Understanding the Relationship Between Biotherapeutic Protein Stability and Solid–Liquid Interfacial Shear in Constant Region Mutants of IgG1 and IgG4

ROUMTEEN TAVAKOLI-KESHE,[1,2] JONATHAN J. PHILLIPS,[2] RICHARD TURNER,[2] DANIEL G. BRACEWELL[1]

[1]The Advanced Centre for Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK
[2]MedImmune, Granta Park, Cambridge CB21 6GH, UK

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ABSTRACT: Relative stability of therapeutic antibody candidates is currently evaluated primarily through their response to thermal degradation, yet this technique is not always predictive of stability in manufacture, shipping, and storage. A rotating disk shear device is proposed that produces defined shear conditions at a known solid–liquid interface to measure stability in this environment. Five variants of IgG1 and IgG4 antibodies were created using combinations of two discrete triple amino acid sequence mutations denoted TM and YTE. Antibodies were ranked for stability based on shear device output (protein decay coefficient, PDC), and compared with accelerated thermal stability data and the melting temperature of the CH2 domain (T_m1) from differential scanning calorimetry to investigate technique complimentarity. Results suggest that the techniques are orthogonal, with thermal methods based on intramolecular interaction and shear device stability based on localized unfolding revealing less stable regions that drive aggregation. Molecular modeling shows the modifications' effects on the antibody structures and indicates a possible role for Fc conformation and Fab-Fc docking in determining suspended protein stability. The data introduce the PDC value as an orthogonal stability indicator, complementary to traditional thermal methods, allowing lead antibody selection based on a more full understanding of process stability. © 2013 The Authors. *Journal of Pharmaceutical Sciences* published by Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:437–444, 2014
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INTRODUCTION

Minimizing aggregation in protein therapeutics is a major challenge in drug development, involving a thorough understanding of the factors influencing protein stability over the product lifetime.[1,2] Aggregation is a critical regulatory concern when choosing a lead candidate for production because of the health risks associated with injecting aggregated proteins into patients,[3,4] and their ability to neutralize antibodies and inhibit the efficacy of the product.[5] Current guidelines advise measuring aggregation using the light obscuration method described in the European Pharmacopoeia methods 2.9.19[6] and the US Pharmacopoeia (USP) <788>.[7] These state that particulates greater than 10 μm in size should be controlled at or below 6000 particles per container, whereas particulates greater than 25 μm should be controlled at less than 600 particles per container.[8]

One common approach to mitigate particulate formation and other degradation phenomena is to determine relative stability with the aid of temperature hold studies, often after screening many candidates using differential scanning calorimetry (DSC).[9] Other methods for looking at the stability of candidates, which rely on structural characterization are available such as circular dichroism[10] and spectroscopy techniques.[11,12] These methods give more specific information about the candidates but cannot easily be used to rank antibody stabilities. Alternative methods are emerging that measure protein stability based on degradation by interfacial effects. These methods allow assessment of the impact of solid–liquid[13] or air–liquid[14] interfaces at the same time as ranking protein stability in formulation conditions. At present, however, there is little standardization of such methods and no consensus on which is the best to use.

Significant amounts of aggregates can form around nuclei over timeframes relevant to modern pharmaceuticals and under the stress conditions they are exposed to.[15,16] Stress is commonly used as a generic term unless specifically stated to describe a range of forces (e.g., hydrodynamic, chemical, thermal, and interfacial) exerted on a protein in different environments.[16–18] For example, one study showed that the aggregate produced was dependent upon whether the solution environment was shaken or stirred.[15,19] These external stresses can directly disrupt the native conformation of the protein molecule, causing changes to the surface charge and hydrophobicity of the molecule,[20] allowing binding to other proteins.[21] In general, the maximum shear rate created during downstream processing is around 20,000 s^{-1}.[22] Such mechanical stress is generated by pumping, filtration, mixing, fill-finishing, shipping or shaking,[23] affecting protein stability,[24] and resulting in a loss of soluble protein.[25]

The effect of shear stress on the aggregation rate of proteins has produced conflicting reports seemingly because of the

Correspondence to: Daniel G. Bracewell (Telephone: +44-20-7679-7031; Fax: +44-20-7209-0703; E-mail: d.bracewell@ucl.ac.uk)

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presence or absence of different interfaces. There are some suggestions that shear causes minor conformational changes to the native structure of the monomer.[26] Monomeric mAb showed no aggregation after 30–51 ms exposure to shear rates up to 250,000 s^{-1}, with only reversible aggregates in the 40–60 unit range observed after 300 s at 20,000 s^{-1} shear. A second sample with an aggregate population of 17% was not significantly altered by the shear rate.[22] Although shear alone appears not to cause formation of large aggregates during mixing and adsorption to stainless steel surfaces shows only small levels of aggregation,[27] together the effects of these forces can more be pronounced. Previous work highlights how at solid–liquid interfaces both the physiochemical properties of the solid interface and the shear above that boundary directly impact the rate of antibody aggregate formation.[28] This infers the hydrodynamic environment directly above the surface is critical to rate of aggregate formation. The rotating disk surface adsorption shear device used in this work was developed at UCL to mimic loss of protein to aggregation experienced through solid–liquid interfacial effects particularly during fill-finish operations.[13] Monomer loss from prolonged operation of the device follows first-order exponential decay and is protein specific, dependent on both disk speed and surface material.[28] By having a well-controlled shear and interface environment and using the coefficient of decay of nine sample points over 2 h of shear device operation, reproducible results can be achieved using this method.

The most prominent class of biopharmaceuticals are IgG antibodies, which are globular plasma glycoproteins with a mass of approximately 146 kDa[29] consisting of two light and two heavy chains, folded to form domains of two beta sheets that create a sandwich shape held together by conserved disulfide bonds and other noncovalent interactions. The two identical γ heavy chains contain around 450 amino acids and consist of three constant and one variable domain. The light chains contain around 215 amino acids and again consist of a constant and a variable domain. These variable domains are the origin of protein specificity.[30] The IgG4 subtype structure differs from the IgG1 subtype in that they are unable to cross-link two antigens because of the monovalent behavior.[31] It has been suggested this behavior is because of an amino acid change from proline to serine in the core of the hinge of IgG1[32] resulting in less disulfide bond formation. The molecule dissociates from the hinge and produces identical monomeric halves, each containing a light and heavy chain. Bispecificity is observed when two different IgG4 monomeric halves reform to give a whole biclonal antibody. This prevents precipitation of the purified antibodies[33] under normal conditions because they are unable to cross-link two antigens.

Considerable work has gone into defining the role of the hinge on the effector functions of an antibody. For IgG, this hinge can generally be divided into upper, middle, and lower regions. The middle hinge stretches from the first to the last cysteine that form disulfide bridges and is believed to be rigid because of interheavy chain disulfide bridging and the subsequent formation of polyproline helices.[34] It is considered that the hinge region acts as a spacer, effecting segmental flexibility and allowing the Fab arms to move in relation to the Fc.[35] A study showed that changes in the amino acid sequences could affect the rigidity of the hinge, which in turn affected the effector function of the antibody.[36] Further studies show that IgG4 has less flexibility in the hinge region than IgG1,[37]

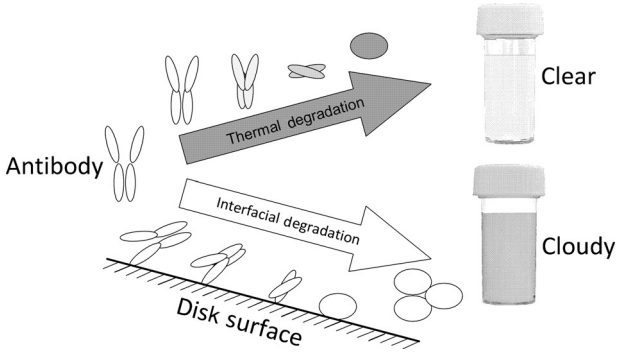

Figure 1. Diagram representing comparability of thermal degradation and interfacial shear-induced aggregation for determining relative stability. The thermal degradation route is based on work on nonnative protein aggregation by Roberts and co-workers,[41,42] whereas the interfacial route builds upon this background with work by Biddlecombe et al.[28]

which correlates with a reduction in stability between the two subgroups.[37,38] This also correlates with a greater hydrodynamic radius of the IgG4 subtype.

In this study, the effects of controlled antibody modifications TM[39] and YTE[40] on a drug candidate's secondary and tertiary structures and overall charge were evaluated. By using the solid–liquid interfacial shear device, the relative stability of protein candidates was measured. This technique was compared with melting temperature (T_m) data from DSC and the results from a 4-week accelerated stability study at 40°C to give relative stability based on thermal parameters. This comparison could be used to determine whether the shear device method could be used as an orthogonal method for lead antibody screening (Fig. 1).

Molecular modeling techniques[43] were then employed to help determine the effect of the modifications on the proteins' secondary and tertiary structures, to help explain any trends seen in the relative stabilities of the antibodies using surface charge and spatial aggregation propensity (SAP) information.

MATERIALS AND METHODS

Antibodies

Five sets of human monoclonal antibodies, three IgG1 and two different IgG4, were expressed by CHO cells and purified using protein A and cation chromatography at MedImmune (Cambridge, UK). Candidates were formulated in a stability buffer containing L-histidine and D(+)-trehalose pH 5.5 at concentrations of 1 mg/mL, and with an overall purity of at least 99%. Antibodies were labeled as IgG1—WT, YTE, and TM YTE—and IgG4—WT and YTE.

Size-Exclusion High-Pressure Liquid Chromatography

A TSKgel3000SWXL (TOSOH Biosciences, Tokyo, Japan) size-exclusion column (7.8 × 300 mm^2) was run using an Agilent 1100 HPLC system (California) comprising degasser, solvent pump, injector, temperature controller, and a fixed-wavelength (DAD) UV detector. The mobile phase was 200 mM sodium phosphate buffer pH 7.4 used at a flow rate of 1 mL/min. Samples were centrifuged at 18,000 g for 10 min prior to size-exclusion high-pressure liquid chromatography (SE-HPLC).