody stability determination. Nokwe et al. [23] showed the importance of the conserved residue 2 of antibody V-L domains for thermodynamic stability and their tendency to form fibrils. (Some variants of V-L domains can form pathogenic amyloid fibrils in patients.) They found that hydrophobic amino acids at position 2 stabilized the domain, while charged residues were destabilizing and led to the formation of amyloid fibrils. NMR also showed that amino acid changes at position 2 affected several segments in the V-L domain core. Intriguingly, this molecular switch point occurs in kappa family V-L domains only, and not in V-L lambda or V-H domains.

Mass spectroscopy is an additional tool to probe antibody stability. Using hydrogen/deuterium exchange mass spectroscopy (H/D-MS), Guo and Carta [24] showed that two-peak elution behaviour of a mAb from ion-exchange chromatography correlated with conformational changes in the mAb upon binding to the stationary phase (see also Sections 3.1.1 and 3.1.2 below). Manikwar et al. [25] used H/D-MS to examine the effects of excipients (see Section 4.1 below) on the local flexibility of an IgG1 mAb at 25 °C, pH 6. The same study also employed DSC and SEC to measure conformational and storage stability (aggregation), respectively.

The complexity of antibodies, and of their derivatives and conjugates, often demands the use of orthogonal analytical techniques (i.e. alternative methods based on different principles) in order to

assure their quality (safety and efficacy) and stability. This is well illustrated by a case study of an antibody-drug conjugate. Trastuzumab (Herceptin), a humanized monoclonal IgG1 antibody therapeutic against the HER2 receptor, also exists as a conjugate with the anti-cancer drug DM1. This conjugate is known as Trastuzumab emtansine, or T-DM1. Mohamed *et al* [26], applied a variety of techniques to probe key attributes of T-DM1. SE-HPLC was used to detect antibody fragmentation or aggregation, and these results were correlated with DLS measurements and with reducing/non-reducing SDS-PAGE profiles. (Both DLS and SDS-PAGE can indicate aggregation and/or degradation; see Table 1 and Fig. 2.) DM1 stability within the T-DM1 antibody-drug conjugate was examined by RP-HPLC (the amount of unconjugated drug is a key quality attribute that relates to product safety) and the drug-antibody ratio was measured using spectrophotometry. Special attention was given to those factors that can induce fragmentation and/or aggregate formation (and, subsequently, formation of anti-drug antibodies when administered to patients). Stress factors applied to both T-DM1 and to unconjugated Trastuzumab included pH (4.0–10.0), temperature (–20–37 °C), agitation and repeated freeze/thaw cycles, according to ICH Q2 (R1) guidelines [27]. It became clear that the conjugate was less stable than the parent mAb protein. Although both products were relatively stable against freeze/thawing, short-time agitation and low temperatures, strongly acidic and basic pH values caused formation of high molecular weight aggregates for both Trastuzumab and T-DM1. Mohamed *et al.* [26] propose that their testing regime is applicable to stability and quality measurement of other antibody-drug conjugates.

Other examples of the use of multiple complementary and orthogonal techniques include CD, DLS and tryptophan fluorescence studies [28], SEC-HPLC for aggregates, CD and FTIR for structural integrity, DSC for thermodynamic stability, DLS for colloidal interactions and SEC-HPLC/capillary isoelectric focusing for fragmentation and deamidation [29]. Ito and Tsumoto [30] used SYPRO Orange as a dye probe together with differential scanning fluoroscopy, CD and DSC in a heat-stress study of chimeric, humanized and human antibodies. Alsenaidy *et al.* [31] outlined a two-step high-throughput approach for stability investigation of mAb glycoforms utilizing, as a first step, CD, fluorescence spectroscopy and static light scattering over a wide range of conditions (10 – 90 °C; pH 3 – 8). Second-stage analysis took place between pH 4.0–6.0 and over smaller increments of temperature, with the use of DSC and DSF. Wang *et al.* [32] used a similar range of techniques in a study of salt concentrations on the stability of an Asp-rich Fab, together with ion-exchange chromatography to determine Asp isomerization.

Persistence of biological activity (e.g. binding to antigen, measured by ELISA and/or SPR) should, wherever possible, be monitored in addition to the use of biophysical methods. The ideal stability measurement technique (or combination of techniques) should closely mirror the retention of the antibody's biological performance throughout production steps and on storage. It should provide accurate readings in 'real-time', or with a very short delay. It should be non-destructive or, if this is not possible, require very small analytical quantities of precious and highly valuable antibody material.

3. The main factors that influence antibody stability

Since an antibody's format or structure, its state of aggregation and susceptibility to proteases all greatly affect its stability, manipulation of these factors can enable the optimization of antibody stability.

Table 1
Some stability measurement techniques for use with antibodies.

Technique	Basis	Attribute Measured	Biological/Physical Stability	Equipment Needed	Outline of Method	Strengths	Weaknesses	Reference(s)
ELISA	Antigen-antibody binding interactions	Binding affinity & specificity	Biological (i.e. molecular function)	Microplate reader, microplates	Fig. 2A	Index of biological function. Widely practised. Large range of reagents and equipment available.	Requires numerous reagents and procedural steps.	[6–8]
Western blot	Antigen-antibody binding interactions	Binding specificity; thermal stability; purity	Physical (Biological: correct folding)	Western blot electrophoretic transfer cell	Fig. 2B	May indicate biological function. Widely practised. Large range of reagents and equipment available.	Requires numerous reagents and procedural steps.	[9]
Biacore	Antigen-antibody binding interactions	Binding affinity & specificity	Biological (i.e. molecular function)	Biacore instrument, sensor chip	Fig. 2C	Index of biological function. Rapid technique: results available in 'real-time'.	Requires costly instrumentation and specialized reagents.	[10,11]
SDS-PAGE	Electrophoretic separation of unfolded proteins	Purity; aggregation; degradation	Physical (polypeptide chain integrity)	Electrophoresis; discontinuous polyacrylamide gel	—	Widely practised. Large range of reagents and equipment available.	Uses denatured sample, so does not measure biological function.	[12]
Size exclusion chromatography (SEC)	Separation based on molecular radius	Purity; aggregation; degradation	Physical	Size exclusion chromatography column	Fig. 2D	Widely practised. Large range of reagents and equipment available.	Dilution of sample may occur.	[13]
Dynamic light scattering	Determination of the mean protein size by detecting and measuring fluctuations of the scattered light at a fixed angle	Purity; aggregation; degradation	Physical	Dynamic light scattering instruments	Fig. 2E	Characterizes aggregates. Rapid technique.	Requires specialized instrument.	[14,15]
Differential scanning calorimetry	Measurement of heat-induced transformations by measuring the temperature difference between a sample and a reference	Unfolding; thermal stability	Physical	Differential scanning calorimeter	Fig. 2F	Sensitive method, independent of sample's optical properties. Measures (un)folding transitions/stability.	Requires costly instrumentation.	[16,17]
Differential scanning fluorimetry; dye binding	Quantification of thermally-induced antibody denaturation by measuring of fluorescence changes in a protein-bound dye	Unfolding; thermal stability	Physical	Real-time PCR device	Fig. 1G	Sensitive method, measures (un)folding transitions/stability.	Requires specialized real-time PCR device.	[18–20]

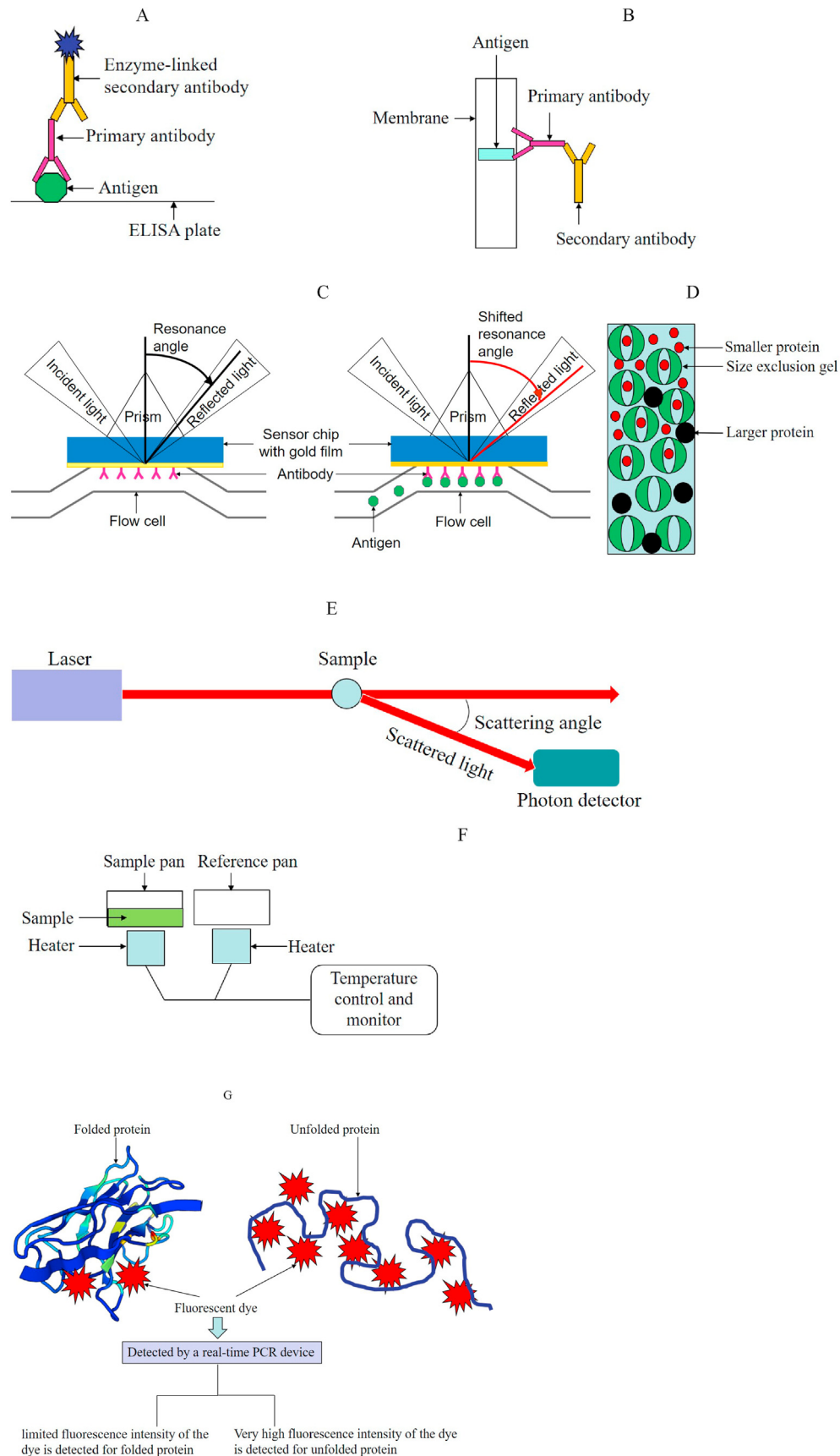


Fig. 2. Various techniques for analysing and monitoring antibody properties and stability. (A) An example of ELISA. The target antigen is firstly immobilised on a ELISA plate, followed by an antigen-specific primary antibody. Then, an enzyme-linked secondary antibody (which recognises the primary antibody fragment crystallizable (Fc) region without

3.1. Issues in expression and production

Bayat et al. [33] examined the influence of expression vector design on IgG1 antibody expression levels from CHO cells, together with quality and stability. Comparison of the efficiency of different vector designs indicated that a dual-promoter single-vector system gave the highest expression level and the highest productivity.

Maltose binding protein (MBP) is a stable monomer which does not induce dimerization or aggregation when fused as a tag on a recombinant protein. Sarker et al. [34] found that use of a MBP tag significantly improved the *in vitro* refolding yield of a scFv, compared with the alternative tags hexa-histidine or glutathione-S-transferase. Interestingly, a mild solubilisation procedure (involving the use of mild denaturing agents such as 2 M urea, 5% (v/v) dimethylsulfoxide, 5% (v/v) isopropanol, 4 M oxidized glutathione in 50 mM phosphate buffered saline pH 7.4) and *in vitro* refolding strategies proved effective in recovery of a soluble ‘tag-free’ scFv from non-classical bacterial inclusion bodies. The mild solubilisation procedure could, with optimization, have potential for efficient and economical recombinant protein production in *E. coli* because it does not require any fusion tag.

3.1.1. Proteolysis/fragmentation

Antibody proteolysis can occur in both mammalian and plant host cells. This is highly undesirable, as it can affect yield and compromise product quality. A therapeutic IgG4 mAb-X was shown (by different orthogonal techniques including CE-SDS, SEC-HPLC, RP-LC-MS and MS/MS) to undergo pH-dependent fragmentation in CHO cells, mostly in the hinge region (see Fig. 2A). Proteolysis increased as the pH decreased from 6.0 to 4.0, while use of pepstatin A in mAb-X formulation samples greatly decreased the degree of fragmentation. These observations indicated that a residual host-cell acidic protease was to blame. Fragmentation of mAb-X was eliminated following optimization of a Protein A chromatographic step by the use of 0.05 M Tris containing 0.5 M arginine (pH 8.5) as the post-wash buffer in the mAb-X purification process. This additional step more effectively removed the residual host cell protease, helping to ensure the stability and efficacy of the mAb-X product. Yang et al. [35] noted that residual host cell proteases should receive careful attention as a critical quality attribute (CQA) from the earliest development stages of mAb manufacturing processes, so as to optimize quality implementation by design. This is especially important, since the presence of residual host cell proteases might become evident only by the occurrence of product degradation over an extended time period (ICH, Q5E) [36].

Working with a mouse IgG1κ monoclonal antibody (mAb Guy's 13), expressed in the plant *Nicotiana tabacum*, Hehle et al. [37] noted the presence of smaller fragments together with the whole protein. These fragments resulted from proteolytic cleavage. Investigations revealed a small number of sites in both the heavy and light chains that were vulnerable to proteolysis. These sites were situated predominantly in the antibody's interdomain (between variable and constant domains in the light and heavy chains) or in solvent-exposed regions. A site-directed mutagenesis approach led to improved antibody-versus-fragments ratio, but did not eliminate

proteolysis entirely.

3.1.2. Aggregation

Antibodies have a tendency to aggregate, due to the various chemical interactions that maintain their structure (combining van der Waals forces, hydrophobic interactions, disulfide linkages and hydrogen bonds). Generally, there are two types of aggregation: native (reversible, with retention of protein structure) and non-native (irreversible, with changes to protein structure). Extensive aggregation can lead to the formation of visible particles and even to antibody precipitation [38]. Aggregation is one of the major factors affecting antibody stability during production processes, delivery and storage. Antibody aggregation also leads to low expression yield from host cells, affecting biological activity, and may increase immunogenic responses [39]. Clearly, such adverse effects must be prevented.

Arosio et al. [40] reported that pH and salt concentration are among the main parameters affecting monoclonal antibody aggregation. Low pH (pH < 4.0) and presence of salt led to reversible aggregation to oligomers, together with an increased content of beta-sheet secondary structure. Famm et al. [41] noted that aggregation is usually caused by interactions between unfolded or partially unfolded antibodies, and often takes place when antibodies are denatured by heat or low pH. Aggregation can also arise from weak thermal stability. It often occurs when mAbs are heated near to their unfolding temperature (at which 50% of the protein is unfolded [42,43]). For mAb production, aggregate formation varies depending on medium compositions, physical properties of mAbs, culture conditions and cell line characteristics. Ishii et al. [44] generated 28 individual trastuzumab-producing cell lines and found that those cell lines with low levels of light chain production yielded significantly lower antibody titres (i.e. poorer productivity) and a higher percentage of aggregates (i.e. poorer quality), since light chains are essential for the correct folding of heavy chains and secretion of mature antibodies. Therefore, correct and efficient antibody folding and assembly in the endoplasmic reticulum are crucial to obtain antibody preparations with low contents of aggregates. These observations may help to improve mAb manufacturing processes. The manner of aggregate formation also differed between the two cell lines. Aggregates from the high-titre/high-quality cell line resulted mainly from *covalent* interactions, while those from the low-titre/low-quality cell line were predominantly due to *hydrophobic* interactions. Ishii et al. [44] also reported, for the first time, the presence of half-antibody + CH₂ in samples purified from protein A. These half-antibody + CH₂ structures may serve as nuclei for aggregation.

Working with scFv directed against the epidermal growth factor receptor, Lehmann et al. [45] noted that some scFv contained a fusion of lambda 3 and lambda 1 V-region (LV3 and LV1) genes that led to sub-optimal biophysical protein properties (decreased thermodynamic stability and increased tendency to aggregate), resulting in poor production and limited application. They then adopted a structure-based approach to re-design an anti-epidermal growth factor receptor scFv by exchanging lambda sequences with a more stable kappa3 framework (KV3) within the V_L domain

inhibiting the binding of primary antibody to antigen) is added, followed by the enzyme's substrate (chromogenic, fluorimetric or luminescent). Washing steps take place between the stages indicated. (B) Strategy of Western Blotting. The antigen, which is electrically transferred onto a membrane, is typically probed using an antigen-specific primary antibody, followed by a primary Fc region-specific secondary antibody for signal amplification and visualisation. (C) Biacore detection system. Target antigens are bound by the specific antibodies previously immobilised onto the sensor chip surface in a flow cell. The mass change caused by antibody-antigen binding leads to a shift of resonance angle that is proportional to the binding antigen concentration. (D) Size exclusion chromatography. Smaller proteins pass through both smaller and larger pores, while larger proteins enter only the larger pores. Therefore, larger proteins elute sooner and smaller proteins later. (E) Illustration of Dynamic Light Scattering. The sample is illuminated by a laser beam, and fluctuations of the scattered light are detected using a fast photon detector at a known scattering angle. (F) Differential Scanning Calorimetry. The temperatures of sample and reference pans are measured. Heat flow is monitored as a function of temperature. (G) Differential Scanning Fluorimetry. Fluorescent dye binding to folded protein is limited, while unfolded protein binds larger amounts of dye.

(containing the original lambda DE-loop). This led to increased thermodynamic stability with retention of binding affinity. Such a structure-guided sequence-switching redesign strategy provides a novel means to enhance the stability and potential therapeutic applicability of problematic scFv at an early stage of the engineering and selection process.

Identification of aggregation-prone regions could help to predict and prevent antibody aggregation [46]. Use of an aggregation prediction tool to screen for structurally-feasible residue mutations enabled re-engineering of antibodies with reduced aggregation and improved stability [47]. Jespers et al. [48] created human antibody variable domains resistant to heat aggregation by selection on phage through heat denaturation. Later, this team successfully selected domains resistant to aggregation under acid conditions [41]. Perchiacca et al. [49] reported that antibody stability can be improved by molecular grafting of CDR loops, and that replacement of a wild-type antibody's CDR1 with the corresponding CDR1 from an aggregation resistant antibody, Hel 4, decreased susceptibility of the 'swapped' antibody to aggregation. In addition, systematic mutagenesis analysis of CDR loops could identify charged mutations that would enhance the reversible unfolding tendencies of antibodies and related proteins (see also section 3.2.1). Hernández-Jiménez et al. [50] recently studied the effects of forced degradation (by exposure to high temperature, to an acidic medium, to a basic medium and to an oxidative medium) on aggregation of five IgG1 therapeutic mAbs. The mAbs showed different patterns of instability, but storage at refrigerated temperatures (rather than frozen) was generally beneficial and protein concentration was a key factor. Detailed studies of this sort could help prevent undesirable conformational and colloidal instabilities in antibodies, thus assuring the quality and safety of antibody products. It is reported that positively-charged residues are more likely to promote recombinant protein aggregation in the cytoplasm of *E. coli*, hence limiting protein solubility in comparison with negatively-charged residues. This effect depends on the surrounding water and on the protein's net charge [51]. (However, a protein's overall hydrophobicity is the main factor influencing its tendency to aggregate [51].) Carballo-Amador et al. [52] undertook surface patch analysis to predict amino acid changes that would affect the solubility of *E. coli*-expressed recombinant human erythropoietin, then generated and expressed these mutants. Variants with a greater number of negative surface charges showed increased solubility, while one with an introduced positive surface charge was notably less soluble. Neither of these studies focused on antibodies as examples, but Kuhn et al. [53] reported improvements in solubility (and other properties) of two IgG1 monoclonal antibodies following targeted point mutations, based on computational rational design. The mutations aimed to smoothen the electrostatic surface distribution of each mAb. Interestingly, the beneficial mutations involved the removal of negative charges, or the insertion of positive charges, at particular sites.

The reports above described electrical charge alterations within the target proteins themselves. Addition of positively-charged peptides (e.g. poly-His, poly-Arg) to the N- or C-terminus of a recombinant protein is often practised to enable purification by immobilised metal affinity chromatography. Such terminal peptide tags can also benefit the protein's solubility. Attachment of a short 12-residue arginine-rich solubility enhancing peptide at the C terminus of an anti-epidermal growth factor receptor [EGFR] scFv led to decreased aggregation and the enhanced solubility [54]. The unmodified scFv expressed in the insoluble fraction (thus requiring refolding) and was prone to aggregation at 37 °C and pH 6. In contrast, a scFv with the 12-mer peptide was expressed with 85% in the soluble fraction and its total yield was approximately 3-fold higher than that of the unmodified form. Light scattering and

circular dichroism measurements indicated greater solubility and higher thermal stability for the peptide-extended form of the scFv. These benefits had no adverse effect on the scFv's ability to bind to EGFR.

Guo and Carta [24] examined the elution behaviour of a mAb from cation-exchange chromatography media. CD analysis indicated a correlation between the chromatographic behaviour of different mAbs and their tendency, upon thermal stress, to either precipitate or to form stabilizing intermolecular structures. Comparison of six stationary phases revealed notable two-peak elution with tentacle and polymer-grafted resins but scarcely any with macroporous materials. Increases in the buffer pH and concentration (leading to weaker mAb binding) decreased the extent of two-peak elution. Replacement of sodium by alternative counterions could increase or decrease the two-peak effect.

A hazard of phage-display selection strategies for scFv is the possible occurrence of V_H and V_L domains with decreased folding stability and increased tendency to aggregate. Lehman et al. [45] devised a means of exchanging lambda with a more stable kappa3 framework (KV3) within the V_L domain. ScFv prepared in this way were more thermodynamically stable and were more easily produced from bacterial culture, with little effect on scFv binding properties.

Different forms of a recombinant antibody may display different properties (e.g. Ma et al. [10] found that a scAb was notably more stable than its Fab and scFv counterparts). Rao et al. [55] compared scFv and Fab structural variants of a scFab (single chain Fab) that could detect *O,O*-diethyl organophosphorus pesticides. The scFab was configured in two orientations of the heavy (Fd) and light (kappa) chains with the $(Gly_4Ser)_3$ linker. Expression, specificity and long-term stability of the three antibody forms were measured. IC_{50} values (50% inhibition of binding; measured by indirect competitive ELISA) for the pesticides coumaphos and parathion were 2.5-fold and 2.7-fold lower, respectively, for the scFab in kappa-linker-Fd orientation versus the reverse Fd-linker-kappa arrangement. IC_{50} values for the kappa-linker-Fd and the Fab were similar, but those of the scFv were higher. FAB, scFv and scFab showed similar concentration in the expression extract against the antigen but the concentration of Fab was lower. Following nine days of incubation at 37 °C, the scFab and Fab retained good antigen-binding activity but the scFv had scarcely any remaining affinity, indicating that the Fab and scFab had greater stability than the scFv.

3.2. Effects of antibody structure, formats and engineering on stability

Whole or complete mAbs (typically ~150 kDa; Fig. 1A), which contain both variable and constant domains of light and heavy chains, are generally more stable than antibody fragments (i.e. scFv, scAb, Fab and sdAb; see Fig. 1) which are much smaller. Moreover, glycosylated mAbs are more stable than unglycosylated forms, which aggregate more easily [56]. (Glycosylation is the post-translational attachment of carbohydrate structures (glycans) to newly-synthesized protein molecules. These glycans aid protein folding and stabilize the folded structure, while protecting against proteolysis and aggregation [57]. However, it is harder to characterise the potential modification, degeneration and aggregation of glycosylated mAbs during manufacture, storage and application due to their larger size and greater number of functional groups [58].

Thermodynamic stability of scFv varies widely, and most mAb-derived scFv have poor to moderate stability without modification/protein engineering. This, combined with patent issues, means that scFv have had limited application to date for diagnostics. scFv stability can, however, be improved by various strategies; for

instance, protein engineering (e.g. point mutations, grafting of their CDR regions onto stable variable domain frameworks, chain shuffling and addition of various tags [59]), optimization of storage conditions and reduction of the potential for aggregation.

3.2.1. Grafting of CDRs to enhance antibody stability

In some cases, conversion of scFv to scAb, Fab or IgG can enhance antibody stability; for example, Quintero-Hernández et al. [60] found improved stability after converting a scFv to a Fab antibody format. Honegger [59] concluded that, as the intrinsic thermodynamic stabilities of antibody variable domains vary widely, engineering of suboptimal variable domains (e.g. by a limited number of point mutations and/or grafting the antigen specificity domains onto more-stable variable domain frameworks) can enable improved antibody stability (and folding efficiency), while maintaining specificity and affinity. Borras et al. [61] dramatically improved scFv stability by minimalistic grafting of rabbit CDRs onto a human scFv scaffold, and provided an effective and workable method to humanise and stabilize rabbit variable domains. Kügler et al. [62] also stabilized and humanized a scFv (against human lymphocyte antigen CD19) by point mutations and CDR-grafting onto a human framework. Jung and Plückthun [63] generated a scFv by transplanting the CDR region from an insoluble antibody to the framework of a humanized antibody, which resulted in better folding *in vivo*, significantly increased solubility and much improved thermodynamic stability (measured by urea denaturation). Zabetakis et al. [64] proved that some single domain antibodies (with notably high melting points of $\sim 84^\circ\text{C}$) can be further stabilized ($>6^\circ\text{C}$) by addition of disulfide bonds.

Various reports have shown that addition of kappa or lambda light chain constant domain (C_L) to scFv (yielding a scAb) can increase scaffold stability, probably due to improved folding and/or decreased tendency to denature [10,65,66].

Certain scFvs are known to fold satisfactorily in high yield in *E. coli* cytoplasm, independent of disulfide bond formation. Such disulfide independence is a key property for production of soluble scFv, and it is important to know the relative contributions of the antibody framework and of the complementarity-determining regions (CDRs) to disulfide-independent folding. Gaciarz & Ruddock [67] exchanged CDRs between four scFv, two with disulfide-independent folding, and two with disulfide dependent folding. Cys-to-Ala mutations were made to confirm disulfide independence. Yields, thermal stability and secondary structure of solubly-produced scFv were compared. For those produced in good yield, binding studies were performed (by western or dot blotting, or by surface plasmon resonance). Both the CDRs and the framework influenced soluble scFv production, but the CDRs had the larger effect. No correlation emerged between thermal stability and the dependence of folding on disulfides, while there was only a weak correlation between protein yield and thermal stability.

A Fab (~ 50 kD; Fig. 1B) structure is usually more stable than a scFv (~ 25 kD Fig. 1C) or scAb (~ 37 kD; Fig. 1D), because (i) the additional interface of the constant domain and (ii) the hydrophobic interfaces exposed in scFv are buried by the constant domain in Fab [68]; also proven *in vivo* by Quintero-Hernández et al. [60]. However, Ma et al. [10] reported that a scAb showed greater stability (under defined conditions) than the equivalent Fab (both the scAb and Fab were derived from the same scFv), indicating that the C_H - C_L interaction is only one factor contributing to higher stability.

Interestingly, the smallest intact antigen-binding fragments, namely single domain antibodies (sdAbs: camelid variable heavy-chain antibody fragment (V_{HH} ; ~ 15 kD) and shark variable new antigen receptors (V_{NAR} ; ~ 12 kD); see Fig. 1E), showed specificity and affinity similar to scFv and Fab - but higher stability and

solubility, which may be because their simple structure enables higher refolding efficiency [69,70].

3.3. Role and insertion of disulfides

Antibody fragments containing disulfide bonds tend to show greater stability [71,72]. Flygare et al. [73] reported that cysteine modification (which causes interchain disulfide reduction) led to the permanent loss of structural disulfide bonds, thus decreasing the stability of an antibody-drug conjugate *in vivo*. It has been reported that a disulfide bond and/or Cys residues within the CDR-H3 region contributes to antibody stability [74]. Furthermore, Ma et al. [10] performed Cys-to-Ala mutations in the CDR-H3 region for three formats of an antibody, namely, scFv, Fab and scAb. The overall decreased storage stability at 37°C for all the three antibody formats was observed following mutation of Cys in CDR-H3 region, which could be due to the removal of inter- or intra-CDR disulfide bond(s). Kawade et al. [75] reported that the inter-domain disulfide bond between the variable and constant domains of kappa chain contributed to the thermal stability of the rabbit antibody. By introducing a disulfide bond within a single domain antibody, the conformational stability can be generally increased [76]. Moreover, by insertion of a second disulfide bond, Zabetakis et al. [64] further stabilized a single domain antibody (A3, a high affinity anti-Staphylococcal Enterotoxin B sdAb) with an already high melting point ($T_m \sim 84^\circ\text{C}$) by $> 6^\circ\text{C}$. Information from a recently-available crystal structure was used to identify target sites for disulphide insertion. The wild-type A3 had a single Cys22-Cys99 disulfide, while the most-stabilizing second disulfide ($T_m > 90^\circ\text{C}$) was that introduced at A49C-I73C. Zeng et al. [77] noted that insertion of additional disulfide bonds in CH2 and/or CH3 domains (one engineered disulfide bond in CH2 or CH3 domain and two engineered disulfide bonds in CH2 and CH3 domains respectively), significantly increased antibody thermal stability, while the Fc-mediated functions were maintained. It is reported that even the disulfide variants in IgGs may alter drug substance stability and functional activity [78]. Moreover, various arrangements of the inter-chain disulfide bonds can lead to differences in the mAb higher-order structure, thus affecting thermal stability [79]. Therefore, Resemann and colleagues [79] developed a rapid LC-MALDI-TOF/TOF system which enables the identification of the IgG2 disulfide linkages and semi-quantitative assessment of the distribution of the disulfide isoforms. Furthermore, Baker et al. [80] successfully generated a rapid and accurate nonreduced peptide map method (together with machine learning) for mapping-out expected disulfide bonds and cysteine-related modifications in half-antibodies and bispecific antibodies. Liu et al. [81] genetically encoded non-canonical amino acids containing long side-chain thiols which can pair with cysteines to form extended disulfide bonds and allow cross-linking of protein distant sites and distinct domains, which led to significant enhancement of protein thermal stability.

Single-domain antibodies (sdAb), recombinant variable domains derived from heavy-chain-only camel antibodies, show promising stability characteristics. Building on previous work, Henry et al. [82] described stabilized sdAbs. They incorporated a second Cys48-Cys64 disulfide (Kabat numbering) into a phage-displayed synthetic human V_L library that already contained a highly conserved Cys23-Cys88 disulfide linkage. Both the 'double' and 'single' disulfide V_L libraries produced soluble V_L s that bound to an antigen (maltose binding protein) with micromolar-range affinities. Notably, the double-disulfide V_L s showed T_m values typically 10°C higher, and remained more functionally diverse after selection with Protein L, than their single-disulfide counterparts. This 'additional disulfide' approach could be broadly applicable for the generation of stabilized human V_L s via phage display (although

lower expression yields of such protein scaffolds could impact on manufacturability).

Although this section has emphasized the important contribution of disulfides to the (thermodynamic) stability of antibodies and their fragments, some studies on removal of the conserved disulfide Cys22-Cys97 from V_{HH} fragments (Fig. 1E) have revealed interesting results. Mutants lacking this disulfide have lower thermal stability but may retain antigen binding affinity. Akazawa-Ogawa et al. [83] examined single-, double- or no-disulfide variants of two different V_{HH} s. (The second, non-natural, disulfide was engineered between wild-type Ala 49 and Ile 69.) Heat resistance (i.e. retention of binding activity following heating/cooling cycles, or irreversible denaturation) decreased as the number of disulfides increased (except for one mutant). A greater heat-denaturation effect was observed in mutants with two disulfides, indicating that disulfide shuffling may influence the unfolding of multiple-disulfide proteins. Intriguingly, disulfide bond removal can moderately increase heat resistance, regardless of its adverse effect on equilibrium thermodynamic stability (reversible unfolding). Using single-molecule force spectroscopy, Liu et al. [84] showed that the mechanical strength of a disulfide-free V_{HH} in complex with its antigen was similar to that of the wild-type V_{HH} -antigen complex, despite the mutant V_{HH} 's decreased thermal stability.

3.3.1. Other protein engineering approaches

Based on screening amino acid variations found in functional antibody variable domains, Stevens [85] disclosed a method for identifying amino acids for substitution in target proteins (especially those with an immunoglobulin-like fold), so as to control their stability and alter their shelf-life and/or half-life. Such an approach was used to optimize the stability of scFv for biosensors, which successfully maintained function after incubation for >2 h at 70 °C. Kettenberger et al. [86] improved antibody stability via a structure-based strategy to identify and remove asparagine and aspartate degradation sites in antibody peptide sequences. (Asparagine's side-chain is prone to deamidation which may, in turn, lead to peptide chain cleavage or formation of iso-aspartic acid [57].) Burton [87] engineered recombinant antibodies with significant stability to proteases and intestinal digestion by identifying the structural features of proteolysis-resistant antibodies found in the early colostrum of ruminants.

Shriver-Lake et al. [88] studied the 'melting' temperatures and production yields (from *E. coli* shake-flask cultures) of fourteen sdAb against the seven serotypes of botulinum neurotoxin (BoNT). Upon insertion of four framework 1 site mutations (Q1E or D, K3Q, Q5V, A6E) that lowered the iso-electric point and were known (from previous work) to be stabilizing, thermal stability increased by 5 – 9 °C. This approach, which avoids the insertion of additional disulfides, may provide a means of stability improvement for other sdAbs. Interestingly, one clone with five "framework 3" changes (S83A, R84K, N86 M, D92 N, Q95K) in addition to the four "framework 1" mutations above recognized BoNT E, expressed at up to 4 mg/L and showed a T_m of 80 °C.

3.3.2. "PEGylation"

The antibody stabilization toolbox is not limited to mutational strategies. Chemical modification can be a useful complementary means to influence proteins' molecular properties. For example, covalent conjugation of an antibody (or derivative) to polyethylene glycol (PEG) can greatly benefit stability.

Maruani et al. [89] reported increased the lifetimes of antibody fragment-drug conjugates *in vivo*, by attachment of a blood serum-stabilizing functionality (e.g. PEG, albumin or albumin-binding functionality) using a versatile "dual-click" technology. Pyridazinediones with two orthogonal 'clickable' handles were

inserted into native disulfide bonds in the antibody protein. Subsequent orthogonal reactions enabled the attachment of two distinct moieties to generate antibody-drug conjugates.

Chapman et al. [90] improved antibody stability by site-specific chemical modification of antibody fragments with polyethylene glycol. They attached PEG to Fab' specifically at the hinge-region cysteine residue, resulting in longer half-lives *in vivo* and full retention of antigen-binding properties. Johnson et al. [91] devised antibody-stabilizing formulations comprising an antibody fragment, covalently attached via a Cys-linked succinimide moiety to one (or more) non-proteinaceous polymer (e.g. PEG, preferably 20 kDa but possibly ranging 5 – 50 kDa) in an aqueous buffer (pH range 3.5 – 6.0, ideally pH 4.8). The formulation should be isotonic, with an ideal antibody concentration range of 150 – 220 mg/mL.

Roque et al. [92] undertook site-specific attachment of PEG to the hinge of a monoclonal Fab'. Spectroscopic techniques indicated that physical stability of the Fab' was maximal at pH 6 – 7 – but with no discernible difference between the control and "PEGylated" forms. However, PEGylation was very stabilizing against temperature-induced and surface-induced aggregation, most likely due to a steric hindrance mechanism.

4. Presentation and storage for optimal antibody stability

It is well known that storage conditions (pH, buffer type, stabiliser, bacteriostatic agents, protease inhibitors, temperature and freeze/thaw cycles) play crucial roles in antibody stability [93].

4.1. Formulation and excipients

Excipients can greatly influence the stability of antibodies and their derivatives. Laptoš and Omersel [5] noted that those excipients which maintain pH (e.g. Tris, acetate, histidine and citrate buffers) and prevent oxidation (e.g. sugars and polyols) can enhance protein stability. Manikwar et al. [25] examined the effects of sucrose and arginine (0.5 M each) on an IgG1 mAb versus a control preparation in 0.1 M NaCl. Sucrose increased the folding stability and led to a slower reduction in monomer content (i.e. slower aggregation). Sucrose also decreased local flexibility across much of the mAb structure and the formation of insoluble aggregates. In sharp contrast, arginine lowered the mAb's conformational stability and notably increased local flexibility in specific parts of the structure, particularly in the CH2 domain. Arginine also increased the levels of soluble and insoluble aggregates, with an increased rate of monomer loss.

Bhambhani et al. [94] studied dilute (1 mg/mL) and highly concentrated (up to 100 mg/mL) solutions of a humanized IgG1 kappa mAb in a systematic approach to formulation design and high-throughput excipient selection. CD, fluorescence techniques and light scattering were used as probes of secondary structure, tertiary structure and aggregation respectively. "Generally regarded as safe" substances were chosen for the excipient library. Conditions of pH 4.5 and temperatures close to 60 °C were used for the excipient screening assay. Notably stabilizing effects were observed in the presence of (i) 20% glucose, (ii) 20% sorbitol and (iii) 5% glucose plus 10% sorbitol. Stability gains were similar at 50 mg/mL and at 1 mg/mL mAb concentrations.

In view of "cold chain" restrictions on mAbs and other biologicals (i.e. the need for continuous and validated storage at low temperatures ranging –80–20 °C), Bansal et al. [95] attempted to devise formulations that would ensure stability at higher temperatures (25–55 °C), thus avoiding the need for cold storage. They examined the effects of peptide dendrons in formulations of an IgG1 mAb and noted that a third-generation lysine dendron L6 had notably stabilizing effects, as shown by numerous biophysical

techniques. The dendrons were shown to be biocompatible and SPR analysis showed that antigen-binding activity was unaffected.

Buffers can have various effects on mAb conformational stability and can lead to fragmentation. Kubiak et al. [96] found that ruthenylated reagents for anti-drug antibody assays had lower aggregation levels and produced lower baseline responses when stored in a histidine-sucrose buffer (HSB) compared with PBS. Zheng et al. [58] reported that both pH and buffer species affected the degradation process of an IgG 1-subtype therapeutic monoclonal antibody A, with pH the more important. Degradation was slower at higher protein concentrations. Therefore, antibody storage concentration should be 1 mg/mL or higher and the protein BSA (2–5%, w/v) is often used as a stabiliser in antibody storage [97]. Chavez et al. [98] described an optimised buffer (200 mM arginine, 50 mM histidine, and 100 mM NaCl at a pH of 6.5) for improved long-term storage stability of murine IgG3 after multiple freeze/thaw cycles. Absorbance at 280 and 410 nm with SEC were used to monitor antibody solubility, opalescence and aggregate formation. Cini et al. [99] enhanced antibody long-term storage stability (thermal, conformational and colloidal stability) by using buffered formulations (a buffer comprising an acetate salt (1 mM range), mannitol (around 200 mM), glacial acetic acid (in the 20 mM concentration range), sodium chloride (about 24 – 28 mM), polysorbate 80 (range 0.1% v/v) and mildly acidic pH in the range 5.1 – 5.3).

Correia et al. [100] devised a metal-free histidine buffer system, based on the finding that iron, in presence of histidine, increases fragmentation of an antibody (containing a lambda light chain) through the cleavage in the hinge region. (This effect was not observed in antibodies containing a kappa light chain.) They successfully increased the stability of immunoglobulins containing a lambda light chain using buffer formulations of pH 5 – 7 that contained histidine (1 – 100 mM range) and methionine (1 – 50 mM) among other components. Lambda light chain prepared in these cocktails had shelf-lives beyond least 24 months, or the ability to withstand at least 5 freeze/thaw cycles.

Bacteriostatic agents (e.g. sodium azide and/or thimerosal, in situations where these toxic chemicals are permitted) and protease inhibitors are usually added to prevent bacterial growth, thus increasing the antibody's storage stability. Note that thimerosal (0.01% w/v) contains mercury, while azide (0.02% w/v) will inhibit heme-containing proteins such as peroxidase, and may present an explosion hazard if not handled with care [101].

Practical hints on the use of salts, osmolytes and other protein-stabilizing additives are outlined in Ref. [101].

4.2. Storage temperature

Common storage temperatures for antibodies are 4 °C, –20 °C and –80 °C [102]. Enzyme-conjugated antibodies should be stored at 4 °C, as freeze/thaw stresses (e.g. crystallization of buffer or non-buffer components, ice formation, cold-denaturation, phase separation, thawing time, redistribution of solutes and pH fluctuation) will reduce enzyme activity. Notably, an IgG3 antibody isotype should always be stored at 4 °C as it will undergo aggregation during thawing [102,103].

Antibody aggregation has been observed during freeze/thaw processes [104] and can potentially be induced by freeze/thaw through the complex physical and chemical changes in the solvent/solute conditions due to freeze/thaw stresses [103]. Therefore, reducing the number of freeze/thaw cycles should limit the occurrence and extent of aggregation. Many cryoprotectants are rich in hydroxyl groups and are preferentially excluded from the vicinity of protein molecules. The protein becomes preferentially hydrated and hence is stabilized, because denaturation would lead to increased - but thermodynamically unfavourable - protein-

additive interactions [101]. Moreover, antibody aggregation can be reduced by cryoprotectants through delaying ice crystallization and eutectic transition of an antibody solution [105]. Thus, cryoprotectants can be applied for the inhibition of freeze/thaw-induced antibody aggregation.

Generally, storage at –20 °C and –80 °C are far more appropriate for longer term stability (years) compared to 4 °C (typically weeks or months). For storage at –20 °C, addition of 50% (v/v) glycerol or ethylene glycol can be used to prevent the damage caused by freezing/thawing. Aliquots of antibody stock solutions should be dispensed in small volumes before freezing, so as to avoid multiple freeze/thaw cycles. One or more small volumes of antibody can be thawed for use at any one time, while the rest of the stock is kept frozen. In this way, a given antibody aliquot undergoes only one freeze/thaw cycle. Fast thawing can denature proteins up to 11-fold more than slower thawing, indicating that slower thawing (i.e. on ice) should be applied to minimize antibody instability [104].

Very low storage temperatures often benefit stability. Kukis et al. [106] reported that a monoclonal antibody (Lym-1, an anti-lymphoma mouse IgG2 alpha) still retained structural and functional integrity after 8.5 years storage at –70 °C. Michaut et al. [107] found that immunotherapeutic antibodies remained stable in undiluted serum samples after storage at –80 °C for at least 3.5 years and 3 – 12 freeze/thaw cycles. Generally, however, one should avoid frequent freeze/thaw cycles, which can lead to antibody degeneration and aggregation [108].

4.3. Dried preparations

Daugherty and Mrsny [109] stated that protein stability can be enhanced by exclusion of water from pharmaceutical forms. A common method for water removal is freeze-drying (or lyophilisation), with addition of cryoprotectants [101]. Maintenance of a glassy state is key to ensuring the long-term stability of freeze-dried proteins. Park et al. [110] formulated an IgG1 mAb with mannitol at three different pH values (3.0, 5.0, 7.0) with and without sucrose. They then monitored stability at different temperatures over one year. A variety of spectroscopic and other techniques was used to probe physical and covalent degradation, and protein secondary and tertiary structure. Glassy state dynamics were observed via solid-state proton NMR. The mAb's secondary structure became notably perturbed at pH 3.0 and in the absence of sucrose. Structural changes were least at pH 5.0 with sucrose, and correlated with long-term stability (in terms of aggregate formation).

Notably, Ferraz et al. [111] proved that mAbs (isotypes IgG and IgM) retained stability after drying on filter paper and storage for over 1 year at –20 °C, which can be applied generally and easily in antibody-based diagnosis.

5. Conclusion and recommendations

Antibodies are vital and versatile molecules combining a constant modular scaffold with hypervariable regions that can generate immense diversity for binding to myriad antigens.

In vivo, the entire molecule mediates complex biological processes and responses, in addition to straightforward binding to its antigen.

Much of the antibody structure, however, is unnecessary for human-devised applications. "Stripped down" antibody derivatives are increasingly used in diagnostics and therapeutics, where they can have distinct advantages over the native structure.

Protein stability issues can arise for these smaller antibody derivatives, since they lack the rigid and stabilizing molecular scaffold

that is the hallmark of the intact antibody.

To overcome these stability limitations, one should aim to:

- Eliminate, prevent or minimize proteolysis, fragmentation or aggregation during expression, production and purification of the antibody derivative.
- Choose the antibody format that is best fit for purpose, and aim to stabilize this at the molecular level against adverse physical and chemical events. This may involve sequence or domain swapping, CDR grafting, insertion of additional disulfide(s), PEGylation/conjugation/chemical modification, or a combination of these methods.
- Devise a formulation that will maximize protein stability and ensure a long shelf-life. This will require careful choice of buffers, additives and excipients. Common protein stabilizers include 5% (w/v) BSA, suitable pH, protease inhibitors and perhaps a bacteriostatic agent [112].
- Pay close attention to the prospective storage conditions: container, temperature, and the question of wet or dry product presentation. The use of small aliquots and, for frozen preparations, slow/gentle thawing and a minimal number of freeze/thaw cycles, should be considered.

We hope that, with the aid of this review, researchers may find it easier to solve (or at least relieve) antibody stability problems in research and in the manufacture of antibody-based diagnostics and therapeutics.

Author contributions

Conception of the work: RO'K, HM, C'F, Collection of data: HM, C'F, Analysis of data: HM, C'F, RO'K, Writing of manuscript: HM, C'F, RO'K, Resources and funding acquisition: RO'K, HM, All the authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgments

This work was supported by Science Foundation Ireland [CSET Grant No. 05/CE3/B754, 10/CE/B1821] and by Enterprise Ireland [RECENT Grant No. CF/2015/0105].

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