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Protein solubilization: A novel approach

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ABSTRACT

Formulation development presents significant challenges with respect to protein therapeutics. One component of these challenges is to attain high protein solubility (>50 mg/ml for immunoglobulins) with minimal aggregation. Protein–protein interactions contribute to aggregation and the integral sum of these interactions can be quantified by a thermodynamic parameter known as the osmotic second virial coefficient (*B*-value). The method presented here utilizes high-throughput measurement of *B*-values to identify the influence of additives on protein–protein interactions. The experiment design uses three tiers of screens to arrive at final solution conditions that improve protein solubility. The first screen identifies individual additives that reduce protein interactions. A second set of *B*-values are then measured for different combinations of these additives via an incomplete factorial screen. Results from the incomplete factorial screen are used to train an artificial neural network (ANN). The "trained" ANN enables predictions of *B*-values for more than 4000 formulations that include additive combinations not previously experimentally measured. Validation steps are incorporated throughout the screening process to ensure that (1) the protein's thermal and aggregation stability characteristics are not reduced and (2) the artificial neural network predictive model is accurate. The ability of this approach to reduce aggregation and increase solubility is demonstrated using an IgG protein supplied by Minerva Biotechnologies, Inc.

1. Introduction

Protein therapeutics is the fastest growing class of drugs in the pharmaceutical industry [1]. As basic research reveals biological pathways, protein deficiencies/abnormalities and key protein targets involved in disease states, we gain a greater understanding of how protein molecules can be used to influence those diseases. The first clinically demonstrated example of a protein therapeutic involved replacement of human insulin for diabetes treatment [2]. Even in those cases that do not involve direct protein replacement, engineered immunoglobulins or other proteins have been demonstrated to influence disease pathways [3].

To achieve an effective protein dose using a small injection volume, the concentration of clinically approved protein drugs typically exceeds 100 mg/ml [4]. Physical stability of the protein at these high concentrations is a critical concern due to potential for an immune response to protein aggregates [5]. Therefore, solubility and physical stability are key variables that must be addressed when developing a protein therapeutic. The International Conference on Harmonization (ICH), whose objective is to harmonize requirements for safety, effectiveness and quality for pharmaceutical products, includes molecular characterization as one factor of a stability indicating profile [6]. To maintain physical stability at high concentration, a large number of different formulations must be evaluated followed by additional optimization of promising candidates—a process that may require several months, consuming significant quantities of protein. A process is needed to efficiently evaluate solubility behavior of protein drug formulations. The following presents a general method to improve protein colloidal stability of protein solutions based on a novel high-throughput HPLC system and a sequential design of experiments (DOE) that provides rational screening of formulations (beginning with individual buffered additives and progressing to complex formulations).

Evaluation techniques used to determine protein solubility behavior can be grouped into two broad categories; (1) detection of aggregates and (2) measurement of protein–protein interactions.

Abbreviations: ANN, artificial neural network; BCA, bicinchoninic acid; DLS, dynamic light scattering; Fab, antibody fragment region; FDA, food and drug administration; GLM, general linear model; HSC, high-throughput self-interaction chromatography; IgG, immunoglobulin G; Mab, monoclonal antibody; MES, 2-(*N*-morpholino) ethanesulfonic; PBS, phosphate buffered saline; R_h, hydrodynamic radius; RMSE, root mean square error.

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Examples of aggregate detection methods include size-exclusion chromatography, analytical ultracentrifugation and dynamic light scattering (DLS). The integral sum of protein–protein interactions (as opposed to site-specific interactions) is typically quantified by a thermodynamic parameter known as the osmotic second virial coefficient (also referred to as the *B* value) [7–10]. The *B* parameter is correlated with both protein solubility [11–13] and the ability of a protein to crystallize [11,13–15,15–17] depending on whether the protein–protein interactions are repulsive (positive *B* values) or attractive (negative *B* values), respectively. Several methods exist to measure *B* values, including osmotic pressure [18], analytical ultracentrifugation [19], static light scattering [14,20] and self-interaction chromatography [8] (SIC).

We previously described the development process of a novel, high-throughput instrument that uses an established technique, self-interaction chromatography (SIC) to rapidly measure *B*-values [8,9,21,22]. SIC requires that the protein of interest is randomly bound to column media (static phase) while the column is equilibrated with the formulation being tested. A small bolus of protein (1 μl) is injected onto the column and its elution volume/time measured by UV absorption. The volume required to elute the protein of interest from the column is related to the protein–protein interactions of the injected bolus of protein with randomly oriented stationary protein. Other variables affecting *B*-value calculations such as the concentration of bound protein and nonspecific protein–media interactions are discussed in the methods section. The initial protein-media binding step requires up to 2 mg of purified protein with each experimental *B*-value measurement in different test formulations consuming an additional 1 μg of protein. The time required for each measurement is approximately two hours (~30–45 min for protein elution and an additional hour for re-equilibration in a new formulation condition). The advantages of SIC over other methods to calculate *B*-values include: (1) relatively straightforward, automatable experimental protocol that does not require specific operator expertise/experience, (2) low total protein consumption, (3) dead column reference and multiple *B* value measurements per run, (4) ability to use the technology for both aqueous and membrane proteins.

In addition to inherent benefits of SIC, the high-throughput self-interaction chromatography (HSC) system includes several key technological improvements including: (1) simultaneous use of four columns, (2) miniaturization of column dimensions resulting in a significant reduction in total protein consumption, (3) a high capacity formulation reservoir with automated robotic dispensing of different formulation conditions, (4) automated data acquisition. This lab previously reported the hazard analysis process which was used in the development of the HSC [23]. Use of the HSC instrument is combined with an approach that includes multiple levels of formulation screens and validation steps to assess: (a) additive influence on protein thermal stability, (b) protein–protein interactions and (c) identify formulations with improved solubility.

2. Material and methods

2.1. Multi level additive screen

The novel approach described in this paper provides a rapid, cost effective method to determine solution conditions that optimize protein solubility. It requires 10–30 mg of protein and two to six weeks depending on the number of conditions needed to optimize solubility. The approach includes multiple levels of high throughput screening along with validation steps at each stage of the process. The overall goal of the approach is to determine which combination and concentration of additives minimize protein–protein attraction, thereby increasing protein solubility.

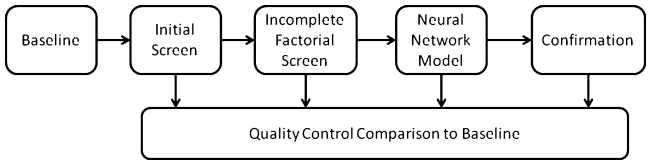

Fig. 1. Experiment flow. Flow chart of the experiments conducted to identify formulations with reduced aggregation. Most steps are followed by two actions: validation and comparison with baseline and the next experiment in the screening process.

In the first stage, additives are screened individually via experimentally measured *B*-values to identify those most effective at reducing protein–protein interactions. More than 300 additives are approved by the FDA for use in injectable drug formulations as inactive ingredients [24]. If all 300 were to be assessed, just three concentration levels of two additives at three pH levels would result in over 1.2 million possible formulation conditions. Even with high-throughput hardware the physical evaluation of this number of formulations is infeasible. The initial screen measures *B* values of the protein in each formulation consisting of the storage formulation along with one additional additive. This initial screen permits rank ordering of the individual additives with respect to their individual effect on the protein under study. This is an important new addition to the screening process and allows for the selection of not only additives known to improve general protein stability, but also those which may be more specific to the protein of interest.

The individual additives identified as producing the most positive *B* values in the initial screen are combined in the incomplete factorial screen using an orthogonal array (assuring combinations of additives are equally represented throughout the screen). These more complex formulations are based on a balanced combination of additives at different concentrations. For each protein studied a numerical model of how additives affect protein–protein interaction (*B*-value) is created by training an artificial neural network (ANN) using experimental data generated from the balanced screen. The neural network model is used to predict the *B*-value of the full factorial of screened additives. The following experimental results demonstrate the ability of an HSC system combined with this multi-tiered screening process to rapidly determine formulations with significantly improved solubility behavior for a candidate IgG protein therapeutic compared to its original formulation.

The flow chart presented in Fig. 1 shows that the entire formulation screen is performed in five distinct phases: baseline measurements, initial screen, incomplete factorial screen, neural network modeling and confirmation with integrated quality control steps. Expanded baseline measurements include SIC as well as DSC and DLS. These measurements are referenced throughout the screening process to identify false positive *B* values. Each step is described in more detail in the following sections, beginning with the HSC instrument that enables automated parallel data collection.

2.2. High-throughput self-interaction chromatography (HSC)

HSC requires covalent attachment of the protein of interest (~2 mg) to media contained in a chromatography column. The same protein is injected into the mobile phase and retention times measured. Protein retention time measurement in each formulation requires approximately 1 μg of additional protein per column. Each experiment requires approximately two hours using traditional column sizes and a precision HPLC. To reduce experiment time and protein consumption compared to traditional *B* measurement techniques, this laboratory reduced column dimensions (0.5 mm × 180 mm) and developed an

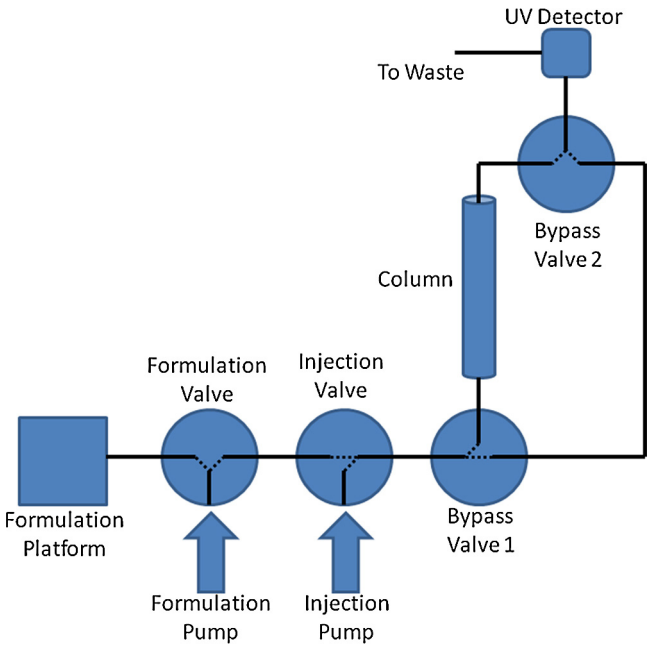

Fig. 2. Schematic drawing of the HSC system. For clarity, this figure shows only a single flow path. The valves, platform and detectors handle four channels in parallel.

automated high-throughput screening system to enable uninterrupted, simultaneous data acquisition from four chromatographic columns.

A schematic drawing of the HSC system is shown in Fig. 2. Four valves control fluid flow throughout the system including the formulation, injection, and bypass valves. The formulation valve controls access of the formulation pump to the reservoir or other portions of the system. This pump withdraws formulations from the reservoir to the formulation syringe which then extrudes these solutions over one of the four columns. Bypass valves enable flushing the entire system with new formulations. This process is fully automated, accommodating 48 different formulations in 10 ml reservoirs. After column equilibration with new formulations, the protein is automatically injected over each column via the injection valve. The retention time of the eluted protein is determined using a UV280 detector.

2.2.1. Baseline measurements

Protein purity is initially confirmed via SDS PAGE and staining with BioSafe Coomassie. Protein *B*-value measurements are established using a "reference buffer" solution which provides a baseline reference for subsequent comparisons to identify formulation improvements. The reference buffer is typically a previously identified "good formulation" solution, judged by the extent of non-specific protein aggregation using size exclusion chromatography (SEC) and/or dynamic light scattering (DLS). To ensure conformational consistency of the protein, the unfolding temperature is measured using a MicroCal VP-capillary differential scanning calorimetry system. The *B*-value for the protein in the reference buffer can be measured via SIC using either a Shimadzu HPLC or the custom HSC system. This *B*-value measurement is the primary metric by which additives and formulations are evaluated. Baseline measurements are repeated between screens to ensure that the integrity of the column is not adversely affected due to changes in capacity (due to irreversible protein binding) and degradation of the media and/or media packing (caused by excessive back-pressure, non-specific irreversible protein binding). The reference buffer for the Fab protein presented in this paper is phosphate buffered saline (PBS—diluted 1× contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4).

2.3. Self interaction chromatography

From a broad perspective, self-interaction chromatography is an affinity chromatography technique that involves binding the protein of interest to chromatography media, injecting a bolus of protein over the column, and measuring the retention time of the protein as it passes through and interacts with the protein bound to the media. Longer retention times are associated with increased protein–protein interaction [7,22]. The following sections provide a detailed description of the SIC method including media selection (based on evaluation of the concentration of bound protein), column preparation, retention time measurement and *B*-value calculation based on protein retention time.

2.3.1. Binding test

There are multiple protein binding chemistries available to covalently bind protein to media such that the point of attachment between the protein's surface and the media is random (this is accomplished by using free amine or carboxyl functional R-groups associated with amino acids). To identify optimal binding conditions, four different chemistries available from TosoHaas are considered: formyl, tresyl, amino and carboxy. For each media type, 20 μl of beads are washed three times with 1 ml of the appropriate binding buffer for the given media (MES or phosphate buffer at pH 6.0 or 8.0 depending on the media). The binding tests are performed by adding 20 μl of 5 mg/ml protein to each sample of washed beads. Reagents are added according to the given chemistry. Table 1 displays the media options tested, their binding formulations, reagents required and binding times. After the binding process is complete, the media, now bound with some quantity of protein, is washed again three times with 200 μl of the binding buffer. A BCA assay is performed on each media sample to precisely quantify the amount of bound protein. This process requires ~0.4 mg of protein (20 μl × 5 mg/ml × 4 tests). In addition to identifying the media with the highest binding capacity, DSC is performed on the protein in the binding buffer, as a quality control step, to confirm that the binding buffer does not destabilize or partially denature the protein. The binding chemistry with the highest quantity of bound protein, as determined by the BCA assay, is used for subsequent preparation of the SIC columns.

Table 1
Chromatography media binding test.

	Binding group	Binding pH	Reagents	Binding time	Blocking	Blocking time
Formyl	$-NH_2$	6.9–9.0	$NaBH_3CN$	4 h	Ethanolamine	1 h
Tresyl	$-NH_2$, –SH	7.0–9.0	None	24 h	Ethanolamine	4 h
Amino	–HOOC, –OHC	4.5–6.0	EDC[a]/NHS[b]	24 h	3HP[c]	4 h
Carboxy	–NH	4.5–6.0	EDC[a]/NHS[b]	24 h	Ethanolamine	4 h

[a] Ethyl(dimethylaminopropyl) carbodiimide.
[b] n-Hydroxysuccinimide.
[c] 3-Hydroxypropionic acid.

2.3.2. Binding and packing a column

For each protein investigated, the protein chemical binding process is scaled up 15-fold to support simultaneous use of three "live" columns ("live" column = column with protein). A fourth "dead" column ("dead" = column without protein) is prepared to serve as a control to assess protein–media interactions. The binding process for the live columns consists of adding 300 μl of 5 mg/ml protein to 300 μl of media. The appropriate reagents are added to the protein media solution followed by placement of samples on a rotating mixer for an appropriate amount of time according to Table 1. After completion of the protein binding step, the media is washed three times with 2 ml of the binding buffer. Using the identical binding reaction, remaining active sites of the media are capped with a capping reagent (0.5 M ethanolamine or 3-hydroxypropionic acid) used in place of the protein solution. At this point, 100 μl of media is prepared with the capping reagent (no protein) to produce a capped-only "dead" column media. It should be noted that the covalently bound protein assures several different orientations on the column media due to the fact that the protein's covalent bond is formed with free amine nitrogens (which are typically available at multiple positions along the proteins exterior surface). Thus SIC-determined second virial coefficients are not less due to a specific protein-media orientation that would prevent interaction with various regions on the protein's surface.

The prepared media is packed into 0.02″ i.d., 1/16″ o.d. and 20 cm length columns consisting of teflon FEP tubing (IDEX) sealed at one end with a union (Valco ZU1CFPK) containing a 2 micron frit (Valco 2SR1-10). Two 1 cm sections are cut from the end of each column for protein analysis via Pierce BCA assay to determine the protein concentration bound to the column media. The concentration of bound protein is a critical variable required to calculate B from the measured protein retention time. Generally, for a 150 kDa protein (IgG) a minimum binding concentration of 5 mg/ml is required to yield a sufficient number of protein interactions to provide the sensitivity needed to accurately measure *B*-values for each solution condition. Finally the packed column is sealed with a union containing a 2 micron frit.

To separate injected protein from the storage buffer in which it is contained a desalting "guard" pre-column is also prepared for each live column and the dead column. This ensures the protein is fully equilibrated in the formulation of interest before it passes over the SIC column. The 60 cm, 0.03″ i.d., 1/16″ o.d. teflon FEP guard column uses Sephadex G-25 media. The media is prepared by soaking in a phosphate buffered saline (PBS) solution, pH 7.4, for 4 h and rinsed 4 times with 5 ml of PBS. The media is packed into the column tubing and sealed at both ends as described for the SIC columns.

2.3.3. Retention time measurements

A guard column is positioned prior to each live and dead column to separate absorption peaks caused by salts, buffer and other small molecules contained in the base buffer used to solubilize the protein (these small molecules sometimes absorb at or close to 280 nm and co-elute close to the protein absorption peak). The system is equilibrated in the formulation of interest by passing approximately 5 system volumes of formulation solution through the columns. After equilibration, a standard affinity chromatography experiment is performed by injecting the protein of interest in the mobile phase formulation at a constant 8 μl/min flow rate and protein elution time is measured from the time of injection until the time of elution from each column (protein elution is detected via U.V. absorbance at 280 nm). The retention time is defined as the time required for the protein to pass through the SIC column only—total elution time through both columns minus the elution time through the guard column, measured separately. This process is repeated for each of the additive formulations contained in the initial and incomplete factorial screens.

2.4. DSC confirmation

B-values for protein in each formulation are measured followed by assessment of protein integrity for the nine additives yielding the most positive *B*-values (this is assessed using DSC measurements of protein thermal stability). This step serves as a quality control measure to ensure that protein denaturation is not a significant factor affecting protein retention times. Samples are prepared for DSC by buffer exchange using a centrifugal concentrator. A quantity of 0.25 mg protein from solution is added to the centrifuge tube. Three ml of formulation is added and spun down at 3000 rpm in an Eppendorf-5810R centrifuge to 500 μl and this is repeated four times for a buffer exchange of over 99.9%. The final effluent is used as the blank control for the DSC experiment. If a formulation fails the DSC confirmation step (a drop in unfolding temperature of more than 4 °C) the next most positive *B*-value formulation is evaluated until nine additives are identified that improve *B*-value without significantly reducing protein unfolding temperature.

2.5. B value screens

2.5.1. Initial screen

The initial screen is used to identify individual additives that contribute to protein-protein repulsion (positive *B*-value). The formulations for the initial screen are prepared by combining 10× concentrated phosphate buffered saline (PBS – diluted 1× contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_3PO_4, 2 mM KH_2PO_4), and a single additive from concentrated stock, filled to 90% volume with Millipore H_2O and titrated with NaOH or HCl to pH 7.4. The list of forty additives, each tested individually in the initial screen, can be found in Appendix A. The *B*-value of the protein of interest is measured in each formulation using SIC. The additives most beneficial to reduce protein–protein interactions are not known a priori. Therefore, the purpose of the initial screen is to identify additives that reduce protein–protein interaction for a given protein. Thermal stability of the protein combined with those individual additives exhibiting the nine highest *B*-values is then confirmed using DSC. Three salts and six additives with the highest *B*-values which also maintain thermal stability via DSC (defined as those additives that do not result in more than a negative 4 °C temperature shift) are chosen for the incomplete factorial screen.

2.5.2. Incomplete factorial screen

After completion of the DSC tests, the top nine solutions are combined in an incomplete factorial using an orthogonal array according to the Taguchi method [25]. This method ensures that additive identity and additive concentration are equally represented throughout the incomplete factorial screen. The screen specifies more complex formulations with multiple additive components at high, medium and low concentrations. *B*-values of the protein in each formulation of the incomplete factorial screen can be measured by SIC using either the HSC system or a Shimadzu HPLC.

2.5.3. Neural network training

The measured *B*-values are used to create a numerical model of how specific additives in the formulation affect protein–protein interaction. An artificial neural network (ANN) model [26] is first trained five separate times using a different random set of 4/5 of the data with the remaining 1/5 of the data used for validation. The weights of the neural network are adjusted based on the training set using a standard back-propagation algorithm until the error of the validation set (not used to adjust weights) is no longer improved

by incremental changes to the ANN weights. This is an established method to train and prevent over-fitting of an ANN and is described in Bishop's book, Neural Networks for Pattern Recognition [27]. In a previous publication [28], our lab compared this method to that of a standard general linear model (GLM) and found the ANN to exhibit reduced prediction error compared to the GLM.

2.5.4. Neural network prediction and confirmation

The trained ANN returns a *B*-value given a formulation where the output is based on previously measured *B*-values. After training is complete the neural network is presented with each formulation in the complete factorial combination of all parameters measured. This consists of over 4000 possible formulations that combine one or two additives, a salt and a buffer. The ability of the ANN to predict *B*-values of novel formulations is evaluated by *B*-value measurement (by SIC) of several predicted formulations, chosen throughout the predicted *B*-value range. A root mean square error (RMSE) is determined for the model predictions compared to the actual *B*-values measured. Confirmation measurements serve two purposes: an evaluation of the model predictions and confirmation of predicted formulations with improved *B*-values.

2.6. Thermal stability confirmation

The predicted formulations with increased *B*-value measurements are experimentally validated followed by DSC confirmation of protein thermal stability in these new formulations.

2.7. Indirect solubility testing

Dynamic light scattering (DLS) is used to indirectly test the solution solubility characteristics of the protein by evaluation of the aggregation properties of the protein as protein concentration is increased. Due to a limited supply of protein (the proprietary Fab protein, provided by Minerva Biotechnologies, Inc., used for these experiments was of limited supply), DLS measurements were restricted to the most positive *B*-value formulation (by prediction and measurement) and the most negative *B*-value formulation from the initial screen. The rationale is to determine if more positive *B*-value predicted formulations produce significant improvements in solubility behavior. Each protein formulation is filtered through a 0.22 micron filter into a 10 μl quartz 90° scattering cell. Scattering counts are measured using a Wyatt DynaPro Titan DLS system to confirm that there is not a significant scattered light signal from the buffer itself. The protein is buffer-exchanged into each formulation using centrifugal concentrators. Diffusion coefficients and apparent hydrodynamic radii of the protein are measured in the solution at a fixed concentration. The Fab sample is concentrated with DLS measurements taken at various points during in the concentration process. The aggregation behavior of the protein is compared as a function of protein concentration for each formulation. The minimal use of protein required for DLS measurements and the fact that protein can be recovered for additional tests resulted in this being the method of choice for comparing aggregation behavior of the protein.

3. Calculation of *B*-value

The standard method of *B* value calculation, published by Tessier, Lenhoff and Sandler [8], is used in this research:

$$B = \frac{N_A}{MW^2}\left(V_{HS} - \frac{k'}{\varphi\rho}\right)$$

In this equation, N_A is Avogadro's number (molecules/mol) and MW is molecular weight (g/mol). V_{HS} (ml/molecule) is the hard sphere volume of the protein of interest (calculated as a globular protein based on molecular weight). The parameter, φ (cm^2/ml), is the phase ratio of the media defined as the ratio of the surface area available to that of the volume available to a mobile phase protein passing through the media. Phi is a characteristic of the media used and molecular weight of the protein and has been determined for several media types including TosoHaas affinity media [29]. The parameter ρ (molecules/cm^2) is the amount of protein per unit area bound to the media (determined via the BCA assay as described previously).

The final parameter k' is the retention factor. While the other parameters are fixed for a given column, the k' measurement is the primary variable associated with changes in protein–protein interactions. k' is calculated according to the following [8]:

$$k' = \frac{(V - V_0)}{V_0}$$

V is the retention volume of the protein over the live column and V_0 is the retention volume of an equivalently sized non-interacting marker. Previously an acetone marker has been used as the non-interacting marker and a correction was used to adjust for the small size of the acetone marker compared to the protein [8,9,30]. However, the addition of the guard column used to equilibrate the protein in the mobile phase substantially shifts the acetone marker with respect to the protein marker. In the multi-column system, the dead column is used to identify the non-interacting retention volume. Therefore, V_0 is the retention volume of protein eluted from the dead column in the formulation of interest. This method has the added benefit of accounting for changes in protein–media interactions in the presence of different formulations.

4. Results

Fab protein was subjected to the novel multi-tiered screening process. The specific antigen is proprietary and not known to our laboratory.

4.1. Minerva Fab-initial screen

The primary goal of the Minerva project was to produce a highly soluble storage solution for the protein, not a solution for direct injection. Therefore, this screen contains additives and concentrations not approved for human use. Identifying such a formulation is useful for long-term storage of protein produced on a large scale and for preliminary formulations that can be used with other structure–function analysis methods as well as for *in-vivo* animal studies. With different initial screen components the screening methodology and high-throughput technology are applicable to preparation of solution conditions for pre-clinical evaluation. Minerva provided our lab with ~25 mg of the Fab portion of a proprietary monoclonal antibody (Mab) being considered for future clinical trials. It was assumed that if improved solubility conditions could be discovered for the Fab, these conditions would also exhibit improved solubility for the complete monoclonal antibody. Components and concentrations of the additives used in this screen can be found in Appendix A.

Table 2 shows the additives producing the nine highest *B*-values chosen from the initial screen. This includes the additives that failed DSC confirmation (1,6-hexanediol and Li_2SO_4). These two additives were replaced with those producing the next most positive *B*-values, NaCl and Glutamic Acid. The additives chosen from the initial screen are applied to an orthogonal array [31] to determine the additives and concentrations used for each formulation condition in the incomplete factorial screen. A full list of the 36 formulations in this phase of the screen can be found in Appendix B and the most positive *B*-values identified in the screen are in Table 3.

Table 2
Most positive *B*-values of Minerva Fab initial screen.

Additive name	*B*-value[a] measured	Delta T_M (°C)
Default Buffer-PBS	0.2	+0.0
1. 0.1 M Arginine	5.8	+0.6
2. 400 mM LiCl	5.1	−1.6
3. 400 mM Na Thiocyanate	4.4	−3.7
4. 0.1 M Arg., 0.1 M Glu. Acid	3.2	NA
5. 400 mM $LiSO_4$	1.2	−9.0
6. 0.1 M Glucose	0.8	−0.6
7. 400 mM Na Citrate	0.1	+2.5
8. 0.1 M Trehalose	0.0	−0.1
9. 10% (w/v) 1,6 Hexanediol	0.0	−10.5
10. 400 mM NH_4 Citrate	−0.6	−2.3

[a] ($\times 10^{-4}$ mol ml/g^2).

Table 3
Most positive *B*-values from minerva Fab incomplete factorial screen.

Formulation	*B*-value[a] measured
Default Buffer–PBS	0.2
1. 0.1 M Phosphate pH 7.4, 0.15 M NaCl, 0.2 M Na Citrate, 0.6 M NH_4 Citrate	5.6
2. 0.1 M Phosphate pH 7.4, 0.15 M NaCl, 0.1 M Trehalose, 0.05 M Glucose	4.1
3. 0.1 M Tris pH 8.0, 0.05 M LiCl, 0.4 M Na Citrate	2.7
4. 0.1 M Phosphate pH 7.4, 0.05 M NaSCN, 0.4 M NH_4 Citrate, 0.15 M Arginine	2.1
5. 0.1 M MES pH 6.1, 0.1 M NaCl, 0.05 M Glutamic Acid, 0.15 M Trehalose	1.9
6. 0.1 M Phosphate pH 7.4, 0.05 M LiCl, 0.15 M Trehalose	1.4
7. 0.1 M Tris pH 8.0, 0.1 M NaCl, 0.1 M Trehalose, 0.15 M Arginine	1.4
8. 0.1 M MES pH 6.1, 0.15 M NaSCN, 0.1 M Glutamic Acid	1.4

[a] ($\times 10^{-4}$ mol ml/g^2).

From the 27 different neural networks trained, the 5 × 2 topography provides the smallest validation error across all validation sets for the Minerva Fab protein. The average validation error is 1.2 B units. The trained neural network produces a range of *B*-value predictions from −5.4 to 4.3 *B* units and 4 formulations from the top quartile of *B*-values are chosen to yield improved formulations. Different topologies represent a different number of variables considered for influence on *B*-value. It is expected that some topologies (those that consider too few or too many variables) would produce lower validation errors than others. The evaluation of multiple topologies is automated and does not require additional effort and accounts for the fact that the number of variables which influence *B*-value are expected to differ from protein to protein. The measured confirmation of *B*-value by SIC and change in unfolding temperature by DSC are given in Table 4.

The restriction on protein quantity received (25 mg) limits the maximum solubility that can be determined for a given formulation. In the case of the Minerva Fab the formulations submitted to the company were tested by the company with larger protein quantities. Minerva concentrated the complete monoclonal antibody (Mab) in each formulation until visible precipitation was observed. These results are shown in Fig. 3.

Table 4
B-value confirmations and DSC unfolding temperatures for Fab.

Formulation	*B*-value[a] predicted	*B*-value[a] measured	Delta T_m (°C)
1. 100 mM Tris pH 8.0, 100 mM NaCl, 150 mM Trehalose, 400 mM NH_4 Citrate	3.4	0.8	+5.4
2. 100 mM Tris pH 8.0, 150 mM NaSCN, 100 mM Trehalose 600 mM NaCitrate	1.6	1.6	+5.0
3. 100 mM Tris pH 8.0, 150 mM LiCl, 150 mM Arg-HCl, 50 mM Glucose	1.0	0.9	+0.2
4. 100 mM Phosphate pH 7.5, 150 mM NaCl, 100 mM Trehalose, 100 mM Glutamic Acid	4.1	2.1	+1.9

[a] ($\times 10^{-4}$ mol ml/g^2).

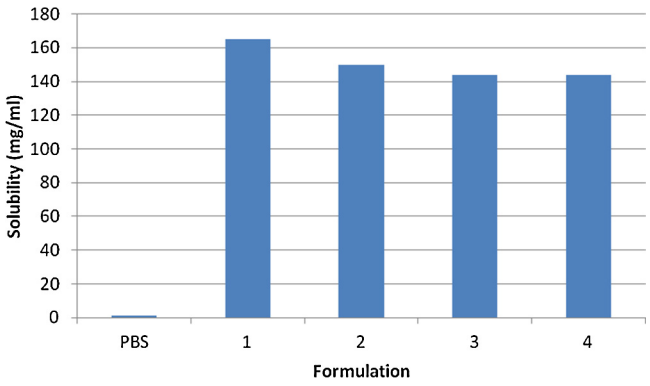

Fig. 3. Solubility estimates of Fab from Minerva.

5. Discussion

Each step in the screening process is an important part of determining improved formulation conditions. The following discussion compares the results of the protein evaluation. The following subsections are focused on a single step in the screening process outlined in Fig. 1.

5.1. Baseline

Baseline measurements are important for both quality assurance (of the initial quality of the protein) and quality control (of formulation improvements). The baseline unfolding temperature provides a reference to quantify shift in unfolding temperature for protein equilibrated in each formulation. In the case of denatured protein, DSC does not result in a positive heat capacity signal and can be used to identify formulations which denature the protein. An additional quality assurance step includes evaluation of protein binding to a small quantity of each media type to identify the optimum binding chemistry for a particular protein. Both proteins bound to Tresyl media at greater than 7 mg/ml and demonstrated typical IgG unfolding temperatures from 60 °C to 90 °C. This ensures that the best media is chosen for protein binding.

Regarding use of PBS (pH 7.4) as the baseline formulation, it should be noted that proteins close to their isoelectric point are generally less soluble; a pH of 7.4 is close to the IgG isoelectric point of 8.2. In the case of the Minerva protein the simple task of reducing pH of the buffer did not increase solubility. This protein and most other proteins evaluated are generally “problem” proteins (proteins that exhibit low solubility). The starting point for the formulation screen for Minerva’s Fab was the best available based on information provided by Minerva (the company approached Soluble Therapeutics due to their prior difficulty improving the solubility of the protein). Obviously, no single screening process can *always* produce and guarantee the maximally optimal formulation. That would require a complete factorial testing of the search space. As noted in the introduction, even with severe limitations on the dimensionality of the search space (2 additives, 3 concentration and pH levels) an exhaustive search of the space is not feasible. Soluble Therapeutics’ screening process is designed to improve formulations with an approach that begins with identification of individual additives that influence protein–protein interactions followed by expansion to more complex formulations that contain multiple additives and

additive concentrations. After individual additives are selected, an incomplete factorial additive screen is used to improve the chance of identifying combinations of additives that work well together.

5.2. Initial screen

The initial screen of individual additives includes additives not in the FDA database. A wide range of additives was chosen in order to evaluate the overall system's capability to predict *B*-values that modify solubility behavior. Each additive formulation is screened using SIC to identify the *B*-value associated with the additive. Individual additives with the highest *B*-values from the initial screen are identified in Tables 2 and 5 for the Minerva Fab. Formulation optimization experiments performed with proprietary proteins (both IgGs and other protein classes), using FDA-approved additives, resulted in alternative additives and additive concentrations.

As noted earlier, PBS was both the starting point for the initial screen and the "best" formulation available (defined as the base buffer) at the time. This is usually not the case. However, when a more optimally soluble formulation is available it is not necessarily the best starting point for the initial screen. Beginning with a complex formulation augmented with additives is likely to result in a search space located around a local maximum (but not necessarily the global maximum). Identification of a minimal buffer (such as PBS) allows for the inclusion of individual additives that may have a significant impact on protein–protein interactions; this may not be distinguishable in an already complex formulation. Thus, the minimal buffer formulation enables broadening of the search space to include more diverse formulations for the incomplete factorial screen.

Although some individual additives, such as trehalose, are known to improve thermal stability [32], the primary metric of the HSC system is *B* value. This measure of protein–protein interaction serves as the ranking criteria for all additives in the screen, with DLS and DSC measurements acting as secondary quality control measurements. Additives which increase *B* value are not necessarily the same as those which improve thermal stability. On the contrary, there has not been a published direct correlation between thermal stability and solubility or thermal stability and *B* value. These are independent measurements and because the primary goal of the screen is to identify a formulation with increased colloidal stability, prior knowledge of thermal stability is not necessary or sufficient to identify improved solubility formulations.

5.3. Incomplete factorial

The incomplete factorial screen design is based on experimental results from the initial screen. Additives identified in the initial screen (a different set for each protein) are combined using an orthogonal array to ensure equal representation of each additive throughout the screen. Each formulation in the incomplete factorial is evaluated by SIC to determine the *B*-value of the protein in the formulation. Appendices 3 and 4 identify all additives in the incomplete factorial for the Minerva Fab. The formulations from the incomplete factorial with the most positive *B*-values are listed in Table 3.

5.4. Neural network

Neural network training produced a Fab validation error 1.2 *B* units and a percent validation error of 12.4%. After training, prediction of the complete factorial of additive combinations resulted in both positive and negative *B*-value predictions.

The range of *B*-values predicted for the Minerva protein was −5.4 to +4.3 *B* units. Even before empirical confirmation, the broad range of these *B*-values included many formulations that were expected to have high solubility. Literature on the empirical relationship between *B*-values and solubility [11–13,33], shows that protein solubility increases with *B*-value. The increase in solubility is nonlinear with respect to *B*-value with a more rapid increase above zero. The solubility reported by Minerva (Fig. 3) confirms that the formulations with high positive predicted *B*-value have improved solubility.

6. Conclusion

The results demonstrate a significant improvement for the predicted formulations versus the original formulation with respect to both increased *B*-values and increased protein solubility in the top four predicted formulations. The approach utilizes our novel technology (HSC) and design of experiments to evaluate multiple tiers of additive formulations. The results from these screens are evaluated with an artificial neural network model to identify formulations with improved solubility behavior. The formulations identified for the Minerva Fab improved solubility one-hundred fold over the existing baseline formulation and enabled the protein to advance to animal trials using two of the best predicted formulations.

The novel contributions of this paper are three-fold. (1) The addition of an initial screen to previous methodologies which allows a large number of individual additives to be ranked without a-priori knowledge of how those additives will affect *B* value for a given protein. This was demonstrated with the inclusion of both expected (trehalose) and unexpected (LiCl) additives in the incomplete factorial. (2) The use of DSC and DLS as quality control methods in a larger multi-tiered screening process. This permits the exclusion of false-positive *B* values due to protein denaturation as well as the inclusion of a formulation. (3) Demonstration that the first two contributions enabled the identification of a formulation with a 100-fold increase in solubility for a pharmaceutical protein.

It is important to emphasize the incorporation of baseline checks throughout the process. Differential scanning calorimetry is essential to exclude false positives. After the initial screen of formulation additives, it is common to have at least one or two additives which reduce the unfolding temperature of the protein or result in denaturation, but result in positive *B*-values due to size exclusion effects. One could argue that a reduction in unfolding temperature of 2–3 °C is not significant in the thermal stability of a protein—especially if those temperatures are around 80 °C. However, a strong relationship between unfolding temperature and biological protein activity has not yet been evaluated for a significant number of proteins. In this screening process a cautious approach is taken to eliminate additives which could alter activity due to a conformational change. This is acceptable due to the large number of additives evaluated and the small incremental cost due to additional protein consumption. As more evidence is gathered regarding the nature of unfolding temperatures, solubility and activity, operating procedures will be adjusted. The evaluation presented in this paper provides a snapshot of a current screening technique to improve solubility behavior of therapeutic proteins. An aspect of this research that must be emphasized is that of solubility behavior vs absolute solubility. Absolute maximum solubility is difficult to measure for a protein solution because of the ability of a protein to super-saturate and result in significantly different solubility maximums in slightly different formulation conditions. Therefore, one approach that may prove useful is to evaluate the tendency of the protein to aggregate at increasing concentrations. Laser light scattering (and an observed A_{280} signal decrease) can be used to assess the tendency of different formulations to produce increasing protein aggregation. This technique allows protein solubility assessment with a small amount of protein. To our knowledge, a method does not

exist (when only small quantities of purified protein are available) to evaluate absolute protein solubility for proteins exhibiting a high maximum solubility.

The improvement of Mab solubility based on Fab screening suggests that problematic protein domains could be formulated separately to improve solubility of the complete protein. This result is supported by other work in our lab that involves determination of solution conditions that improve the solubility of cystic fibrosis transmembrane conductance regulator (CFTR) protein. Due to the difficulty in expressing a sufficient amount of this integral membrane protein to support self-interactions studies, one of CFTR's nucleotide binding domains (NBD1), was used to determine solution conditions that also improved solubility of the full-length protein [34]. Although there is insufficient evidence to assume that this might apply to all proteins that exhibit poor solubility, when a specific domain is known to cause solubility problems (such as with NBD1 or a specific Fab) solubility improvements of the domain is a valid starting point.

An alternative method to improve solubility is mutagenesis of specific amino acids. This technique was successfully utilized by Bethea et al. and protein–protein attraction was evaluated primarily with the use of cross-interaction chromatography [35]—an identical technique to self-interaction chromatography with the exception that the mobile phase and stationary phase consist of different molecules (Fab and Mab) [36]. In the Bethea publication Fab and Mab were both shown to have significantly negative B values, although the Fab did not display a population of different size aggregates due to postulated formation of stable and homogeneous tetramers. Conditions that reduced the interaction/attraction between the Fab and the Mab were demonstrated to also reduce Mab–Mab interactions. As the protein–protein interaction is detectable in both the Mab and Fab fragments it is expected that the formulation screening technique presented here (performed using self-interactions for one domain of a protein) could be used to identify additives which reduce Fab protein–protein interactions, conditions that may also reduce Mab–Mab interactions.

As a method to enhance comprehensive formulation screens (multiple tiers of additives and an associated hardware) the HSC system could benefit from improvements that would reduce protein consumption and experiment time. Improvements to each step in the evaluation process can potentially reduce protein use and time. In addition to efficiency (time and protein) optimizations, we are exploring expansion of applications. For example an additional improvement would include use of circular dichroism as a reference to ensure that the protein structure is not significantly changing. This would be beneficial for therapeutic proteins at the pre-clinical stage in which end point results are undergoing additional analytical evaluations (e.g. the Minerva Fab).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.09.003.

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