# Abstract

Monoclonal antibodies are one of the fastest growing classes of pharmaceutics. Although they possess an almost completely human aminoacidic sequence, special attention needs to be paid to limit adverse immune responses, which are, due to invasive application, most often caused by aggregates of different sizes. To ensure efficacy, safety, and quality of biological drug products until the end of their shelf life, it is therefore essential to find conditions that efficiently slow down aggregation already in the early development. Evaluation and optimization of long-term stability is thus a crucial step in development of biologics, which is still long-lasting and introduces substantial risk to the development. To date it hasn't been unambiguously shown that increasing thermodynamic and colloidal stability, a primary strategy in the recent years, really leads to lower aggregation rate. On the other hand, with aim to accelerate development, aggregate increase in different formulations is being evaluated at higher temperatures, which doesn't necessarily reflect changes until the end of shelf-life at storage temperature, usually three years at 5 °C.

In this work, I present several approaches for shortening time needed for aggregation assessment and, thus, accelerating formulation optimization aimed specifically at slowing down aggregation at storage conditions. For the group of antibodies used in oncology, immunology, rheumatology, for therapy of osteoporosis, and blood diseases, I managed to successfully describe aggregation data from wide temperature and concentration range by applying a kinetic aggregation mechanism comprised of two pathways, low- and high-temperature pathway. Additionally, model accurately forecasts aggregate fractions up to three years based on data obtained in much shorter period.

Kinetic aggregation mechanism only implies existence of two distinct, temperature-dependent pathways. Therefore, I isolated several aggregate fractions of the selected antibody mAb1. Analysis of antibody's chemical modifications in separate fractions with cIEF and PepMap-MS methods and analysis of activation energy values revealed that aggregates formed at temperature typical for low-temperature aggregation importantly differ from aggregates formed at temperature typical for high-temperature aggregation. Besides differences in oxidation and deamidation level, these results, with aid of available crystal structure of human IgG antibody, indicate that high-temperature aggregation is accompanied by the partial antibody unfolding.

Analysis of aggregation on two pathways requires extensive study for every assessed formulation, which is highly impractical in pharmaceutical development. Simplified model that considers aggregation as a (pseudo) first order kinetic reaction enables large acceleration and simplification in comparing aggregation susceptibility of formulations, when target

concentration is known beforehand. Based on data from at least three temperatures, at which high-temperature pathways do not contribute to total aggregation, we can calculate long-term aggregation rate at any temperature with satisfactory accuracy. If we choose low enough temperature, we can directly compare formulations between themselves and thus in a most efficient way narrow down set of excipients and conditions successfully decreasing aggregation at storage temperature.

Apparent standard Gibbs free energy of denaturation obtained by analysis of antibody chemical denaturation is a parameter of thermodynamic stability, which is a good indicator of antibody's aggregation propensity. A constructed aggregation phase space revealed that we can effectively slow down aggregation of antibodies with low thermodynamic stability by its increase. This is explained by decreasing pool of initial aggregating intermediate. On the other hand, for antibodies with high thermodynamic stability, its value primarily affects kinetic constant for formation of this intermediate, with the effect being much weaker in comparison. Nevertheless, analysis of thermal denaturation offers a useful information for the development of biopharmaceuticals. Namely, temperature, up to which aggregation through high-temperature pathway doesn't contribute substantially, is approximately 15 or 25 °C lower than the thermal denaturation transition temperatures of $CH_2$ and Fab domains, respectively.

Strategy combining kinetic and thermodynamic analysis not only improves efficiency of biologic drug product development and production but contributes to a better understanding of molecular mechanisms of aggregation as well.