The Novel System for Quantification of Aggregated Antibodies

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1. Introduction

Antibody drugs have become a focus of attention in recent years as therapeutic drugs for diseases such as cancer, and their global market has been expanding rapidly [1]. Antibody drugs have complex protein structures of far larger molecules in contrast to small molecule drugs such as aspirin, which have been commonly used, and antibodies in the drugs are known to be partially denatured by various stresses including shaking, agitation, and heat, and then to form aggregates [2-4]. Concerns have been raised that such aggregated antibodies may reduce therapeutic effects or cause adverse effects such as allergic reactions [5, 6], and the necessity of quantification of aggregated antibodies has been increasingly recognized worldwide as a key factor of quality management, as can be seen from the description in the Food and Drug Administration (FDA) guidelines [7].

However, aggregated antibodies, which can range widely from a few tens of nm to several tens of μm, need to be measured using an appropriate method according to their particle size: size-exclusion chromatography for particles of 0.1 μm or less, and microscopy-based methods such as micro flow imaging for particles of 2 μm or more [8]. For particles ranging from 0.1 to 2 μm, available methods are limited to qualitative analysis using the dynamic light scattering method and the laser diffraction scattering method, and even the United States Pharmacopeia (USP) [9] currently recommends analysis using multiple methods and states that a new technology is expected to be developed, indicating that no analysis techniques have been established for particles in such a size range.

In recent years, analysis techniques have been developed by combining quantitative analysis with qualitative analysis or improving quantitativity. For example, Shimadzu Corporation focused on improving the quantitative performance of laser diffraction/scattering by limiting the size range to 40 nm to 20 μm, and then launched in 2013 the Aggregates Sizer, a system that enables evaluation of aggregates in drugs. Malvern Panalytical Ltd. launched in 2018 the Zetasizer Ultra, a system that enables determination of particle concentration using the multi-angle dynamic light scattering method requiring multiple detectors to receive scattering light from different angles, instead of the dynamic light scattering method. However, none of these methods have achieved accurate analysis of polydisperse samples. Moreover, since, with any of these methods, the particle size distribution is determined based on the scattering light from a sample containing particles, quantitative performance is significantly affected by the refractive index and density of particles; however, an accurate determination of these properties of aggregated antibodies is quite difficult. From these perspectives, quantification of aggregated antibodies, especially ranging from 0.1 to 2 μm, is still challenging.

We therefore started to develop a system that enables quantification of particles ranging from 0.1 to 2 μm, a less established area, by first separating particles according to their size and then detecting the separated particles individually. Here, we report the overview of the system we developed and the verification results of quantification of aggregated antibodies in antibody drugs.

We have developed the novel system with the aim of establishing a method with high reliability for quantification of aggregated antibodies, and with the goal of contributing to the society by providing the system as a highly accurate quality management

technology for antibody drugs.

2. Technical Overview

In the process of the development of our novel system for quantification of aggregated antibodies (hereinafter referred to as "this system"), we first established the particle separation technology and particle detection technology separately, developed the new particle separation-detection technology by integrating the established technologies, and then evaluated our technology for this system by comparing it with a competitive method. In the subsequent sections, we report details of our technology and its development process.

[1] Particle Separation Technology

[1-1] Verification of Particle Separation Performance

Pinched-Flow Fractionation [10, 11] (hereinafter referred to as "the PFF method"), which hydrodynamically separates particles according to their size, was adopted as a particle separation technology (**Fig.1**). In the PFF method, a microdevice, on which microchannels with a height and width of micrometers were formed, was used for separation. First, sample solution containing particles and buffer solution not containing particles were introduced from two inlets into the pinched segment (relatively narrow channel). When the flow rate of the buffer solution was set to be enough larger than that of the sample solution, the particles flowed downstream while being pushed and aligned to one sidewall in the pinched segment by the buffer solution. At this time, the centers of the particles were located on different flow streamlines with a slightly different distance from the sidewall according to the particle size. Such distances were amplified in the subsequent broadened segment (relatively wide channel), and the particles were then separated.

In general, the separation performance in the PFF method is significantly affected by the ratio of the widths of the pinched segment and broadened segment. Moreover, our research suggested that since our development only covered particles ranging from 0.1 to 2 μm subject to Brownian motion, the important points were to set a flow rate condition at which particles could flow promptly through the pinched segment and to achieve a design of the microchannel with seamless streamlines between the pinched segment and

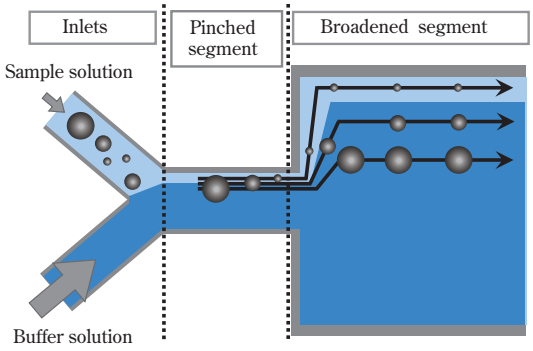

Fig. 1 Principle of Pinched-Flow Fractionation

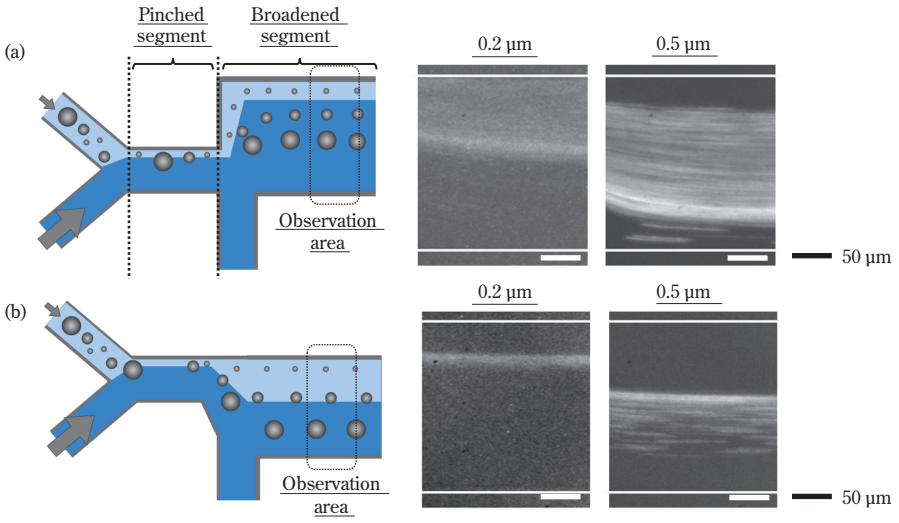

Fig. 2 Separation of particles by Pinched-Flow Fractionation

broadened segment. The overview of particle separation by the PFF method is shown in **Fig.2**. In the PFF method, the microchannel is generally broadened 90° to both sides at the junction of the pinched segment and broadened segment [10]; however, with this structure, a complete separation of fluorescent particles of 0.2 μm and 0.5 μm (Polysciences Inc.) in the sample solution could not be observed under a fluorescence microscope. On the other hand, a complete separation of fluorescent particles of 0.2 μm and 0.5 μm has been achieved by establishing the structure of the microdevice, where the channel is designed not to be broadened toward the sidewall on the sample solution inlet side, or the sidewall to which particles are aligned.

The separation experiment described above was conducted through the following processes.

［1-2］Fabrication of Microdevice

A microdevice used for verification of particle separation performance was fabricated by making a mold and then pouring the resin into the mold and curing it. First, the procedure of fabricating a mold is noted; Photoresist SU-8 3005 (Microchem Corp.) was dropped on a 4-inch bare silicon wafer (Philtech Inc.), and a photoresist thin film was formed using a spin coater (Mikasa Co., Ltd.). At that time, cyclopentanone (Tokyo Chemical Industry Co., Ltd.) was added to dilute SU-8 3005 according to the desired thickness of the thin film to be formed. Subsequently, a desired microchannel pattern on a chrome mask was transferred to the photoresist thin film using a mask aligner (Ushio Inc.) and was then developed using SU-8 Developer (Microchem Corp.) to fabricate a desired mold.

Next, uncured SYLGARD SILICONE ELASTOMER KIT (Dow Corning Toray Co., Ltd.) was poured into the fabricated mold and was then heated at 80ºC for two hours to fabricate Polydimethylsiloxane (PDMS) with the microchannel pattern transferred. The cured PDMS was unmolded and was shaped to a desired size by cutting it with a knife, and then the inlets and outlets were drilled using a puncher. After surface treatment was applied to the unmolded PDMS and a glass slide (Matsunami Glass Ind., Ltd.) using an oxygen plasma generator (Meiwafosis Co., Ltd.), the treated PDMS and glass slide were bonded together to fabricate a microdevice.

［1-3］Particle Separation Experiment

As fluorescent polystyrene beads, Fluorescent Polymer Particles (Green) (Bangs Laboratories, Inc.; absorbance maximum, 480 nm; fluorescence maximum, 520 nm) was adopted for particles of 0.2 μm, and Fluoresbrite BB (Polysciences, Inc; absorbance maximum, 360 nm; fluorescence maximum, 407 nm) for particles of 0.5 μm. As suspension for the particles, phosphate buffer solution containing 0.05% (v/v) Tween 20 was adopted. Before experiment, foreign particulate matters were removed from the buffer solution using a syringe filter with a pore size of 0.45 μm (Merck Millipore Ltd.), and fluorescent particles of 0.2 μm and 0.5 μm were diluted with the buffer solution, respectively, to 3.3 ng/mL and 7.5 ng/mL. The flow rate was adjusted with a syringe pump (KD Scientific Inc.) while introducing sample solution and buffer solution into the microchannel. Fluorescence images were obtained separately at the corresponding wavelengths, using an inverted microscope IX71 (Olympus Corporation) by irradiating the mercury lamp excitation light to the observation area and then shooting video of the target area for 2 seconds using a digital CMOS camera ORCA-FLASH (Hamamatsu Photonics K.K.).

［2］Particle Detection Technology

［2-1］Overview of Particle Detection

The Coulter method [12], which electrically measures individual particle, was adopted as particle detection technology (**Fig.3**). The Coulter method is a technology that enables electrical measurement of particles on a device in which a pair of electrodes is placed across a fine hole called an aperture. In the method, the slight decrease in the electrical current when particles pass through the aperture is used. The decrease range of the electrical current value (when particles pass through the aperture) and the decreased signal pulse count are dependent on, respectively, each particle size and particles concentration.

With the Coulter method, particles can be measured individually and accurately even if their inclusion rate is low; however, there is an issue that the measurable range of particle size (dynamic range) is relatively small. Specifically, in this method, the dynamic range of particles is said to be 2% to 60% of the aperture diameter

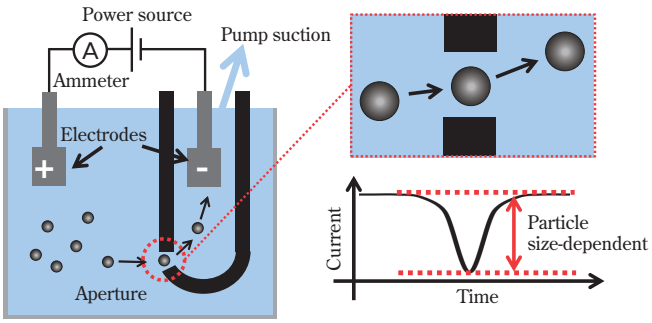

Fig. 3 Principle of Coulter method

in general. Moreover, particles larger than the aperture diameter needs to be eliminated before measurement using a filter with a pore size smaller than the aperture diameter. This is because the aperture may be clogged by such particles, if any, and then subsequent measurements are prevented.

Based on the issue above, we subsequently established the Coulter method in a microdevice in which multiple apertures with the size of the particles to be separated are placed, to develop a system that can individually detect particles ranging from 0.1 to 2 μm, a less established area. In this section, we report the process of establishing the Coulter method in the fabricated microdevice.

[2-2] Development of Particle Detection Channel

Three sizes of apertures were used to detect particles ranging from 0.1 to 2 μm: for detection of small particles, medium particles, and large particles. A schematic overview of the Coulter method is shown in **Fig.4**. The adopted structure was as follows: the channel in which the separated particles flow (particle collection channel) branched off downstream into two channels with a fine structure (aperture) at each branch point, each of which communicated with a downstream outlet. In this structure, each particle collection channel had two apertures. The size of the two apertures was 1 μm (width) × 0.4 μm (height) × 10 μm (length) each for small particles, 2 μm (width) × 0.8 μm (height) × 10 μm (length) each for medium particles, and 3.5 μm (width) × 4.5 μm (height) × 20 μm (length) each for large particles. A platinum electrode was inserted into each outlet at the downstream of the aperture, and DC power supply and a current amplifiers (current to voltage converter; NF Corporation) were placed between the two platinum electrodes to form a circuit. The output of the current amplifiers (current to voltage converter) was connected to an A/D convertor, to which a PC was connected to obtain electrical signal using a self-made software set up with LabVIEW.

[3] Particle Separation-Detection Technology

[3-1] Integration of Particle Separation Technology and Particle Detection Technology

A schematic overview of our developed microdevice where particle separation technology and particle detection technology are integrated is shown in **Fig.5**. With this system, particles ranging from 0.1 to 2 μm can be evaluated individually by first separating the particles according to their size using the PFF method and then introducing the separated particles into the three particle collection channels with the apertures of the corresponding particle size. The detection results

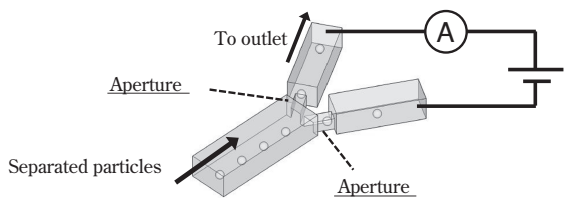

Fig. 4 A schematic overview of the microfluidic Coulter method

of a sample containing mixed particles of 0.1, 0.2, 0.5, 1, 2 μm are shown in **Fig.6**. Signal pulses of particles of 0.1 and 0.2 μm were detected at the apertures for small particles as shown in **Fig.6** (a), signal pulses of particles of 0.2 and 0.5 μm at the apertures for medium particles as shown in **Fig.6** (b), signal pulses of particles of 0.5, 1 and 2 μm at the apertures for large particles as shown in **Fig.6** (c) and (d). These detection results of particles ranging from 0.1 to 2 μm obtained at the three sizes of apertures are integrated into a histogram (**Fig.7**). As can be seen from the five peaks formed in the histogram, the particles were separated according to their size. As a sample containing mixed particles, Duke Standards (Thermo Fisher Scientific K.K.) were adopted, which were diluted with phosphate buffer solution containing 0.05% (v/v) Tween 20 so that the final concentration could be 5 μg/mL.

[3-2] Comparative Evaluation between Our Method and the Laser Diffraction/Scattering Method

A comparative evaluation was conducted by comparing our developed method shown in **Fig.7** with another technology, Aggregates Sizer (Shimadzu Corporation), using a sample containing mixed particles of 0.1, 0.2, 0.5, 1, 2 μm. As can be seen from the histogram in **Fig.8**, the peaks of 0.2 μm and 2 μm were lost and a peak size of 0.1 μm was increased, indicating that an accurate measurement of samples containing mixed particles was difficult.

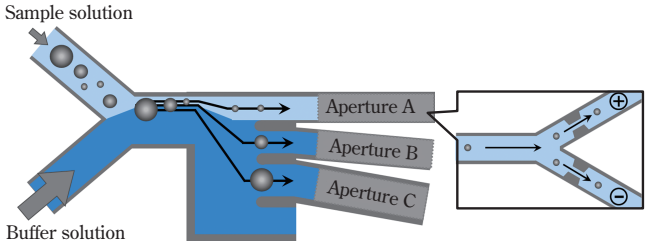

Fig. 5　A schematic overview of the developed microdevice

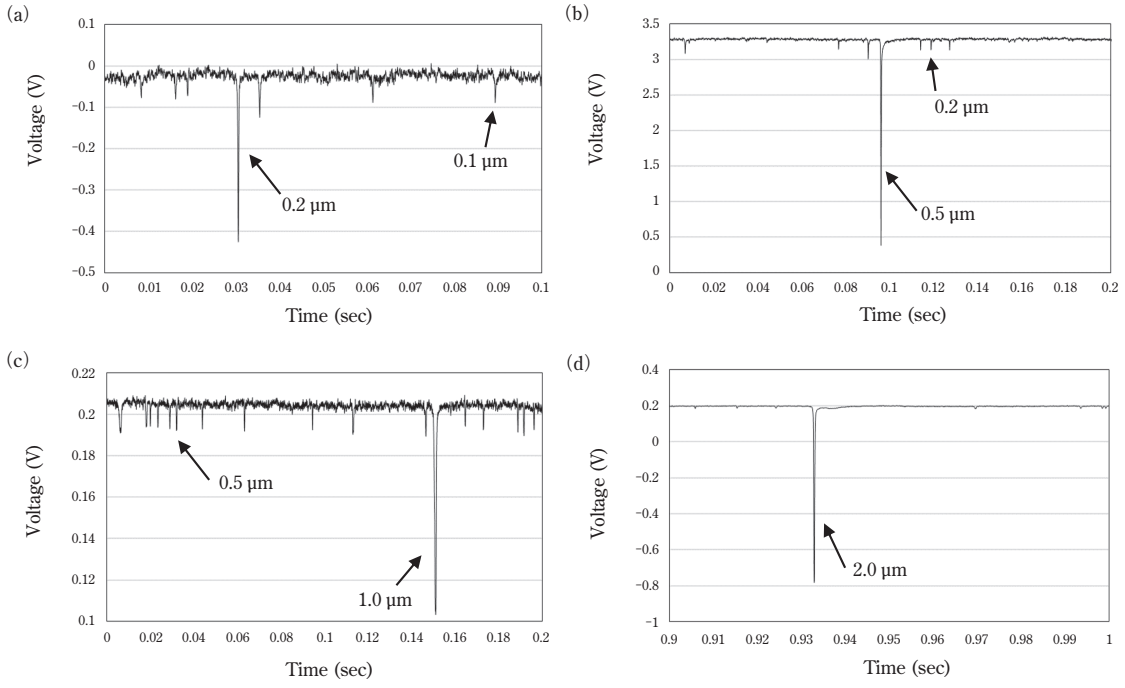

Fig. 6　Electrical signal detected from each aperture

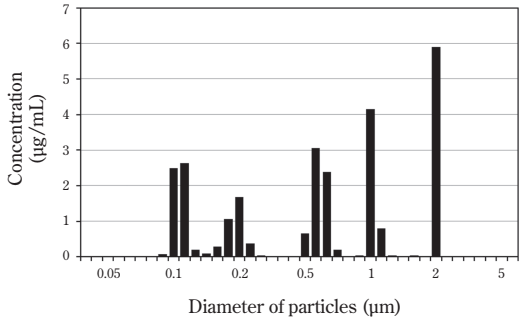

Fig. 7 Resolution of PS standard particles (0.1, 0.2, 0.5, 1.0, 2.0 μm) by developed method

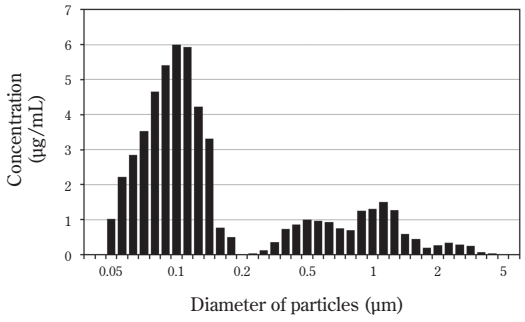

Fig. 8 Resolution of PS standard particles (0.1, 0.2, 0.5, 1.0, 2.0 μm) by Laser Diffraction

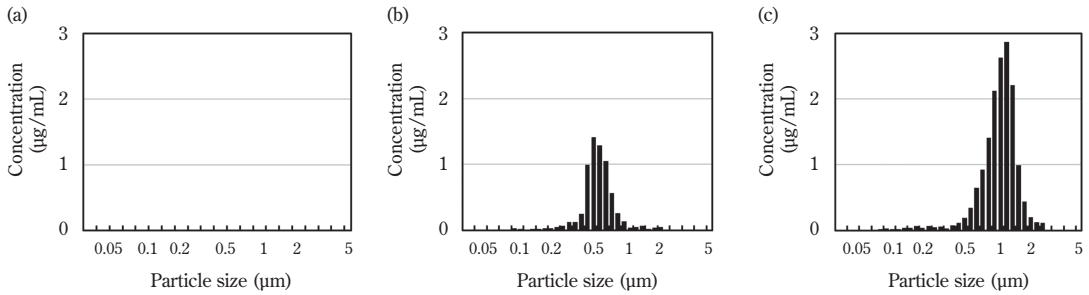

Fig. 9 Time course of protein aggregates (a) 0 min (b) 10 min (c) 120 min

3. Detection of Aggregated Antibodies in Antibody Drugs

The following three types of samples were prepared using a commercially available antibody drug: (a) a control sample (the drug to which no treatment was applied), (b) a sample of the drug that was heated at 60℃ for 30 minutes and then left at room temperature for 10 minutes, (c) a sample of the drug that was heated at 60℃ for 30 minutes and then left at room temperature for 120 minutes. Each of three samples was measured using our developed technology. As can be seen from the histograms in **Fig.9**, while no particles were detected from the control sample, the peak of particles that was suspected to be aggregates were detected from the heat-treated samples, and peak top position was shifted over time to the larger side of the particle size.

4. Conclusion

As described above, we have achieved the novel system that enables quantification of particles ranging 0.1 to 2 μm, a less established area, by developing the microdevice in which the particle separation technology using the PFF method and the particle detection technology using Coulter method are integrated. With this system, aggregated antibodies in commercially available antibody drugs can be detected and their aggregation process over time can be evaluated, providing the possibility of using this system for quality management of antibody drugs. This system is expected to contribute not only to improving the quality of antibody drugs because it enables quantification of a small amount of aggregates in antibody drugs, but also to improving the performance of antibodies used for antibody drugs because it enables verification of the stability of antibodies in antibody drugs by applying a stress (e.g. thermal degradation test).

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