#### **RESEARCH ARTICLE**



# An Interlaboratory Study to Identify Potential Visible Protein-Like Particle Standards

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### Abstract

Visible protein-like particle standards may improve visual inspection and/or appearance testing practices used in the biotechnology industry. They may improve assay performance resulting in better alignment and more standardized training among different companies. The National Institute of Standards and Technology (NIST) has conducted an interlaboratory study to test whether the standards under development mimic typical proteinaceous particles found in biotherapeutics and if they can be implemented during the visual inspection process. Fourteen organizations from industry and government have participated. A total of 20 labs from these 14 organizations participated with analysts from 6 formulation, 7 analytical, 4 quality control, and 3 manufacturing labs. The circulated samples consisted of abraded ethylene tetrafluoroethylene (ETFE) particles or photolithographic particles. The results consist of qualitative ratings, which varied substantially among organizations and within labs. Polydisperse ETFE particle suspensions, containing particles enriched in greater than 150 µm in size, were rated more favorably than the photolithographic particles by formulation and analytical scientists. The largest monodisperse photolithographic particles (approximately 300 µm in size) were favored equally compared to ETFE by all scientists. Solution modifications to decrease the settling rate or to alter optical properties of the ETFE solutions yielded lower ratings by the analysts. Both particle types received mixed ratings for their usability and for their application for visual inspection and for training purposes. Industry feedback will assist NIST in developing reference material(s) for visible protein-like particles.

**Keywords** reference standards · visible particles · visible protein particles · visual inspection

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# Introduction

Visible particles in biotherapeutics may pose safety or quality concerns; therefore, it is essential to be able to detect, characterize, and minimize their formation [1–4]. They range in size from approximately 100  $\mu$ m to a few millimeters and can be seen by the human eye under appropriate environment conditions [5]. According to United States Pharmacopeia (USP), they can be classified as inherent, intrinsic, and extrinsic particulates, depending on the source of the particles [6–8].

Regulatory guidelines require each product batch for parenteral administration be "essentially free" or "practically free" of visible particulates [6, 9, 10] even though these phrases have generated much discussion [5, 11, 12]. Some protein therapeutics can undergo protein aggregation and particle formation during storage and manufacturing, and since these types of inherent particles (e.g., visible aggregates of the active ingredient or formulation components) may not be completely avoidable, they must be controlled [13, 14]. A comment in the monoclonal antibodies for human use monograph states that some biotechnological products "may contain proteinaceous visible particles that are intrinsic to the product" [14]. This manuscript is focused on proteinaceous, inherent particles.

To detect and classify visible particles consistently, analysts should be trained using training sets that contain particles or other defects (not discussed in this work). These training kits can be commercially purchased, customized, or produced in-house that are representative of degradants found in the product of interest [6, 15]. Including real degradants as part of the training kit is ideal, but when those degradants are proteinaceous particles, it may be a challenge to isolate and stabilize particles to construct internal training sets and ensure that their size distribution does not alter over time. Labs could fabricate standards to mimic proteinaceous particles [13, 15], as we have explored previously with one of our industrial collaborators [16]. Images or videos of proteinaceous particles may also be used for training purposes. This work focuses on a small subset of the training set those particles that visually mimic inherent proteinaceous particles; the larger training set should contain additional defects representative of the respective process and product.

Human particle detection is inherently subjective to some extent and depends on factors related to the operator, lighting conditions, observation time, and nature of the particle (i.e., size, shape, translucency, etc.) [5, 6]. It is a probabilistic assay with the probability of detection mainly increasing with increasing particle size [12]. A "visible" particle does not mean that it has a 100% detection rate. A spherical 150-µm particle with high refractive index in a clear, glass vial has been demonstrated to have a 70% probability of detection (POD), which is higher than a similarly sized translucent, low refractive index, and fibrillar proteinaceous particle in a similar configuration [17]. This 70% POD is generally regarded as the industry standard for inspection qualification [12]. Defining "visibility" of particle in general is challenging as a clear size cut-off cannot be defined. Therefore, what might be defined by detection probability as "visible" for beads (with 70% POD) may not be considered "visible" for irregularly shaped, translucent, and proteinaceous particles.

Previous work showed that analysts perceived different particle attributes (e.g., shape, size, movement, translucency, etc.) with varying degrees of importance while making decisions on the "protein-like" appearance of particles in solution [16]. This provides insight into the inspection process and how certain physical particle properties impact how the particles are perceived. Since perceptions change, visual aspects such as these should be documented to ensure long-term reproducibility of the inspection process. In this work, analysts were also asked to comment on the attributes they observed while rating the samples. Analysts' ratings were primarily based on the size, morphology, dispersity, buoyancy, texture, and settling behavior of the particles, as seen by their comments.

Visual assessment, especially as it relates to particles, is performed by different analysts at various stages of drug development and manufacturing. When developing a standard that could be widely implemented, it is important to know how different analysts perceive these particle candidates based on their experiences and day-to-day activities and determine if these particle types might be a useful training tool for them. For this study, the samples were assessed by analysts from formulation development, analytical development, quality control (QC), and manufacturing work.

While multiple industry members support the development of visible particle standards, there are many differing perspectives on visible particle assessment [13, 18]. Numerous articles discuss the need for better, universal visible particle standards that can be used for training analysts [7, 13, 15, 18]. A universal training kit would allow a more harmonized approach for visible particle assessment across the industry, foster collaborations to improve the process, and facilitate interactions with regulatory agencies. It would serve as a harmonized measurement tool to better define the term "visible" and establish a more realistic cut-off for visible, irregular, and proteinaceous particles, thereby reducing some subjectivity associated with the inspection process [13, 15, 16].

The primary aim of this work is to obtain industry feedback that would assist NIST in developing a visible particle reference material mimicking typical proteinaceous particles, especially as there are no widely available visible protein-like particle standards. In this survey, 14 organizations (Table I) volunteered to evaluate samples comprising of particles of either the polymer ethylene tetrafluoroethylene (ETFE) or the photolithographically fabricated SU-8, an epoxy-based photoresist, chosen for their ability to mimic proteinaceous particles in appearance in solution (Fig. 1). Both the ETFE and SU-8 particles are synthetic polymers, not actual proteins. ETFE particles are polydisperse and irregular in shape with their refractive index ( $n \approx 1.40$ ) closer to the refractive index of proteinaceous particles ( $n \approx 1.41$ ) than to conventionally used polystyrene spheres ( $n \approx 1.59$ ) [19–22]. They can be as large as 300  $\mu$ m in size, whereas the SU-8 particles ( $n \approx 1.60$ ) are monodisperse with well-defined size and shape and can be approximately 150 µm, 250 µm, and 300 µm in size [23]. Some scientists might consider these particles to fall in the "grey zone" between visible and subvisible size range due to their low detection probability, translucency, irregular morphology, and size [6, 7]. To achieve the standard 70% POD, a single, irregular, fiber particle needs to be between 500 and 2000 µm in size, which is much larger than the particle sizes used in this study [7, 12]. While this is an important consideration, (1) the particles produced for this study are based on what is feasible to produce since larger particles will be more structurally fragile; (2) this study is focused on the appearance and handling of these particles not on determining detection probability; and (3) each vial contains multiple particles, not single particle.

These standards might support two approaches in the detection of visible particles in biotherapeutics. To establish probabilistic limits with a group of analysts or to assess the sensitivity of an inspection method at a stated particle size, the SU-8 particles might be advantageous because they are monodisperse at defined sizes. To perform a semi-quantitative analysis of inherent particles during formulation development

or for monitoring stability changes in a non-destructive manner, ETFE might be more advantageous, as was described in one of our earlier collaborations [16].

# Materials and Methods

The study consists of the following steps: (1) fabrication and vialing of the ETFE and SU-8 particles at NIST; (2) distribution of the test kits to participants; (3) data collection by the participants; and (4) data analysis and reporting.

#### **Fabrication of Particles and Vialing of the Samples**

The preparation and vialing of the abraded ETFE and the photolithographic SU-8 particles are described in detail in the supplementary section. The ETFE and SU-8 samples including the turbidity and/or viscosity modified samples (ET106, EV106, and ETV106) were serially diluted to have the targeted particle concentrations, as shown in Table II and Supplemental Fig. 7. Flow imaging microscopy was used to confirm that the particle concentrations were within the range desired. For this study, knowing the exact particle concentrations is not crucial; the concentrations chosen should allow easy detection by analysts and be low enough to count with adequate repeatability on a flow imaging microscopy system. The particle content seen in these vials is much higher than what would be seen in a mature drug product, where there would be much fewer particles. Previous (unpublished) work showed that the ETFE and SU-8 particles in various formulations are

Table I List of Participants in the Study	Among Inc. Drosons Daviderment They and Oaks California			
	Angen, n.c., Process Development, Phousand Oaks, Camornia			
	AstraZeneca, Global Operations Biologics, Nijmegen Manufacturing Facility, Nijmegen, Netherlands			
	Biogen, Analytical Development, Cambridge, MA, USA			
	Boehringer Ingelheim Pharma GmbH & Co., Innovation Unit, KG, Biberach, Germany			
	Coriolis Pharma Research GmbH, Martinsried, Germany			
	Eli Lilly and Company, Global Quality Laboratories, Indianapolis, IN, USA			
	FibroGen, Inc. Analytical Development, San Francisco, CA, USA			
	Food and Drug Administration, Office of Pharmaceutical Quality, Silver Spring, Center for Drug Evalua- tion and Research, MD, USA			
	Genentech, Inc., Pharmaceutical Development, San Francisco, CA, USA			
	GlaxoSmithKline, Drug Product Development, Rockville, MD, USA			
	Janssen R&D, DPDS BTDS Analytical Development, Schaffhausen, Switzerland			
	MacroGenics, Inc., Analytical Sciences BPD, Rockville, MA, USA			
	Porton Biopharma Ltd., Salisbury, UK			
	Sanofi, BioAnalytics Germany, Characterization, Frankfurt am Main, Germany			

<sup>&</sup>lt;sup>a</sup>Although US FDA laboratory participated in the scientific study and/ or discussion, please note that FDA does not recommend, endorse, or recognize this standard development and further, the content of this communication represents the authors' views and does not bind or obliga te FDA

Fig. 1 Overview of study



stable for the duration of this study, which was anticipated to last between a few months to a year.

#### Sizing Definition Used for ETFE Particles

The size range mentioned in Table II is defined differently for the ETFE and SU-8 particles. Because the abraded ETFE is irregular and polydisperse, it is possible to enrich particles in a certain size range but not possible to eliminate particles that fall outside of this range using our filtering approach. Multiple sieves of different sizes were used to enrich the particle number concentration for ES75, ES106, and ES150 in the size range of interest, as described in the Supplementary section. Since these particles are not spherical, a sieve with a particular opening will not fully eliminate all irregular particles smaller than that sieve size due to the way the particles deposit on the sieve during filtration. The size of the filters used to prepare the ETFE samples determined the targeted size range that was used for the rest of the study, as listed in Table II. A more quantitative sizing system for ETFE will be determined in the future. When analyzing these ETFE samples on a flow imaging microscopy system, the size measurements are obtained in the same equivalent spherical diameter (ESD) range as the sieve size of the filter used to prepare that ETFE sample; i.e., for ES75, the reported ESD particle concentration is between 75 and 150 µm range since that sample was prepared with 75 µm and 150 µm sieves.

#### Sizing Definition Used for SU-8 Particles

The SU-8 particles are monodisperse and are not produced by filtering. The size of the SU-8 particles was defined by the largest circle that circumscribes that particle, equivalent to the length, which is slightly larger than the maximum Feret diameter and larger than the ESD (Fig. 2c). Because this sizing definition is unconventional, maximum Feret diameter and area based diameter will be used in future work. These particles rotate while falling through the flow cell, which impacts their measured size. The targeted size range reflects the range of orientations measured.

# **Distribution of Test Kits**

A test kit consisting of 16 containers with ETFE, SU-8, and diluent solutions was distributed to participants, as shown in Table II and Supplemental Fig. 1, along with study instructions and survey form. The analysts were informed whether their sample was ETFE or SU-8 but were not given any additional sample information.

Figures 2 and 3 show representative images of the SU-8 and ETFE vials and particle images obtained by a semiautomated visual inspection method. Table II displays the particle number concentrations for each sample as obtained by a flow imaging microscopy system. The disparity in number of particles present in the figure images and the particle **Table II** Materials Included in the Test Kit. The Feature of Interest in the Particle or Solution Studied is Size, Morphology, or Background Modification (Turbidity or Viscosity). The Particle Concentrations in
 the Targeted Size Range are Obtained from 4 Separate Measurements (n=4) Reported as the Mean  $\pm 1$  Standard Deviation

Vial label	Comment	Feature of interest in particle/ solution	Targeted size range, <sup>a</sup> (ESD or length) μm	Particle concentration (in targeted size range), mL <sup>-1</sup>
ES75	ETFE	Size	75 to 150	93±8
ES106	ETFE	Size	106 to 150	74±3
ES150	ETFE	Size	150 to 250	$89 \pm 9$
ET106	ETFE	Turbidity	106 to 150	$72 \pm 2$
EV106	ETFE	Viscosity	106 to 150	$76\pm8$
ETV106	ETFE	Turbidity and viscosity	106 to 150	$66 \pm 9$
ES106C	Concentrated ETFE	Size	106 to 150	$137 \pm 18$
ETFE diluent	ETFE diluent	-	-	-
S150M1	SU-8	Morphology-1	100 to 160	$16 \pm 5$
S250M1	SU-8	Morphology-1	160 to 270	19±4
S150M2	SU-8	Morphology-2	100 to 160	$30\pm8$
S250M2	SU-8	Morphology-2	160 to 270	$12 \pm 5$
S300M1	SU-8	Morphology-1	270 to 310	18±3
S250M1C	Concentrated SU-	Morphology-1	160 to 270	$116 \pm 13$
SU-8 diluent	SU-8 diluent	-	-	
Blank	Diluent	-	-	-

<sup>a</sup>The targeted size range is measured on the flow imaging system, as ESD for ETFE and length for SU-8. The targeted size range for the ETFE depended on the sieves used for filtering the particles. The targeted size range for the SU-8 particles was chosen to account for various orientation of the particles as they were imaged by flow imaging



Fig. 2 Representative images of  $\mathbf{a}$  vials containing SU-8 particles (at increased contrast, with circles over representative particles) obtained by a semi-automated visual inspection system and  $\mathbf{b}$  SU-8 particles

obtained by flow imaging microscopy. c Schematic illustrating the two morphologies and the sizing definition used for the SU-8 particles

number concentrations in Table II, for the corresponding sample, can be due to the differences in instrument sensitivity of the methods used to collect the data. The semiautomated system has decreased sensitivity at smaller sizes and therefore is unable to detect smaller particles (i.e., it detects fewer particles in S150 compared to S250 and S300) while the flow imaging microscopy system has a similar detection sensitivity for particles in all these size ranges.



Fig. 3 Representative images of a vials containing ETFE particles (at increased contrast) obtained by a semi-automated visual inspection system and b ETFE particles obtained by flow imaging microscopy

Flow imaging microscopy measurements are more accurate in terms of sizing and counting whereas the semi-automated method was performed for characterization purposes.

The SU-8 particles appear as distinct particles at two morphologies that were produced for this study. The internal holes were added to reduce some of the mirror-like reflections observed with these particles. The ETFE particles appear less distinct and more fiber-like, and the particle movement resembles a tornado.

# **Participants**

The reported data is from 14 organizations. Some organizations had multiple labs participating, leading to a total of 20 labs that were distributed into 6 formulation, 7 analytical, 4 QC, and 3 manufacturing labs. A total of 42 analysts from all these labs participated. All analysts were familiar working with proteinaceous particles.

# **Data Collection**

The survey consisted of non-quantitative, subjective questions. For some questions, the analysts were asked to respond using a five-point scale (i.e., from least likely to most likely). Many analysts also used a reference protein sample (not supplied by NIST) for comparison to the standards while performing the study. The survey questions were sample-specific and did not address specific visual inspection practices of the organization.

# **Data Analysis and Reporting**

The survey was completed by at least one analyst at each organization. If multiple analysts submitted data, the data was averaged for that organization and compared to the average values for the other organizations. Each data point corresponds to a single organization regardless of the number of analysts participating to assure that one organization's results are not weighed more than another based on the number of analysts who participated. This applied when multiple analysts submitted data within the labs as well. The rating system is based on the individual analyst's perspectives, so this work is not intended to be a rigorous statistical evaluation of these subjective results. Our aim is to qualitatively understand what types of samples the analysts think mimic typical proteinaceous particles.

# Flow Imaging Microscopy

Methods described in the Supplementary Section.

### Semi-Automated Visual Inspection (SAVI)

Methods described in the Supplementary Section.

#### Results

This study is composed of two sections. In the first part, participants were requested to inspect each sample vial and rate how similar the particles resemble proteinaceous particles. They were also asked to comment on the attributes they observed while rating the samples. In the second part, they were asked to perform two proof-of-concept exercises to demonstrate the usability of ETFE as a semiquantitative standard and determine if SU-8 can be useful for determining detection thresholds by a group of analysts [16]. The analysts were requested to explain their ratings and any challenges they encountered. The data shown are from 13 (19 labs) or 14 (20 labs) organizations depending on how many data sets were received for each question.

In part 1, analysts from 13 organizations rated each sample on a scale from 1 to 5, with 1 indicating that the sample looks least like containing proteinaceous particles and 5 indicating that the sample looks most like containing proteinaceous particles. Samples look more like proteinaceous particles in solution if their average rating is 3 or greater ( $\geq$  3).

Figure 4 summarizes the ratings for the 6 ETFE and 5 SU-8 samples. The vertical axis shows the number of organizations who have rated the corresponding samples an average rating of  $\geq$  3. Overall, ETFE rated higher than the SU-8 samples. ES150 was rated the highest with 12 out of



**Fig. 4** The graph shows the number of organizations (out of the 13 who submitted this portion of the data) that rated the corresponding sample with a rating  $\geq$  3. Analysts were asked to rate each sample from 1 (least protein-like) to 5 (most protein-like). The **Y**-axis refers to the number of organizations who gave the samples a rating that is  $\geq$  3. ET106 refers to the sample with increased turbidity; EV106 refers to the sample with increased turbidity; and ETV106 refers to the sample with both increased turbidity and viscosity

13 organizations rating it  $\geq$  3. Analysts described ES150 as containing amorphous, translucent particles with particle movement resembling quick-settling, proteinaceous particles in solution. ES106 was rated second most favorably and contained translucent, "snow-like," and flaky particles, with movement resembling swirling sediment. ET106 and EV106, the two solution-modified samples rated moderately. ES75 and ETV106 were rated the lowest with only 4 organizations scoring them  $\geq$  3. ES75 was described as containing small, globular, off-white, translucent, and irregular shaped particles. These particles were fiber-like and heterogeneous in size and shape. While most analysts were able to visualize all the particles, multiple participants stated that particles in the unmodified ETFE samples settled too fast. Particles in the viscous modified sample (EV106 and ETV106) settled slower, more like protein particles, but bringing them into motion was difficult; particles in the opalescent background sample (ET106 and ETV106) were more challenging to inspect due to increased turbidity.

The SU-8 particles were generally larger and analysts had no trouble visualizing these particles. S300M1 was rated the highest with 8 out of 13 organizations rating it  $\geq$  3. Several responses indicated the irregular morphology and slow settling behavior made these particles better mimic protein particles. Some stated that the particles' flat, "flaky" surface reflected light so their "edge" was visible, which is unnatural for proteinaceous particles. The remaining SU-8 particles were rated  $\geq$  3 by 5 or less organizations. Supplemental Fig. 4 shows the results obtained from each of the 13 organizations.

Analysts were categorized into two groups, roughly based on the different stages of product lifecycle they support: 10 formulation and analytical labs (group 1) and 6 quality control and manufacturing labs (group 2). Analysts from both groups rated ETFE as appearing more proteinaceous than SU-8 particles (Fig. 5a and b). ES150 sample was rated the highest ( $\geq$  3 rating) by 9 out of the 10 group 1 labs, followed by ES106. In group 2, ES150 and ET106 each received  $\geq 3$ rating from 5 out of the 6 labs. ETV106 sample was rated the lowest in both groups. In group 2, none of the labs thought the particles present in ETV106 appeared proteinaceous (i.e., rated it at least a 3). It is easier to detect a particle if it is moving, in comparison to a slow moving or stationary particle. Perhaps this is one of the reasons, in addition to the increased turbidity and viscosity of the background solutions, that the analysts were not readily able to detect the ETV106 sample. Group 1 rated S300M1 the highest (5 out of 10 labs rated  $\geq$  3), while group 2 rated S250M1 the highest (4 out of 6 labs rated  $\geq$  3). S150M1 and S250M2 rated worst among groups 1 and 2, respectively. Supplemental Fig. 5 shows the break-down of the ratings obtained from each lab. Data from 6 formulation, 6 analytical, 4 QC, and 3 manufacturing labs is shown in Supplemental Fig. 6. The





**Fig. 5** Number of labs with average ratings of  $\geq 3$  for ETFE and SU-8 samples by analysts from **a** 10 formulation and analytical development labs and from **b** 6 quality control and manufacturing labs. ET106 refers to the sample with increased turbidity; EV106 refers to

the sample with increased viscosity; and ETV106 refers to the sample with both increased turbidity and viscosity. Ratings are from 1 (least protein-like) to 5 (most protein-like). The **Y**-axis refers to the number of labs who gave the samples a rating that is  $\geq 3$ 

formulation group favored nearly all ETFE (except ETV106) more than SU-8. The analytical group had a mixed preference for ETFE but still preferred ETFE more than SU-8; they preferred ES150 and S300M1 the most. QC most favored ET106 and ES150 while manufacturing favored ES150 and S300M1.

Analysts were asked to identify samples that (1) resembled most protein-like, (2) resembled least protein-like, and (3) they would like to see become commercially available. In Fig. 6, the bars represent the frequency of times a sample appeared as a response to the 3 choices above. Since some organizations had multiple analysts and some analysts provided more than 1 response for each of the 3 categories, the frequencies do not sum up to 14, the number of organizations providing the data. If the same response was received from multiple analysts within one company, it was only counted once for that company to ensure that the results submitted from organizations with more analysts are not weighed heavier than those submitted with fewer analysts. ES150 and S300M1 were rated most protein-like and most commercially desirable, according to Fig. 6a and b. S300M1 was simultaneously rated as most protein-like/ commercially desirable from 8 responses and least proteinlike by 7 responses. ES75 and ETV106 appeared at least 9 times as the least protein-like samples. ETFE and SU-8 were listed as not commercially desirable by two and four responses, respectively.

The physical properties such as particle size, particle morphology, and background solution characteristics compared in this study did not significantly impact the ratings, as obtained from 13 organizations. Figure 7a shows the ratings increased with particle size for ETFE (9 ratings increased with increasing particle size *versus* 4 ratings that showed no difference) but not with SU-8. None of the three turbidity and/or viscosity modified samples (ET106, EV106, or ETV106) rated more favorably than the unmodified ES106 sample (Fig. 7b). Morphology did not impact the ratings of the SU-8 particles (Fig. 7c) when particles at approximately the same size were compared at two morphologies (shape 1=0.6 aspect ratio and shape 2=0.3 aspect ratio).



**Fig. 6** Frequency of responses given by analysts from 14 organizations regarding which **a** ETFE samples and **b** SU-8 samples most or least resembles proteinaceous particles and those that they think would be most commercially desirable



Fig. 7 Average analyst ratings based on a particle size b background solution of the ETFE and c morphology of SU-8 particles. For panel b, higher and lower are relative to the solution without solution modi-

In the second part of the study, analysts performed two exercises with the concentrated ETFE and SU-8 samples. The analysts were asked to prepare a dilution set with the ETFE to assess its potential to be used in a semi-quantitative manner, much like what was described in Telikepalli et al. and Cash et al. [15, 16]. For the SU-8, the analysts were asked to seed a glass vial with one 250 µm SU-8 particle and inspect that vial multiple times to see how frequently the particle can be detected. The ETFE dilution task is a typical laboratory task, whereas seeding a single SU-8 particle is technically difficult, time-consuming, and is usually done by specialists. Upon completing these exercises, the analysts were asked to rate the following four statements on a scale from 1 to 5, with 1 implying strong disagreement and 5 implying strong agreement with the following statements. For analysis, anything  $\geq 3$  indicates an agreement with the statement and anything below a 3 indicates an unfavorable agreement to it.

fications, such as the ES106 sample. Data from 13 organizations was used for this analysis. The numbers above the bars represent the label for the bars

- 1) ETFE particles in solution may help reduce some subjectivity and better define "visible" when monitoring proteinaceous particles.
- 2) ETFE particle standard sets are easy to handle and prepare by the analysts.
- 3) SU-8 particles in solution may help reduce some subjectivity and better define "visible" when monitoring proteinaceous particles.
- 4) Single SU-8 particle per vial is easy to prepare by the analysts.

Not all 14 organizations provided responses to these questions. For the first and third questions, only 11 organizations provided responses. For the second and fourth question, 8 organizations responded. The ratings for ETFE are higher than for SU-8. Ten of the 11 respondents agreed that ETFE could better define "visible" (Fig. 8a) but only 8 (Fig. 8b) responded this way for the SU-8 (rating  $\geq$  3). For the ETFE,

Fig. 8 Average analyst ratings of a ETFE particles and b SU-8 particles for two subjective questions. Ratings are given from 1 (least agree) to 5 (most agree) whether the analysts believe the particles can be used to better define "visible" for proteinaceous particles and for their ease of use. Ease of use refers to preparing dilutions for ETFE and preparing seeded vials for SU-8



all 8 respondents rated the ETFE as easy to use, especially once methods are optimized with the protein of interest. For the SU-8, 3 out of the 8 respondents rated similarly based on the exercise. Those participants who rated low for the ETFE stated that dilution series exercise might be more useful if done in comparison to an actual product. For the SU-8, some analysts claimed that seeding a single SU-8 particle was a difficult task.

Participants were then asked their opinions on using both particle types for training purposes. Out of the 14 participants, 7 responses were positive, 2 responses were negative, 1 response was mixed, and 4 provided no responses. In summary, those with a positive response stated that the particle types would be helpful as a teaching tool, especially when handling difficult to detect particles. The particles' irregular morphology and heterogeneous nature make them an attractive reference material as they are better than the polystyrene latex beads currently available. Those with negative responses stated that these particles were too small or their products do not contain sufficient proteinaceous particle loads thus making a semi-quantitative standard unnecessary. Others mentioned that they would favor more optimization of these standards before implementing them and until then, would prefer to use real-world particle sets, images, or video-based technologies.

# Discussion

Industry feedback was collected regarding the feasibility of ETFE and the SU-8 particle standards to be used for training purposes for visible particle assessment in biotherapeutics. This was a qualitative study and the rating variability observed among organizations confirms the subjective nature of this work. The variability could be due to a combination of the (1) organizations' internal practices regarding visible proteinaceous particles and whether they have products with these types of particles and the (2) analyst's function in the organization and their perception of proteinaceous particles.

In general, analysts favored the ETFE particles more than the SU-8 particles according to Figs. 4, 5, 6, and 8. ES150 sample, enriched with particles in the size range of 150 to 250  $\mu$ m (ES150), was the most preferred of the ETFE samples tested. There was a discrepancy in analysts' preference for SU-8 particles. Some analysts favored the S300M1 because they were large and easy to see but to other analysts, the dissimilarities between them and actual proteinaceous particles were magnified due to their large size.

There was only a minor difference in analyst ratings based on their function. Formulation and analytical scientists preferred the ETFE more than the QC and manufacturing scientists. Previous work showed that ETFE is useful in earlier development, where potentially most formulation and analytical work might be performed. These scientists, who may be working with some not-fully-stabilized products that might form visible proteinaceous particles, might envision the utility of a semi-quantitative standard [16].

The preference for the ETFE might be most attributed to the polydispersity of the particles that closely mimics those of proteinaceous particles. Both ES150 and S300M1 were comprised of the largest particles in their respective set. Supplemental Fig. 7 shows that the ETFE concentration in the 3 bins is higher than that of SU-8. However, the differences in particle size distribution or visibility of the two particle types were not the main reasons for the differences in ratings. Even at the lower concentrations, none of the analysts had trouble seeing the SU-8 particles. Factors such as the SU-8 samples' monodispersity, particle reflectivity, and visible edges had a dominant impact on the lower ratings of these particles.

Morphology and background solution did not impact the analysts' ratings for the particle sizes tested. It is possible that the SU-8 particles' orientation can preclude inspection. Morphology 2 particles are narrower with a smaller aspect ratio; therefore, they may "disappear" if they are floating on their side compared to similar-sized morphology 1 particles. This could be one of the reasons that the SU-8 ratings did not always trend favorably with increasing size (i.e., 250 µm particles always rating higher than 150 µm), as shown in Figs. 4 and 5b (Fig. 5a did follow this trend, though). Modifying the background solution to be more representative of high concentration proteins was not favorably rated; this result was surprising even though the viscosity modifier reduced the quick settling rate, which some analysts saw as a limitation of the particles. Previous limited evaluation by industrial analysts suggested that matching the slight opalescence to protein solutions in addition to reducing the settling time of the particles would help to better mimic actual protein particle samples [16] illustrating the diversity of inspection strategies or expectations. If ES150 or larger size ranges were used instead of the ES106 sample, the larger particle size in combination with increased background opalescence and slower settling might have allowed the analysts to better visualize the particles in solution and identify them as more closely resembling proteinaceous particles in solution.

The variability observed reflects the differences among organizations, products, and training practices; but also demonstrates difficulties in achieving production of a rugged, stable particle that closely mimics the transparent and fibrous nature of proteinaceous particles, along with their nearly neutral buoyancy. The candidates used in this study represent compromises of what particles are feasible to produce. Fabrication of particles from fully fluorinated polymer would increase particle transparency but would exacerbate the relatively fast settling time due to the increased density of the polymer and would be harder to stabilize because of their hydrophobic nature. Polydisperse standards are also harder to characterize. One of the challenges with polydisperse samples in this study is the assumption that analysts cannot see particles smaller than 75  $\mu$ m or outside the "target size range." Determining a cut-off is challenge since the human eye resolution can go down to 50  $\mu$ m but if enough particles are present, even the probability of seeing some 75  $\mu$ m particles will increase [24].

The overall assessment of the ETFE and the SU-8 samples gained from this study provides a direction forward for developing a broadly available semiquantitative count and size standard, respectively, for voluntary use by industry. Having particles of highly defined irregular morphology and size is very important, especially since such types of particle standards are not commercially available. While the particle number concentrations are higher than what would be found in biopharmaceuticals, this was to facilitate visual assessment of the different particle types in the comparison. Past work has demonstrated that these types of particle standards are readily diluted [16]. Our intention is to provide a tool that can help the industry better train analysts to monitor visible proteinaceous particles, but it will be up to the individual organizations to decide on how to use this tool, as appropriate for their products. Although the results showed that neither the ETFE nor the SU-8 can be useful for training analysts on whether a particle is or is not proteinaceous, ETFE has potential value in assessing the relative quantity of proteinaceous particles and the SU-8 has potential value in serving as a visual reference for approximate particle size.

# Conclusion

In this interlaboratory study, scientists from formulation, analytical, QC, and manufacturing labs from 14 organizations provided feedback on two types of visible particle standards being produced by NIST. The feedback from the analysts will help identify the utility and challenges associated with these particle types and develop a path forward for their commercial release.

In general, all analysts favored the ETFE particles more than the SU-8 particles, especially in the larger size range. The analysts had mixed responses regarding the sample handling and usability of the two particle types, with some favoring them but others not ready to implement them, largely due to some of the particles being too small, settling too fast, or appearing too reflective with sharp edges. ETFE samples prepared at elevated viscosity and opalescence to mimic high proteinaceous solutions were deemed not proteinaceous enough, in general. The subjective nature behind visual inspection practices and analyst's background could explain some of the variability in the results observed.

Based on the feedback, NIST intends to produce both types of particles but in a staggered timeline. While the ratings suggested that the ETFEs were the most favorable particle types compared to the SU-8, defining a specific size range of interest with a specified number concentration for ETFE, especially as it is heterogeneous over a broad size range, is a challenge. Unlike the SU-8, the ETFE samples also contain very fine particles. Therefore, it is important to identify methods to remove the very fine particles and better define the size range of interest for the heterogeneous ETFE samples before a well-defined reference material can be produced. The SU-8 particles are more straightforward to produce and characterize, as they are very homogeneous. Consequently, while they have a limited role, the SU-8 particles can still be useful for defining particle size. Since the SU-8 particles are easier to characterize, they will be developed first in one morphology, with the primary goal of serving as a training reference for the appearance of irregular particles of a given size. Afterwards, a polydisperse ETFE particle standard of certified particle size distribution, similar to the ES150 sample, is planned. These standards will be used to train analysts to semi-quantitatively track inherent particles in biopharmaceuticals [16]. Standards such as these will be an effective tool that will enable visual assessment of biotherapeutics to be more uniform throughout the industry.

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#### Declarations

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