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Development of capillary size exclusion chromatography for the analysis of monoclonal antibody fragments extracted from human vitreous humor☆

Jennifer C. Rea[a,*], Yun Lou[a], Joel Cuzzi[a,1], Yuhua Hu[b], Isabella de Jong[b], Yajun Jennifer Wang[a], Dell Farnan[a]

[a] Protein Analytical Chemistry, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA
[b] Pharmaceutical Development, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA

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ABSTRACT

Recombinant antigen-binding fragments (Fabs) are currently on the market and in development for the treatment of ophthalmologic indications. Recently, Quality by Design (QbD) initiatives have been implemented that emphasize understanding the relationship between quality attributes of the product and their impact on safety and efficacy. In particular, changes in product quality once the protein is administered to the patient are of particular interest. Knowledge of protein aggregation *in vivo* is of importance due to the possibility of antibody aggregates eliciting an immunogenic response in the patient. Presently, there are few analytical methods with adequate sensitivity to analyze Fab aggregates in human vitreous humor (HVH) because the Fab amount available for analysis is often quite low. Here, we report the development of a highly sensitive capillary size exclusion chromatography (SEC) methodology for Fab aggregate analysis in HVH. We demonstrate a process to perform capillary SEC to analyze Fabs with picogram sensitivity and an RSD of less than 8% for the relative peak area of high molecular weight species (HMWS). In addition, we have developed a Protein G affinity chromatography method to capture Fabs from HVH for capillary SEC analysis. Recovery efficiencies ranging from 86 to 99% were achieved using this recovery method with 300 μL HVH samples containing Fab1. Finally, we demonstrate the applicability of the methodology by quantifying Fab aggregates in HVH, which can potentially be used for aggregate analysis of clinically relevant samples.

1. Introduction

Recombinant antigen-binding fragments (Fabs) are commercially available for the treatment of ophthalmologic conditions and are in development to fulfill significant medical needs [1,2]. The Fab fragment of a monoclonal antibody contains complementarity determining regions (CDRs), which are regions of the antibody that bind to antigens, and are composed of one constant and one variable domain of each of the heavy and the light chains. Fabs are target-specific and well tolerated, contributing to the continued research and development of therapeutic antibody fragments for a variety of indications [3].

Protein aggregates are a carefully monitored product impurity from the earliest stages of clinical development, as aggregates may pose a safety risk to the patient due to the potential for immunogenic response [4–8]. Aggregates may also reduce the potency of the drug product, leading to reduced efficacy [9]. In addition, high molecular weight antibody aggregates in the vitreous cavity may lead to obstruction of aqueous outflow and subsequent elevation of intraocular pressure [10]. Aggregation has been documented as a critical quality attribute and is often monitored by quality control [9,11,12]. During development and manufacturing, size exclusion chromatography (SEC) is commonly used to measure therapeutic protein aggregates [13,14].

Protein characterization studies performed in human vitreous humor (HVH) are of importance because HVH represents the protein environment once the drug is administered to the patient's vitreous cavity. The drug's half-life in the vitreous cavity may be on the order of days to weeks, allowing the drug to undergo modifications while exposed to an *in vivo* environment [15]. Two potential causes of aggregation in HVH *in vivo* are (1) thermally induced aggregation of the protein due to exposure to high temperatures (37 °C), and (2) incubation in an aqueous system that is not formulated to stabilize the protein.

The analysis of Fab product quality in HVH poses several challenges, including (1) Fab recovery from the HVH prior to analysis,

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* Corresponding author. Tel.: +1 650 225 7013; fax: +1 650 225 3554.
E-mail address: rea.jennifer@gene.com (J.C. Rea).
[1] Present address: BioMarin Pharmaceutical Inc., 105 Digital Drive, Novato, CA 94949, USA.

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and (2) inadequate sensitivity in analyzing very low amounts of Fab in HVH samples by conventional analytical methods. Scaling down conventional analytical methods generally increases sensitivity while reducing sample usage, thus allowing for recovered Fabs to be analyzed for product quality using multiple assays, such as potency and purity assays. Capillary SEC has been used for the analysis of monoclonal antibodies (mAbs) extracted from harvested cell culture fluid [16], but has not been demonstrated with Fabs extracted from human-sourced material. Studies performed in an *in vivo* environment or that closely resemble *in vivo* conditions are increasingly important for determining critical quality attributes of protein therapeutics. We report herein a capillary SEC methodology with picogram detection limits for the characterization of Fabs. We developed a Protein G affinity chromatography method to capture Fabs from HVH without introducing sample bias or recovery artifacts for subsequent capillary SEC analysis. This report describes the development of a Protein G affinity sample preparation/capillary SEC method for Fab analysis, which can be used to study protein aggregation in clinically relevant samples.

2. Experimental

2.1. Instrumentation

Chromatographic experiments were performed on a Dionex (Sunnyvale, CA) Ultimate 3000 Nano/Cap/Micro biocompatible liquid chromatography system. Components of the system included dual ternary low-pressure gradient pumps, an autosampler with sample temperature control capability, and a four channel UV–Vis detector. Instrument control, data acquisition and compilation of results were performed using Dionex Chromeleon software, version 6.8.

For the capillary chromatography, the system was equipped with a Dionex FLM-3000 flow manager, configured with a flow splitter cartridge (1:1000 split ratio). The capillary columns were connected directly to a four-port Valco injector (Houston, TX) containing a 20 nL internal loop. The detector contained a Dionex 45 nL, 1 cm path length flow cell. A 50 μm I.D. (~0.55 μL) fused silica needle from Dionex and a low draw speed (20 nL/s) were used to accurately draw nanoliter volumes. A user-defined sample uptake method was used as previously described [16]. For protein concentration measurements using a UV–Vis spectrophotometer, a Thermo Scientific (Wilmington, DE) NanoDrop 2000 spectrophotometer was used.

2.2. Columns, chemicals and equipment

Capillary SEC columns (300 μm × 300 mm) containing Tosoh Biosciences (Montgomery, PA) TSKgel Super SW2000 stationary phase (4 μm particle size, 125 Å mean pore size) were obtained from Dionex Benelux (Amsterdam, The Netherlands). The capillary columns were fitted with a fused silica capillary outlet (20 μm I.D.) for connecting the column outlet to the detector. Conventional scale SEC columns containing Tosoh Biosciences TSKgel stationary phase of dimensions 7.8 mm × 300 mm (TSKgel G2000SWxl, 5 μm particle size, 125 Å mean pore size) and 4.6 mm × 300 mm (TSKgel Super SW2000) were obtained from Tosoh Biosciences.

Sodium hydroxide, potassium chloride, and acetic acid were obtained from Mallinckrodt (Phillipsburg, NJ). Trizma HCl, Trizma base, potassium phosphate (mono- and dibasic) were Sigma brand reagents (St. Louis, MO). All other common materials were obtained from Fisher Scientific (Waltham, MA) unless otherwise indicated.

Consumables used for Fab recovery from HVH and protein concentration include: GE Healthcare (Piscataway, NJ) GammaBind G Sepharose resin, BioRad (Hercules, CA) PolyPrep columns to retain the GammaBind G resin, 2 mL microcentrifuge tubes obtained from E&K Scientific Products (Santa Clara, CA), GE Healthcare PD-10 columns for buffer exchange, Pall (Port Washington, NY) Nanosep 30 kDa MWCO centrifugal devices, and Waters Corporation (Milford, MA) maximum recovery HPLC vials. An Eppendorf (Hauppauge, NY) micro-centrifuge capable of 14,000 × *g* was used to perform all of the centrifugation steps.

Table 1
Summary of mobile phases and buffers used in this work.

Buffer	Composition
SEC mobile phase	200 mM potassium phosphate, 250 mM potassium chloride, pH 6.2
Equilibration – Protein G	1X PBS[a], 0.01% sodium azide, pH 7.2
Elution – Protein G	100 mM acetic acid
Neutralizing – Protein G	1 M Tris, pH 8.0
Column cleaning – Protein G	500 mM acetic acid
Column storage – Protein G	250 mM Tris, 0.02% sodium azide, pH 7.5

[a] 1× PBS composition: 37 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2.

Monoclonal antibody fragments were produced in-house at Genentech (South San Francisco, CA). Oxidized Fab was produced by incubating Fab samples in 75 mM 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) at 40 °C for 24 h. Human vitreous humor was obtained from BioChemed Services (Winchester, VA).

2.3. Mobile phase and buffer preparation

Appropriate amounts of buffer components were dissolved in purified water equal to 90% of the final target volume of the solution. Once dissolved, the solution was titrated to the appropriate pH by the addition of 10 N sodium hydroxide, as necessary. Once titrated, purified water was added to bring the solutions to the required volume. The buffers were then individually filtered through a fresh 0.2 μm nylon filter prior to use. Mobile phases and buffers prepared for this work are listed in Table 1.

2.4. GammaBind G affinity antibody recovery

The following steps were performed to capture Fabs that were spiked into HVH and are discussed in more detail in the following paragraphs: packing and equilibration of the GammaBind G resin in the PolyPrep column, addition of the Fab-containing HVH sample to the equilibrated column, incubation of the column at room temperature to allow the Fab to bind to the affinity column, washing the column to remove unbound HVH components, elution of the Fab, buffer exchange and sample concentration.

To capture Fabs that were spiked into HVH, frozen HVH samples were thawed at room temperature and mixed briefly by pipetting. Approximately 0.5–1.0 mL of GammaBind G Sepharose resin (supplied preswollen as a 50% slurry in phosphate buffered saline, pH 7.0, containing 20% ethanol as a preservative) was packed in each PolyPrep column by gravity. The resin was equilibrated in the PolyPrep column with five to ten column volumes (CVs) of equilibration buffer. Samples containing Fab spiked into HVH were diluted with equilibration buffer to bring the sample volume up to the volume of the resin bed. The diluted samples were then transferred to equilibrated columns. Samples were allowed to flow through the column until a small amount of liquid remained above the resin bed, while the flow-through was discarded. The PolyPrep column was then capped, and the sample was allowed to incubate in the column at room temperature for 20 min. The PolyPrep column was then uncapped and the remaining flow-through was discarded while the GammaBind G Sepharose resin was retained in the PolyPrep column. The resin was then washed by adding five