Protein Aggregation: Pathways, Induction Factors and Analysis

HANNS-CHRISTIAN MAHLER,[1] WOLFGANG FRIESS,[2] ULLA GRAUSCHOPF,[1] SYLVIA KIESE[1]

[1]Formulation R&D Biologics, Pharmaceutical and Analytical R&D, F. Hoffmann-La Roche Ltd., Basel, Switzerland

[2]Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universitaet Muenchen, Munich, Germany

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ABSTRACT: Control and analysis of protein aggregation is an increasing challenge to pharmaceutical research and development. Due to the nature of protein interactions, protein aggregation may occur at various points throughout the lifetime of a protein and may be of different quantity and quality such as size, shape, morphology. It is therefore important to understand the interactions, causes and analyses of such aggregates in order to control protein aggregation to enable successful products. This review gives a short outline of currently discussed pathways and induction methods for protein aggregation and describes currently employed set of analytical techniques and emerging technologies for aggregate detection, characterization and quantification. A major challenge for the analysis of protein aggregates is that no single analytical method exists to cover the entire size range or type of aggregates which may appear. Each analytical method not only shows its specific advantages but also has its limitations. The limits of detection and the possibility of creating artifacts through sample preparation by inducing or destroying aggregates need to be considered with each method used. Therefore, it may also be advisable to carefully compare analytical results of orthogonal methods for similar size ranges to evaluate method performance. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 98:2909–2934, 2009

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INTRODUCTION

The breakthrough of recombinant DNA technology in the mid 1970s has allowed the development of many recombinant therapeutic proteins and thus has resulted in many protein-based products to reach the market.[1,2] The control and analysis of protein aggregation during production of a biotherapeutic drug is an increasing challenge to many pharmaceutical research and development groups and companies. Aggregation is potentially encountered during various steps of the manufacturing process of biopharmaceuticals, which include fermentation, purification, formulation and during storage. Biopharmaceuticals for

H.-C. Mahler and S. Kiese contributed equally to this study.

Correspondence to: Hanns-Christian Mahler (Telephone: 41-61-68-83174; Fax: 41-61-68-88689; E-mail: hanns-christian.mahler@roche.com)

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clinical trials require full characterization including accurate quantification of protein aggregates to meet the drug product specifications. Protein aggregates potentially cause adverse effects, such as an immune response,[3,4] which may cause neutralization of the endogenous protein with essential biological functions leading to a life-threatening situation for the patient and aggregates may also potentially impact the drug's efficacy.[5] The scientific fact base to clearly link specific types and sizes of aggregates to immune responses is however currently still under investigation. A potential increase in immune responses caused by aggregates has been reported previously,[3] whereas in contrast no enhanced immunogenicity was shown for example in the case of aggregated rFVIII.[6]

There are monographs and acceptance criteria/limits in the pharmacopoeias for visible and subvisible particles (i.e., insoluble proteins aggregates)—for example, United States Pharmacopoeia (USP) <788>,[7] European Pharmacopoeia (Ph. Eur.) 2.9.19[8] and Ph. Eur 2.9.20[9]—for parenteral products. However, limits for soluble aggregates have to be set case-by-case as there are no predefined limits laid down in general for biopharmaceuticals within regulatory documents. In order to control protein aggregation to enable safe and successful products, it is important to understand the origin of protein aggregates, and the analytical techniques for characterizing their full size range.

This review article aims to collate and discuss available literature on the major causes of aggregation and the analytical methods/techniques to characterize protein aggregates.

PATHWAYS AND INDUCTION FACTORS

Definition and Mechanism of Protein Aggregation

The term "protein aggregation" has been given many definitions and terminologies within the literature.[10,11] The authors define "protein aggregates" as a summary of protein species of higher molecular weight such as "oligomers" or "multimers" instead of the desired defined species (e.g., a monomer). Aggregates are thus a *universal term* for all kinds of not further defined multimeric species that are formed by covalent bonds or noncovalent interactions.

Different mechanisms that may lead to formation of various types of aggregates are currently under discussion. There is no single protein aggregation pathway but a variety of pathways, which may differ between proteins[12] and may result in different end states. A protein may undergo various aggregation pathways depending on the environmental conditions, including different types of applied stress. Also, the initial state of a protein that is prone for subsequent aggregation may differ. It may be constituted by the native structure,[13] by a degraded[14] or modified structure,[15] by a partially unfolded structure[15,16] or by the fully unfolded state.[12]

The aggregation process in general may lead to soluble and/or insoluble aggregates which may precipitate.[13,17–19] The morphology of these insoluble aggregates may be in the form of amorphous or fibrillar material which is dependent on the protein and its environment. Noncovalent aggregates are formed solely via weak forces such as Van der Waals interactions, hydrogen bonding, hydrophobic and electrostatic interactions[20] whereas covalent aggregates may for example form via disulfide bond linkages through free thiol groups[11,21,22] or by nondisulfide cross-linking pathways such as dityrosine formation.[23] Aggregation may be reversible[24] or irreversible where the irreversible aggregates could be permanently eliminated by preparative separation processes such as filtration techniques.[25] The formation of reversible aggregates is often considered to be caused by the self-assembly of protein molecules, which could be induced by changes in pH or ionic strength of the protein solution.[26–30]

One model that has been applied to describe irreversible protein aggregation is the Lumry-Eyring two state model.[31] According to this model the native protein undergoes first a reversible conformational change to an aggregation-prone state, which subsequently assembles irreversibly to the aggregated state. In this model protein aggregation is thereby controlled by conformational and colloidal mechanisms.[18,25]

In many cases, aggregation was described to follow a nucleation–propagation polymerization mechanism, whereby the nucleus can be formed by an altered monomeric structure or by a multimeric species.[32] New reports also suggest the appearance of heterogenous nucleation which is induced by micro- and nanoparticles of foreign matter, which for example could be shed from the equipment during processing.[33,34] Much insight in protein aggregation pathways is obtained from research in the field of amyloid fiber formation[35] and sickle cell hemoglobin.[36] In the area of