Journal of Pharmaceutical Sciences xxx (2016) 1–10

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

# Prediction of Protein Aggregation in High Concentration Protein Solutions Utilizing Protein-Protein Interactions Determined by Low Volume Static Light Scattering

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ARTICLE INFO

*Article history:*
Received 26 November 2015
Revised 27 February 2016
Accepted 15 March 2016

*Keywords:*
protein formulation
preformulation
protein aggregation
physical stability
light scattering (static)
protein
interaction

ABSTRACT

The development of highly concentrated protein formulations is more demanding than for conventional concentrations due to an elevated protein aggregation tendency. Predictive protein-protein interaction parameters, such as the second virial coefficient $B_{22}$ or the interaction parameter $k_D$, have already been used to predict aggregation tendency and optimize protein formulations. However, these parameters can only be determined in diluted solutions, up to 20 mg/mL. And their validity at high concentrations is currently controversially discussed. This work presents a μ-scale screening approach which has been adapted to early industrial project needs. The procedure is based on static light scattering to directly determine protein-protein interactions at concentrations up to 100 mg/mL. Three different therapeutic molecules were formulated, varying in pH, salt content, and addition of excipients (e.g., sugars, amino acids, polysorbates, or other macromolecules). Validity of the predicted aggregation tendency was confirmed by stability data of selected formulations. Based on the results obtained, the new prediction method is a promising screening tool for fast and easy formulation development of highly concentrated protein solutions, consuming only microliter of sample volumes.



## Introduction

Most of the marketed therapeutic antibodies are intended for intravenous application. If medical indication and drug safety allows self-administration at home, patients' compliance and treatment success can be significantly improved. However, subcutaneous or intramuscular applications, which are preferable than, are limited in injection volumes and often require highly concentrated formulations of 100 mg/mL or more.[1]

Besides solubility and viscosity issues, long-term stability, especially aggregation, is an important concern. Therefore, screening efforts are increased and low volume, high-throughput methods are desirable in highly concentrated protein formulation (HCF) development.[1] Attractive or repulsive forces between protein molecules can increase or decrease aggregation tendency in a given formulation. Protein-protein interactions (PPIs) have been used previously to predict aggregation tendency of proteins in solution. PPI can be determined by applying several methods. Most frequently determined parameters are the second virial coefficient ($B_{22}$) and interaction parameter ($k_D$). Many studies have been conducted where PPIs were investigated in terms of pH and ionic strength in solution.[2-8] In contrast, less data are available for the effect of additional excipients at high protein concentrations, although it is well-known that sugars, amino acids, and surfactants can significantly impact PPI and colloidal stability.[9]

$B_{22}$ values have been clearly demonstrated to correlate to protein aggregation, especially at low[2,10,11] but also at high[4,12] concentrations. Nevertheless, validity of parameters measured in dilute solutions and transferability to HCF clearly depends on molecule properties and cannot be generalized. Predominant PPIs and mechanisms of aggregation at higher concentrations can differ from those at lower concentrations.[13-15] Saluja et al.[16] stated that in certain cases dilute solution analysis fails to capture the effect of the solution "environment" on protein behavior, but analysis at target concentrations may be successfully used to distinguish between effects. On the other hand, Yadav et al. demonstrated that the magnitude of interactions measured at low concentrations using $k_D$ did not correlate to viscosity at high concentrations. In contrast, the same author reported that interactions derived from storage modulus $G'$, measured at high

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http://dx.doi.org/10.1016/j.xphs.2016.03.022
0022-3549/

concentrations, did correlate to viscosity.[15] Kumar et al.[14] showed that electrostatic interactions governed aggregation at low concentrations but short-range hydrophobic forces determined aggregation at high concentrations, and the second virial coefficient correlates to low concentration aggregation behavior only. In addition, interactions in dilute solutions are not as clear compared to high concentrations and relevant effects might be easily overseen.[4,17] In these exceptional cases there is a need for analytical methods to measure PPI at high(er) concentrations.

This work provides an alternative way to investigate PPI directly at elevated protein concentrations. The method targets early drug discovery project needs in HCF development and is applicable for candidate and formulation screenings. The investigations presented in this work are based on nonideal scattering behavior of high solute concentrations due to intermolecular interactions. The concept of nonlinear scattering at increasing protein concentrations was reported previously and different parameters, such as the structure factor, relative hydrodynamic radius, or apparent molecular weight, have been described.[11,17–19] The presented work determines the extent of nonideality at a specific concentration to compare nature and strength of PPI and rank them according to their aggregation tendency.

Several needs in particular for HCF development during early drug discovery project needs are addressed:

1. A simplified approach for fast-track screening and data interpretation was evaluated. Low-volume and high-throughput requirements associated with HCF development are met.
2. Broad applicability was proven, including a wide range of formulation compositions (more than pH and ionic strength), as well as 3 therapeutic molecule classes.
3. The method enables screening at high target concentrations. As a result, potential discrepancies between dilute screening results and true conditions at HCF can be avoided.

Considering the increasing number of recent biologic drugs, such as Fc-fusions (FcFs), cytokine traps, or novel binder constructs, which significantly deviate from the well understood monoclonal antibody (MAb) structures, an early screening option and a deeper understanding of their aggregation tendency in a given formulation is of great interest for high concentration formulation development.

## Materials and Methods

### Material

#### Model Proteins

An immunoglobulin (IgG)1 MAb with an isoelectric point (pI) of 7.5, an FcF protein with an pI of 9.0, and a single domain antibody (SDA) construct with an pI of 8.0 were provided by Merck KGaA (Darmstadt, Germany). Purity of all species was confirmed by size exclusion high-performance liquid chromatography (HPLC) (SE-HPLC) >98% monomer.

#### Reagents

L-arginine, citric acid monohydrate, di-sodium hydrogen phosphate, L-glycine, L-histidine, 1M hydrochloric acid, sodium chloride, sodium dihydrogen phosphate monohydrate, 1M sodium hydroxide solution, sodium perchlorate monohydrate, sucrose, polysorbate 20 (PS 20), and polysorbate 80 were obtained from Merck KGaA. Hydroxypropyl β-cyclodextrine (Kleptose HPB parenteral grade) was obtained from Roquette (Lestrem, France), trehalose dihydrate was purchased from VWR Chemicals (Darmstadt, Germany), and polyethylene glycol 4000 from Sigma Aldrich (Steinheim, Germany). All solutions were prepared with Milli-Q grade water freshly taken from a Milli-Q gradient A10 water purification system (Merck KGaA).

### Methods

#### Sample Preparation

All formulations were prepared using ultrafiltration units from Sartorius AG (Göttingen, Germany) with polyethersulfone membranes and 10 kDa molecular weight cut-off for SDA and 30 kDa for MAb and FcF, respectively. Ultrafiltration was performed in a Heraeus Multifuge 3SR+ (Thermo Fisher Scientific, Inc., Waltham, MA) at 4000 rpm. Content was adjusted on the basis of spectrophotometric measurements at 280 nm (Implen GmbH, Munich, Germany) in disposable polystyrene cuvettes (Brand GmbH & Company KG, Wertheim, Germany). Samples were sterile filtered through Millex GP 0.22-μm polyethersulfone filters (Merck KGaA) and filled in 0.8 mL glass vials (VWR Chemicals) for storage. Vials were manually crimped with aluminum caps (VWR Chemicals). Table 1 summarizes all formulations tested for aggregation tendency and confirmatory stability studies.

#### Prediction of Colloidal Stability

The measurement principle is based on nonideal scattering behavior at increasing solute concentrations. Nonideality is caused by changes in the apparent molecule radius due to changes in molecule shape, packing, or protein clustering. As a result, scattering intensity increases (or decreases) in a nonlinear way at elevated protein concentrations, due to attractive (or repulsive) PPI.

In Figure 1, light scattering signal is illustrated for protein concentrations up to 100 mg/mL. The effect of PPI on scattering intensity accumulates at higher concentrations and sensitivity to apparent interactions increases. However, net interactions or the relative order of interactions screened does not change. A concentration of 40 mg/mL was chosen for further investigations as a compromise of screening sensitivity and protein requirement. The extent of nonideality at 40 mg/mL was then utilized to predict aggregation tendency for different formulation compositions. By definition, prediction is meant here as relative ranking of aggregation tendency or chronological order of beginning of aggregation. A quantitative correlation to absolute aggregation rates was not possible.

Detailed data evaluation and statistics applied were previously described in Hofmann et al.[20] In short, the procedure is as follows. Static light scattering intensity ($I_{LS}$) at 473 nm was determined at a 90° angle for all formulations at 2, 4, 6, 8, and 40 mg/mL for FcF or at 4, 6, 8, 10, and 40 mg/mL for MAb and SDA. Each concentration was measured in triplicate with 9 μL sample volume using an Optim1000 System (Avacta Group plc, Wetherby, West Yorkshire, UK). Concentration ranges of linear and nonlinear scattering were determined by residual analysis in prior experiments. Linear fits of light scattering intensity over concentration were conducted using 4 different concentrations in the proven linear range (up to 10 mg/mL). Subsequently, deviation from extrapolated linear Rayleigh scattering to apparent scattering (Δ%) at a single concentration of the proven nonlinear range (e.g., 40 mg/mL) was calculated based on Equations 1 and 2, respectively:

$$\Delta\%_{\text{attractive interactions}} = \left( \frac{actual\ I_{LS}}{extrapolated\ I_{LS}} - 1 \right) \cdot 100 \qquad (1)$$

$$\Delta\%_{\text{repulsive interactions}} = \left( \frac{\text{extrapolated } I_{LS}}{\text{actual } I_{LS}} - 1 \right) \cdot - \ 100 \qquad (2)$$

where actual light scattering intensity (actual $I_{LS}$) is divided by extrapolated light scattering intensity (extrapolated $I_{LS}$) if attractive interactions are indicated and actual $I_{LS} \geq$ extrapolated $I_{LS}$. Inverse ratio is built, if repulsive interactions are indicated and extrapolated