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Quanlitative Evaluation of Insoluble Particulate Matters in Therapeutic Protein Injections Using Light Obscuration and Flow Imaging Methods

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ABSTRACT

Flow imaging (FI) has emerged as a powerful tool to evaluate insoluble particles derived from protein aggregates as an orthogonal method to light obscuration (LO). However, few reports directly compare the FI and LO method in the size and number of protein particles in commercially available therapeutic protein injections. In this study, we measured the number of insoluble particles in several therapeutic protein injections using both FI and LO, and characterized these particles to compare the analytical performance of the methods. The particle counts measured using FI were much higher than those measured using LO, and the difference depended on the products or features of particles. Some products contained a large number of transparent and elongated particles, which could escape detection using LO. Our results also suggested that the LO method underestimates the size and number of silicone oil droplets in prefilled syringe products compared to the FI method. The count of particles ≥10 μm in size in one product measured using FI exceeded the criteria (6000 counts per container) defined in the compendial particulate matter test using the LO method. Thus precaution should be taken when setting the acceptance criteria of specification tests using the FI method.

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Introduction

Protein aggregates and insoluble particles in biopharmaceutical drug substances and drug products are considered impurities that require adequate assessment and control because of their potential to elicit immunogenicity.[1,2] To control the protein aggregates and sub-visible particles in biopharmaceutical products, size exclusion chromatography is applied for the purity test of drug substances and drug products, and the particulate matter test using light obscuration (LO) method is also conducted for drug products.[3] The particulate matter test for injections, which is internationally harmonized in pharmcopoeia, consists of the LO and microscopic methods.[4] The LO method should be preferentially used in terms of reproducibility. Although this compendial test defines particulate matter to be tested in injections as “extraneous mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.”, actually inherent particles derived from protein aggregates, excipients and manufacturing processes are detected in the test.[5,6]

The evaluation of protein aggregates and insoluble particles using LO has several limitations. In the LO method, the size and number of particles are determined by the amplitude and number of pulse signals that are generated by particles blocking light passage between the source and sensor. The size of particles is calculated using a calibration curve generated from polystyrene standard particles. However, particles derived from protein aggregates are translucent, and they have a lower refractive index than polystyrene standard particles. Thus, it is suggested that LO may underestimate the counts and size of proteinous insoluble particles.[7-12] Therefore, the flow imaging (FI) method has emerged as a useful tool to characterize protein particles and complement the LO method. In the FI technique, the size, number, and shape of particles can be determined by capturing images of particles as a sample stream passes through a flow cell, and the edges of particles are determined using the contrast between particles and the dispersion liquid. Thus, the FI method has higher sensitivity for detecting nearly transparent particles.[9,13,14] However, the FI method had not yet been standardized or listed in any compendium until the USP chapter mentioned below has been published.

We recently conducted a collaborative study to measure and count sub-visible particles in three different samples using the LO

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and FI methods to assess the applicability of the standardization of the FI method.[15] Our study revealed that consistent particle counts are obtained using FI instruments from the same manufacturer, particle counts may slightly vary by manufacturer, which would be acceptable considering the observed intra-laboratory variability, and the FI method has comparable intra- and inter-laboratory variability as the LO method. Considering our data, using FI as a quality control test would be feasible to precisely analyze the counts of sub-visible particles for therapeutic protein injections, although a validation study should be conducted on each product. Recently United States Pharmacopoeia has published a general chapter <1788.3> Flow imaging method for the determination of sub-visible particulate matter.

From these circumstances, the use of the FI method as additional characterization method to complement quality control tests may increase.[16-18] However, to our knowledge, few reports have compared insoluble particles in commercial therapeutic protein injections detected using FI and LO, which could hinder biopharmaceutical companies from applying the FI method in quality control tests. In this study, we measured insoluble particles present in commercially available therapeutic protein injections using both the LO and FI methods to reveal particle characteristics (size, shape, and number) and compare the detectability of the FI and LO methods. Furthermore, we discussed setting an acceptance criterion when using the FI method from our measurement results.

Materials and Methods

Materials and Methods

Ten therapeutic protein injections, Product A–J, as presented in Table 1, were used in this study. ENBREL® 10 mg for S.C. Injection (lot no. 15M01A, Pfizer Inc., New York, NY, USA), ESPO® subcutaneous injection syringe (lot no. 16804T, Kyowa Kirin Co., Ltd., Tokyo, Japan), Filgrastim BS 75 μg Syringe for Inj. MOCHIDA (lot no. AA18A, MOCHIDA Pharmaceutical Co., Ltd., Tokyo, Japan), GRAN® SYRINGE (lot no. 16103L, Kyowa Kirin Co., Ltd.), HERCEPTIN® for Intravenous Infusion (lot no. 16D070E, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), Infliximab BS for I.V. Infusion 100 mg [NK] (lot no. 250060, Nippon Kayaku Co., Ltd., Tokyo, Japan), MIRCERA (lot no. 16G010Z, Chugai Pharmaceutical Co., Ltd.), NESP injection plastic syringe (lot no. 16719N, Kyowa Kirin Co., Ltd.), Rituximab BS Intravenous Infusion 500 mg [KHK] (lot no. 17L01E, Kyowa Kirin Co., Ltd.), VECTIBIX® injection for intravenous infusion (lot no. H127, Takeda Pharmaceutical Co., Ltd., Tokyo, Japan), and OTSUKA distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) were purchased from commercial suppliers. Products were used prior to their expiration dates for each test. Lyophilized formulations were dissolved in a given solution or distilled water, and then tests were conducted on the day when the products were dissolved.

Light Obscuration (LO)

KL-04A (Rion Co., Ltd., Tokyo, Japan) was used for LO experiments. The instrument was qualified according to Japanese Pharmacopoeia <6.07> Insoluble Particulate Matter Test for Injections [19]. Based on our previous study regarding a reduced test volume in the LO method, the LO measurement was conducted with a sample volume of 0.2 mL four times. The tare volume was set at 0.2 mL. Four results were collected, and three results were analyzed after discarding the first result. The counts of particles were obtained from the particle numbers in the ranges of 2.0–100 μm.

Flow Imaging (FI)

FlowCam 8100 (Fluid Imaging Technologies, Inc., Scarborough, ME, USA) was used for FI experiments. Before the measurements, the performance of the instrument was verified by confirming that the particle counts and size of 10 μm polystyrene standard particles (COUNT-CAL Count Precision Size Standards CC10-PK, Thermo Scientific, CA) are within the certificated value. The flow rate, sample volume, autoimage rate, sampling efficiency, and segmentation threshold (dark/light) were set for 0.1 mL/min, 0.2 mL, 16 frames/s, approximately 70%, and 10/10, respectively. The value of the segmentation threshold was based on our previous report to appropriately detect more proteinous insoluble particles.[15] The instrument was flushed with distilled water and, if necessary, 1% Tergazyme (Alconox Inc., White Plains, NY, USA) solution, at a flow rate of 5 mL/min, ensuring there were no major particles in the flow cell. After setting the instrumental parameters, to assess the qualification of each instrument, 700–1000 μL of distilled water were tested to confirm that fewer than 100 particles >2 μm in size were present in each 1 mL of fluid and ensure the absence of any particles >10 μm in size. In a single assay, 400 μL of the sample were applied to the instrument and measured three times. Three results were collected and analyzed. The counts of particles were obtained from the particle numbers in the ranges of 2.0–100 μm.

Preparation of Silicone Oil Droplets

Silicone oil droplets were generated by dropping 1mL syringes (Terumo 1mL syringe SS-01T) filled with PBS for 20 times from the height of 20 cm, and collected into a tube. Subsequently the solutions were left to stand for 2 hours to remove bubbles.

Results and Discussion

The insoluble particles in commonly used therapeutic protein injections including six vial products and four prefilled syringe products were evaluated by LO and FI. The particle counts of therapeutic protein injections are presented in Tables 2 and 3, and the relationship between the particle counts obtained by the two methods is presented in Supplemental Fig. 1a and 1b. As a whole, the particle counts measured by FI were higher than those measured by LO. However, the ratio of the particle counts measured by FI and LO varied significantly among products. For vial products, Product D had the highest particle count ratio between the two methods, as the particle count (≥10 μm) was 100-fold higher using the FI method than using the LO method. Approximately 10–50-fold differences in particle counts between the methods were observed for Products A–C and F. Contrarily, only a 2–4-fold difference in particle counts was detected for Product E. For prefilled products, the difference in particle counts between the methods generally ranged 2–10-fold, excluding Product G where the difference was 8-25 fold.

Representative images of particles detected using the FI method are presented in Figs. 1 and 2. Among the vial products (Products A–F), most particles observable in Product D were elongated and transparent (Fig. 1). On the contrary, other products had relatively dense and round particles in addition to elongated and transparent particles. Many round or ellipsoidal particles were observed, especially in Products A, C, and F. Product E mainly contained dense particles that were relatively distinct in outline and fewer elongated and transparent particles. This may be one of reasons for lack of a significant difference in the detected particle number of Product E between the LO and FI methods. When measuring particles using the FI and LO methods, no needles or syringes coated with silicone oil were used in this study. However, particles that look like silicone oil droplets were observed in Products A, E and F. The rubber stopper lubricated with

Table 1
Therapeutic protein injections tested in this study.

	Dosage forms		Administration	
Product	Containers	Content	Route	Excipients
A	vial lyophilized	10 mg/1 mL	SC	D-Mannitol Sucrose Trometamol Trometamol hydrochloride
B	vial lyophilized	60 mg/3 mL	IV	Trehalose hydrate L-Histidine hydrochloride hydrate L-Histidine Polysorbate 20
C	vial lyophilized	100 mg/10 mL	IV	Sucrose Polysorbate 80 Sodium dihydrogen phosphate monohydrate Sodium monohydrogen phosphate
D	vial liquid	100 mg/5 mL	IV	Sodium chloride Sodium acetate hydrate pH modifers
E	vial liquid	150 μg/0.6 mL	IV	Polysorbate 80 D-Mannitol pH modifers
F	vial liquid	100 mg/10 mL	IV	Citric acid hydrate Sodium chloride Polysorbate 80 pH modifers
G	pre-filled syringe	100 μg/0.3mL	IV	L-Methionine Sodium sulfate Polyoxyethylene (160) Polyoxypropylene (30) Glycol Tonicity agent (D-Mannitol) Buffering agent pH modifers
H	pre-filled syringe	20 μg/0.5 mL	IV	Polysorbate 80 L-Methionine Sodium dihydrogen phosphate Tonicity Agent pH modifers
I	pre-filled syringe	24,000 IU/0.5 mL	SC	Polysorbate 80 L-Arginine hydrochloride Sodium dihydrogen phosphate Tonicity agent pH modifers
J	pre-filled syringe	300 μg/0.7 mL	SC	Polysorbate 80 D-Mannitol Sodium acetate hydrate Acetic acid

silicone oil or manufacturing proccess such as filling process may introduce silicone oil droplets into solution in vial products. Some reports suggested the existence of smaller particles derived from excipients in therapeutic protein injections.[19,20] Therefore, the other round or dense particles may be derived from excipients. Detailed analyses such as elemental analysis using energy dispersive X-ray analysis, vibrational spectroscopy and Raman spectroscopy etc.will be needed to identify their origin in the future.

For prefilled syringe products (Products G–J), as illustrated in Fig. 2, most particles visually observable from the pictures were round, and they were considered to be derived from silicone oil lubricated on syringes. There were no elongated particles more 10 μm in size. To elucidate the difference in particle counts of silicone oil droplets themselves between the two methods, the number of silicone oil droplets was measured using the LO and FI methods. Silicone oil droplets were generated by dropping of 1 mL of PBS in syringes. The results confirmed that the particle counts measured using the FI method were 2–8-fold higher than those obtained using the LO method (Table 4 and Supplemental Fig. 2).

To characterize the particles present in each product, we analyzed the particle parameters calculated by the FI instrument based on the captured images. Fig. 3 presents the contour plots of circularity to sigma intensity. These parameters were chosen to effectively visualize the features of particles in each product. A circularity value of 1.0 denotes a perfect circle. The sigma intensity is a standard deviation of the grayscale values of images. If the image of a particle includes both light and dark parts, the variability of grayscale values in a single particle is large, resulting in higher sigma intensity. In Fig. 3, the contour plot of Product D clearly illustrated that particles with lower circularity and sigma intensity were more readily detected than other particles, indicating that these particles escape detection using the LO method (Supplemental Fig. 2). It was confirmed that all other vial products also contained particles with lower circularity, although small particles with higher circularity and sigma intensity were also detected, as presented in Fig. 1. Conversely, silicone oil droplets exhibited higher circularity (closest to 1) and sigma intensity (approximately 30) because a single picture of silicone oil droplets has both dark and light parts. The contour plots of Products G–J visually indicated that almost all detected particles in prefilled syringe products were distributed in the same region as silicone oil droplets, although some elongated particles were observed in Product J. Because the FI method permits the measurement of morphological and optical characteristics, some reports demonstrated that FI method is one of useful method to classify silicone oil droplets and

Table 2
Particle counts obtained by Light obscuration and Flow Imaging for vials (product A-F).

Product	Size	Light Obscuration particles/mL[a] ave	SD	Flow Imaging particles/mL[a] ave	SD	FI/LO
Product A	> 2 μm	403.3	133.6	4583.7	1272.6	11.4
1 mL/vial	> 5 μm	68.3	25.7	992.7	467.4	14.5
	> 10 μm	16.7	10.4	278.7	181.0	16.7
	> 25 μm	0.0	0.0	49.4	41.4	≥49.4
Product B	> 2 μm	375.0	92.6	4188.7	1164.6	11.2
3 mL/vial	> 5 μm	68.3	25.7	556.0	64.0	8.1
	> 10 μm	20.0	8.7	146.7	91.9	7.3
	> 25 μm	1.7	2.9	11.1	12.7	6.5
Product C	> 2 μm	1948.3	306.9	17571.5	1082.6	9.0
10 mL/vial	> 5 μm	375.0	134.5	2768.5	632.9	7.4
	> 10 μm	55.0	21.8	523.0	87.7	9.5
	> 25 μm	0.0	0.0	32.0	0.0	≥32
Product D	> 2 μm	4500.0	576.6	56202.7	10739.4	12.5
5 mL/vial	> 5 μm	915.0	107.6	21964.0	3488.6	24.0
	> 10 μm	91.7	24.7	8702.3	1144.3	94.9
	> 25 μm	0.0	0.0	837.3	103.3	≥837.3
Product E	> 2 μm	283.3	41.9	580.7	114.5	2.0
0.6 mL/vial	> 5 μm	136.7	20.2	168.7	62.0	1.2
	> 10 μm	45.0	10.0	63.7	34.4	1.4
	> 25 μm	3.3	2.9	13.8	17.2	4.2
Product F	> 2 μm	678.3	200.4	9154.7	3492.4	13.5
10 mL/vial	> 5 μm	105.0	47.7	1306.7	677.2	12.4
	> 10 μm	10.0	10.0	158.0	39.6	15.8
	> 25 μm	0.0	0.0	16.7	17.0	≥16.7

[a] Value is the mean ± standard deviation of 3 measurements within test.

protein particles by using multiple filters, random forest and convolutional neural metwork.[21–24] A collaborative study of the classification between silicone oil droplets and protein particles detected using the FI method is a next step toward proposing a standardized classifier/model.

Among the products evaluated in this study, the highest difference in particle counts between the LO and FI methods was observed for Product D. From the pictures of particles presented in Fig. 1 and the contour plots presented in Fig. 3, the difference in particle counts between the methods increases with increases in the number of elongated particles in the products. This tendency is consistent with the result of our previous collaborative study using shared samples of protein aggregates, as well as other articles.[9,14,15,25] Our studies demonstrated that the ratio of particle counts between the FI and LO methods ranges from 2-fold to more than 100-fold depending on the features of particles contained in the tested products. In another study, the ratio of cumulative particle counts measured using the FI and LO methods ranged from approximately 3-fold to 50-fold on agitated or heated human albumin and polyclonal immunoglobulin.[14] It was also reported that the cumulative particle counts only slightly differed between the LO and FI methods for a low-concentration therapeutic protein product in a prefilled syringe, in line with the results for Products E and H–J in this study.[26] Our results are in line with these studies. One of the few studies to analyze commercially available therapeutic protein products using the FI method found that the number of insoluble particles in some interferon-β products exceeded 100,000 particles/mL,[27] indicating our results of more than 10,000 particles/mL in Products C and D were not atypical.

Table 3
Particle counts obtained by Light obscuration and Flow Imaging for pre-filled syringes (product G-J).

Product	Size	Light Obscuration particles/mL[a] ave	SD	Flow Imaging particles/mL[a] ave	SD	FI/LO
Product G	> 2 μm	1041.7	64.3	26416.3	554.6	25.4
0.3 mL/syringe	> 5 μm	525.0	102.1	4513.7	307.8	8.6
	> 10 μm	80.0	13.2	815.7	75.2	10.2
	> 25 μm	0.0	0	27.4	17.0	≥27.4
Product H	> 2 μm	701.7	95.7	5361.3	341.6	7.6
0.5 mL/syringe	> 5 μm	255.0	62.6	1186.7	30.1	4.7
	> 10 μm	70.0	21.8	250.0	67.9	3.6
	> 25 μm	5.0	5	11.0	9.5	2.2
Product I	> 2 μm	8250.0	892.1	34053.7	1332.2	4.1
0.5 mL/syringe	> 5 μm	5058.3	574.6	9998.0	810.2	2.0
	> 10 μm	2211.7	287.9	3223.0	408.8	1.5
	> 25 μm	125.0	31.2	223.7	43.8	1.8
Product J	> 2 μm	3846.7	216.6	19025.3	1466.7	4.9
0.7 mL/syringe	> 5 μm	1801.7	257.5	5074.0	456.4	2.8
	> 10 μm	463.3	58.4	1285.3	639.2	2.8
	> 25 μm	3.3	5.8	5.7	9.8	1.7

[a] Value is the mean ± standard deviation of 3 measurements within test.

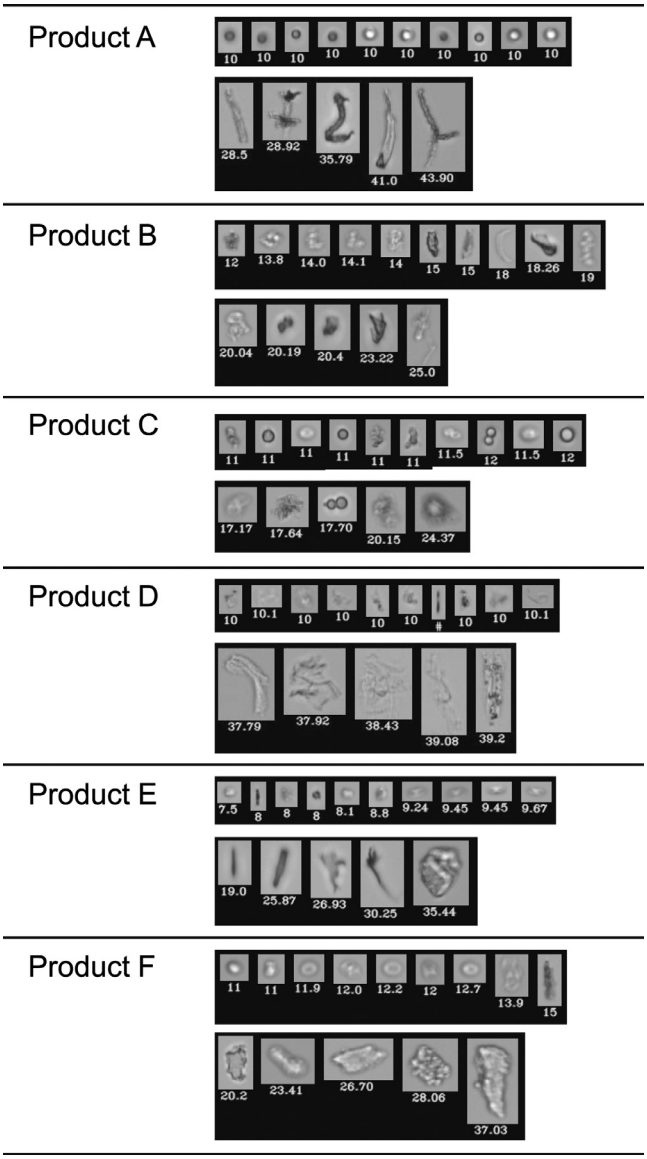

Fig. 1. Representative images of particles contained in vial products (Product A–F) detected using flow imaging.

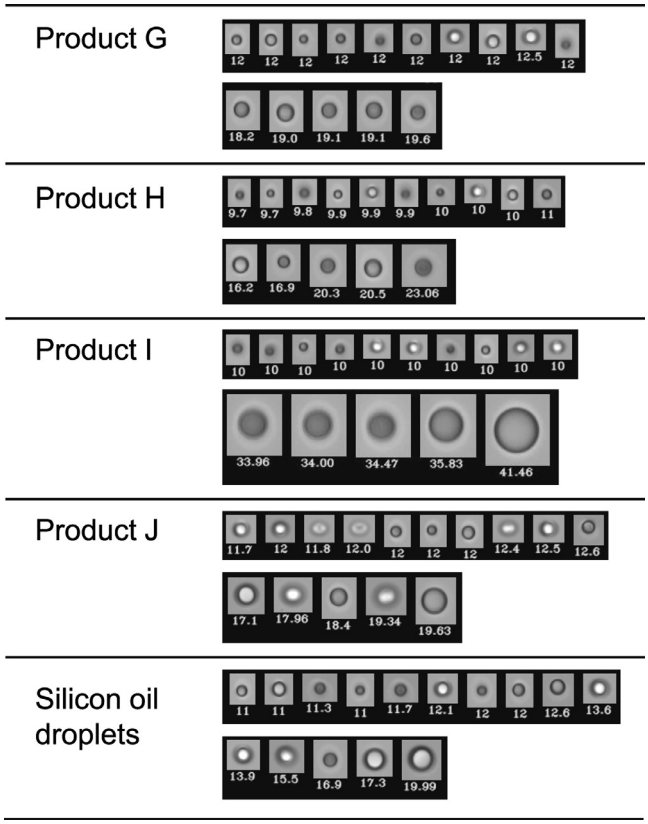

Fig. 2. Representative images of particles contained in prefilled syringe products (Product G–J) and silicone oil droplets detected using flow imaging.

The acceptance criteria of the current compendial particulate matter test using the LO method are defined as follows: "does not exceed 6000 per container equal to or greater than 10 μm and does not exceed 600 per container equal to or greater than 25 μm".[4,28] When calculating the particle counts per container for all products from the results presented in Table 2, all particle counts detected using the LO method met these criteria. Conversely, the protein count (≥10 μm) per container of Product D measured using FI method far exceeded the criteria. The number of particles actually administered to patients would be considerably smaller than the detected particle count using the FI method in this study because Product D should be injected through an in-line filter. Considering the results of this study using commercially available therapeutic protein products, setting acceptance criteria for individual product would be reasonable rather than setting an unified acceptance criterion in reference to the criterion of the LO method when using the FI method as quality test. The accumulation of measurement data measured by LO and FI methods for multiple commercially available therapeutic protein products (e.g. enzyme, monoclonal antibody etc.) with and without stressed conditions is indispensable toward the practical application of the FI method, and our study is anticipated to greatly contribute to this aim.

In this study, we compared the detectability of the FI and LO methods for commercially available products and discussed the

Table 4
Particle counts of silicone oil suspension obtained by Light obscuration and Flow Imaging.

		Light Obscuration particles/mL[a]		Flow Imaging particles/mL[a]		FI/LO
	Size	ave	SD	ave	SD	
Silicone oil	> 2 μm	26725.0	2047.6	117356.3	1887.1	4.4
	> 5 μm	11696.7	1392.7	30821.0	427.9	2.6
	> 10 μm	2415.0	438.6	6717.0	150.3	2.8
	> 25 μm	33.3	2.9	245.3	38.7	7.4

[a] Value is the mean ± standard deviation of 3 measurements within test.

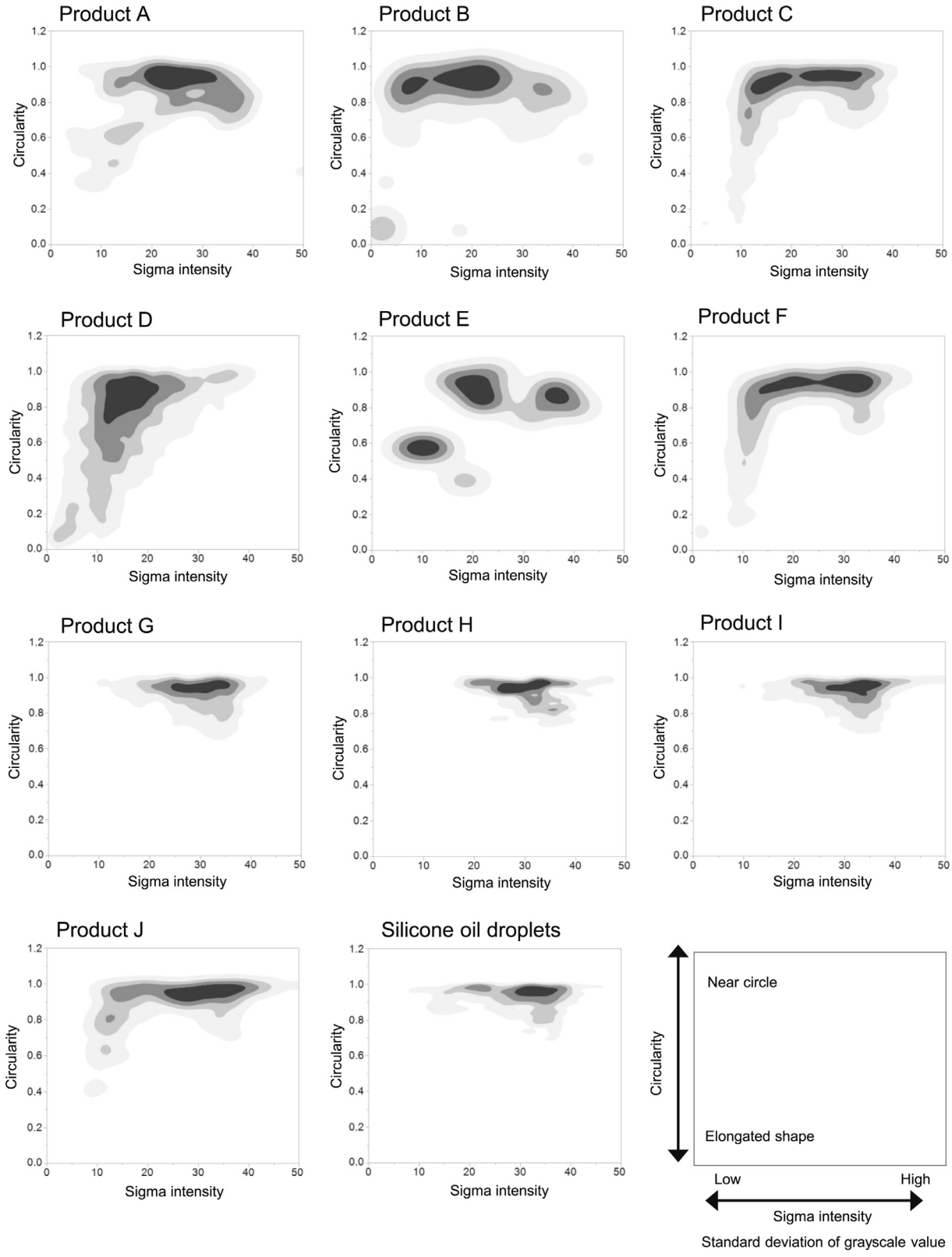

Fig. 3. Contour plots of particles measured using flow imaging. The circularity of particles was plotted against sigma intensity. Circularity: a shape parameter calculated from the perimeter and the filled area of each particle, in which a circle has a value of 1.0. Sigma intensity: standard deviation of grayscale values.

acceptance criteria for the FI method. Protein aggregates contained in therapeutic protein products continuously distribute from nanometer scale to micrometer scale, and sub-micron protein aggregates are recommended to be evaluated as a part of characterization to assess the immunogenicity risk of biopharmaceutical products.[29] The utility and analytical performance of techniques for evaluating sub-micron

particles such as nanoparticle tracking analysis and resonant mass measurement was discussed in a cross-industry collaborative study.[30] Accumulating data for protein aggregates distributed from nanoscale to microscale in each therapeutic protein product would contribute to the establishment of more robust and rational control strategies for protein aggregates in the future.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.xphs.2021.09.047.

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