Application of a High-Throughput Relative Chemical Stability Assay to Screen Therapeutic Protein Formulations by Assessment of Conformational Stability and Correlation to Aggregation Propensity

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Received 28 October 2014; revised 12 January 2015; accepted 2 February 2015

Published online 10 March 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24408

ABSTRACT: In this study, an automated high-throughput relative chemical stability (RCS) assay was developed in which various therapeutic proteins were assessed to determine stability based on the resistance to denaturation post introduction to a chaotrope titration. Detection mechanisms of both intrinsic fluorescence and near UV circular dichroism (near-UV CD) are demonstrated. Assay robustness was investigated by comparing multiple independent assays and achieving r^2 values >0.95 for curve overlays. The complete reversibility of the assay was demonstrated by intrinsic fluorescence, near-UV CD, and biologic potency. To highlight the method utility, we compared the RCS assay with differential scanning calorimetry and dynamic scanning fluorimetry methodologies. Utilizing $C_{1/2}$ values obtained from the RCS assay, formulation rank-ordering of 12 different mAb formulations was performed. The prediction of long-term stability on protein aggregation is obtained by demonstrating a good correlation with an r^2 of 0.83 between RCS and empirical aggregation propensity data. RCS promises to be an extremely useful tool to aid in candidate formulation development efforts based on the complete reversibility of the method to allow for multiple assessments without protein loss and the strong correlation between the $C_{1/2}$ data obtained and accelerated stability under stressed conditions. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:1632–1640, 2015

Keywords: chemical denaturation; protein folding/refolding; circular dichroism; calorimetry (DSC); intrinsic fluorescence; guanidine-HCl; urea; protein aggregation; formulation; robotic automation

INTRODUCTION

Biologic therapeutic formulation development continues to be a challenging environment in which any predictive tool that can provide the insight needed to make better early-stage decisions and guide development to a more stable molecule is desirable. The use of proteins as therapeutic agents has been in practice now for decades. Insulin was first introduced as a treatment for diabetes in the 1920s. The approval for the first therapeutic antibody, rituximab (Rituxan; Genentech, Inc. and Biogen Idec Inc.), used in the treatment of B-cell non-Hodgkin's lymphoma was gained in 1997, and antibodies have since become the fastest-growing class of human therapeutics. Protein-based therapy has become an important strategy that has greatly benefited people suffering from various disorders including cancer, Crohn's disease, diabetes, mucopolysaccharidosis, and multiple sclerosis.[1] Large pharmaceutical companies have begun to increase their focus on protein therapeutics, opening the doors for exciting development opportunities. With each new biologic target of interest there remains an increasing formulation design challenge.

Development of biologic formulations pose stability, manufacturing, and delivery challenges related to the propensity of proteins to aggregate.[2] From a process development viewpoint, the propensity for protein aggregation plays a crucial role on the attention to filtration mechanisms, controls for reducing shear stress, container selection, mixing parameters, and storage both short and long term.

One of the largest challenges, specifically on developing ideal formulations, is obviating the propensity for protein aggregation during process development as well as commercial packaging and shipping. Formulation stability studies involve long-term stability at various temperatures in order to mimic real product shelf-life conditions and obtain empirical data for filing. These studies involve multiple scientific disciplines and many resources. The ability to use any predictive assessment on protein aggregation continues to be critical for making early decisions on clinical candidates and/or optimizing formulations early in development thereby saving both time and resources.

Luckily, the formulation development tool-box when it comes to assessing stability of proteins within a given candidate formulation is ever increasing. Size-exclusion chromatography (SEC) has long been the most common method of assessing aggregate formation. For assessing protein thermal stability data, differential scanning calorimetry (DSC) and dynamic scanning fluorimetry (DSF) methodologies have been shown to be effective. DSF, in particular, has an extensive application to protein science in the last decade, ranging from the identification of optimal crystallization parameters,[3] the determination of binding constants,[4] and screening of stabilizing buffer conditions.[5–7] Recently, an application of a kosmotrope-based solubility assay has been used to assess and predict therapeutic protein aggregation propensity.[8] These methods help build the data required for better formulations, yet additional orthogonal methods that are amenable to industry laboratories should be looked upon for

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Journal of Pharmaceutical Sciences, Vol. 104, 1632–1640 (2015)

decision making. One challenge of all of the above mentioned techniques is that in most cases they modify the protein to an irreversible extent. This impedes generation of complete protein thermodynamic data. As unfolding utilizing certain chaotropes is reversible and can be performed at mild temperatures, this orthogonal technique can provide unique thermodynamic data unable to be achieved with other methodologies.

In this study, we developed a high-throughput relative chemical stability (RCS) assay which uses a chaotrope-based stability assessment to orthogonally screen proteins for conformational stability.

It has been demonstrated that solvent-induced protein unfolding can be detected as a function of denaturant concentration by use of any performance measures giving a detectable difference between the native and unfolded states of the protein.[9] Multiple publications have discussed chaotropic denaturation as a technique to assess protein thermodynamic stability at relevant temperatures to their typical use. The Gibbs energy has been experimentally shown to follow a linear dependence with the denaturant concentration[9–13]:

$$\Delta G = \Delta G^{\circ} - m\,[\text{denaturant}]$$

where ΔG^{o} is the Gibbs energy of stability of the protein at zero denaturant (the base stability of the protein at that temperature and matrix free of denaturant influence), and m is universally referred to as the m value but corresponds to the cooperativity of the unfolding reaction.[11–14]

The mid-point of a chemical denaturation curve is dependent upon both the innate stability of the protein independent of denaturant and also upon the denaturing proficiency of the particular denaturant utilized.[10] For our chemical unfolding curves, we label the curve mid-point as the $C_{1/2}$ value, which has been utilized throughout this study as the primary fitting parameter to rank-order protein formulations.

Herein, we describe an automated adaptation of a chaotropic denaturation technique and assess four monoclonal antibodies, a monoclonal antibody fragment, and a small (~6 kDa) therapeutic peptide. We demonstrate the assay concept, investigate multiple detection mechanisms [fluorescence and near-UV circular dichroism (CD)], compare chaotropic agents, and provide assay robustness and reversibility. In addition, we rank-order protein formulations of a mAb by RCS, which has been shown to correlate well with protein aggregation by SEC under accelerated stability conditions (4 weeks at 50°C). The $C_{1/2}$ values used for correlation were determined from chaotrope mid-point (in molar concentration of denaturant) using sigmoidal curve fitting to the entire titration data set. This correlation implies a path forward for predicting conformational stability very early in development with a theoretically zero loss of protein per assessment.

MATERIALS AND METHODS

Reagents

All buffer constituents and HPLC-grade water were obtained from Fisher Scientific (Pittsburgh, Pennsylvania). Urea and guanidine-HCl (GuHCl) were purchased from Sigma–Aldrich (St. Louis, Missouri). Four monoclonal antibodies, a monoclonal antibody fragment, and a therapeutic peptide were utilized for these studies as representatives of common scaffolds found in pharmaceutical development. mAbs 1–4 were produced in mammalian cells. The mAb fragment (50 kDa) and peptide (~6 kDa) utilized were created using proprietary Merck technology. All formulations were prepared by an appropriate compounding procedure and then pH adjusted followed by filtering through 0.22 μm PVDF filters prior to use.

Automated RCS Assay

The 8-tip JANUS automated liquid handling system (PerkinElmer, Waltham, Massachusetts) was calibrated for accuracy using liquid class settings most suitable for aqueous formulations. The robotic protocol design was aimed at minimizing sample carry-over, fully homogenizing sample wells, and reducing overall preparation time. To minimize sample carry over, each liquid transfer step within the protocol was written to begin with a new disposable tip which gets discarded upon transfer completion. To ensure homogenous sample wells, mixing procedures of 100% of final dispensed volume were performed after every step in which mixing was required. Overall run time was minimized by optimally designing labware locations to minimize superfluous arm motion, eliminating aspiration of air-transfer gaps, and utilizing all 8 tips at once during operation.

Keeping the final protein concentration per well equal, a titration of GuHCl or urea (Sigma–Aldrich) in designated formulation matrix was plated in a 96-well Costar clear UV microplate (Corning Life Sciences, Tewksbury, Massachusetts) to a final volume of 200 μL from an 8 M concentrated stock solution. The entire dilution scheme can be found in Table 1. The stock concentration used can vary appropriately. The samples are equilibrated on a bench top for 15 min and assayed at ambient temperature. Post robotic preparation, the plate is immediately mixed by an automated mixing function and read on a SpectraMax M5 Spectrophotometer (Molecular Devices, Sunnyvale, California) at an excitation of 280 nm with an emission scan of 1 nm increments from 315 to 375 nm to determine sample intrinsic fluorescence. Curve fitting of the

Table 1. RCS Assay Dilution Scheme

Formulated Protein (μL)	Formulated Chaotrope Stock Stock Solution (μL)	Formulation Buffer (μL)	Chaotrope Stock Dilution Factor
20	0	180	0.00
20	10	170	20.0
20	20	160	10.0
20	30	150	6.67
20	40	140	5.00
20	50	130	4.00
20	60	120	3.33
20	70	110	2.86
20	80	100	2.50
20	90	90	2.22
20	100	80	2.00
20	110	70	1.82
20	120	60	1.67
20	130	50	1.54
20	140	40	1.43
20	150	30	1.33
20	160	20	1.25
20	170	10	1.18
20	180	0	1.11