





























































































































































































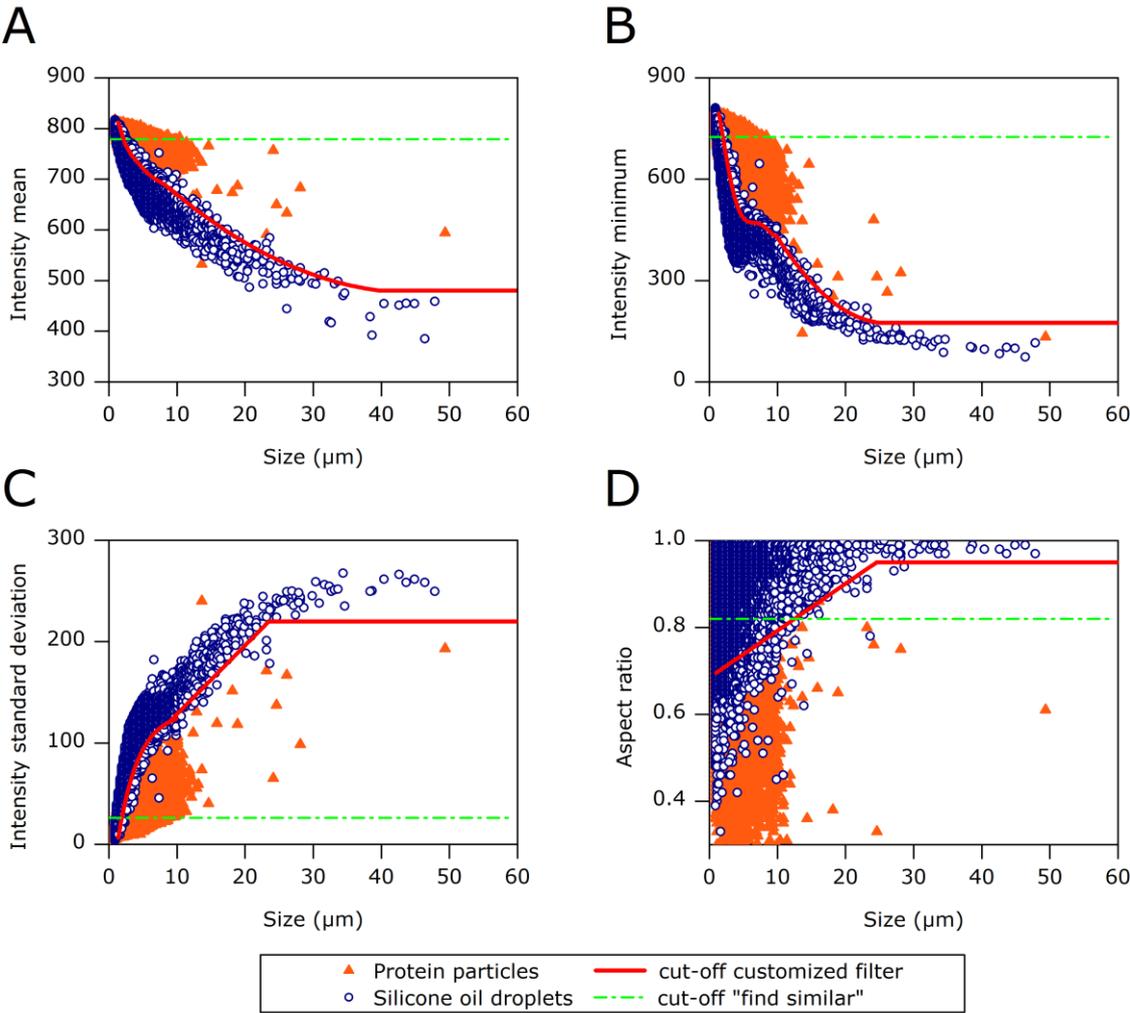




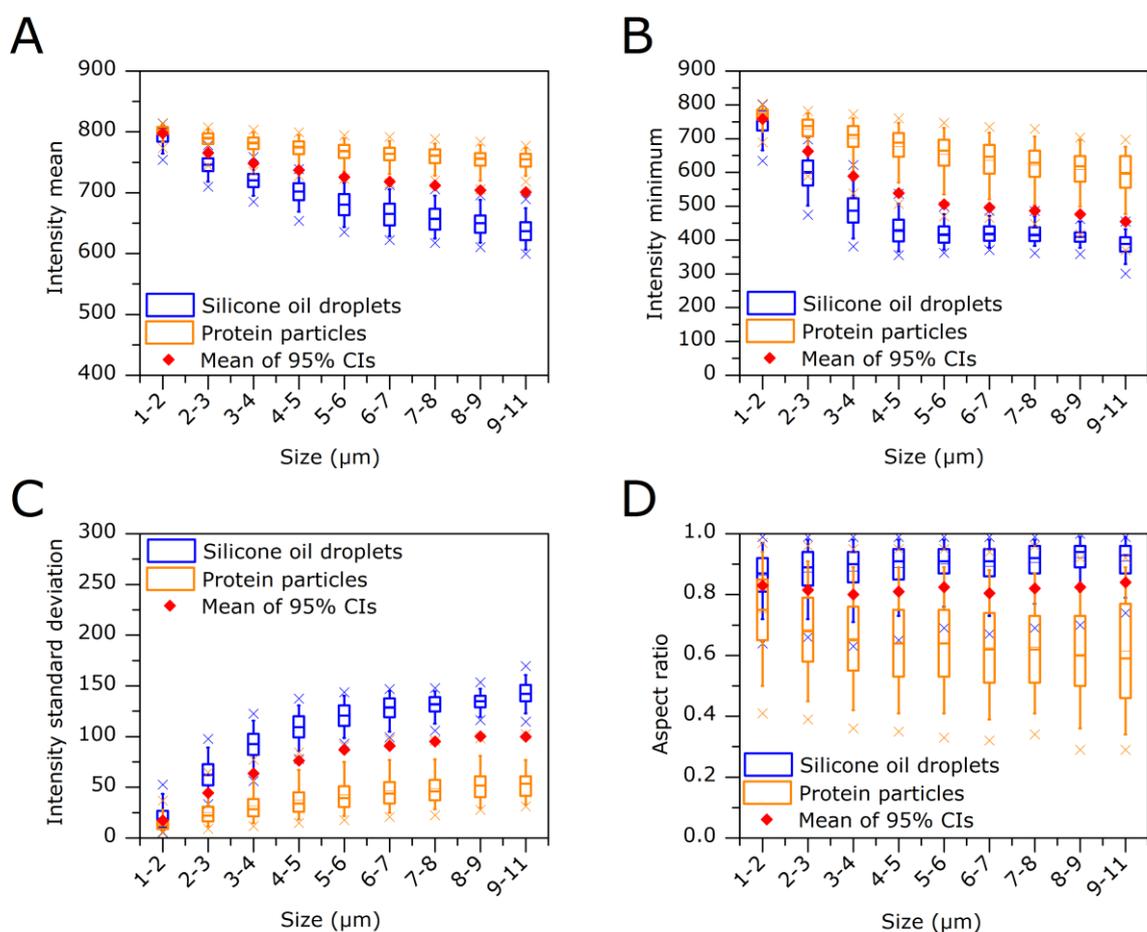


infinite length. For each of the four particle parameters, the individual distributions for silicone oil droplets and protein particles from heat-stressed rituximab were compared as a function of size.

Cut-offs were defined at the mean value of the 95% confidence intervals between the two populations (Figure 3-2). A polynomial function was automatically fitted to these points from 1 to 11  $\mu\text{m}$  and applied for particles from 1 to 9  $\mu\text{m}$ . Above 11  $\mu\text{m}$ , the number of counts acquired was not sufficient for this statistical approach; therefore, the fit was adjusted manually in this larger size range. The automated and the manual fit were overlapped in the size range from 9 to 11  $\mu\text{m}$  to ensure a smooth transition. Since the silicone oil droplet population was more homogeneous than the protein particle population, the customized filter was set to identify objects as silicone oil droplets only when they fulfilled all four cut-off fit criteria. Particles showing values below the cutoff for intensity mean and minimum (Figure 3-1A and B) and at the same time above the cutoff for intensity standard deviation and aspect ratio (Figure 3-1C and D) were marked as silicone oil droplets by the algorithm. Particles fulfilling less than four of these criteria were assigned as non-silicone oil particles, which means in our case protein particles.



**Figure 3-1: Scatter plots of particle parameters (A) intensity mean, (B) intensity minimum, (C) intensity standard deviation, and (D) aspect ratio for individual samples containing only protein particles (heat-stressed rituximab) or only silicone oil droplets analyzed separately by MFI and merged into one graph per particle parameter. The solid red lines illustrate cutoffs as a function of size, generated by our customized fit for the discrimination between silicone oil droplets and protein particles. The dash-dotted green lines illustrate linear cutoffs used by the MVAS software for the “find similar” operation.**



**Figure 3-2: Distribution of the MFI particle parameters (A) intensity mean, (B) intensity minimum, (C) intensity standard deviation and (D) aspect ratio for individual samples of silicone oil droplets and protein particles (heat-stressed rituximab). Box plots show 25/75% (box) and 5/95% percentiles (whisker) as well as minimum and maximum values (X). The mean values of the 95% confidence intervals (CI) were used as a basis to fit the function for the customized filter.**

## 2.7 Resonant mass measurement (RMM)

An Archimedes system (Affinity Biosensors, Santa Barbara, CA) was equipped with a Hi-Q Micro Sensor and controlled by the ParticleLab software version 1.8. The sensor was flushed for 60 seconds with purified water prior to analysis. Subsequently, possible impurities in the system were removed by two “sneeze” operations (liquid in the sensor is pushed into both directions) and the system was flushed again for 60 seconds with purified water. The sample solution was then loaded for 45 seconds. Prior to analysis, the limit of detection (LOD) was determined three times in automatic LOD mode. The mean value was then set

fixed for each measurement. Samples of 150 nL were analyzed (n=3) and fresh sample solution was loaded for each of the triplicate measurements.

Size determination of particles by RMM is based on the frequency shift  $f$  which is proportional to the buoyant mass  $M_B$  and depending on the sensitivity  $S$  of the resonator (Equation 3-1).

$$M_B = \Delta f * S$$

**Equation 3-1**

The conversion of buoyant mass  $M_B$  into dry mass  $M$  (Equation 3-2) and diameter  $D$  (Equation 3-3) is then based on the density of the particle,  $\rho_{\text{particle}}$  (1.32 g/mL for protein particles, based on the density estimation of pure protein<sup>37</sup> and the recommendation of the manufacturer; 0.97 g/mL for silicone oil, according to the supplier) and the density of the fluid,  $\rho_{\text{fluid}}$  (calculated based on the sensor frequency relative to the frequency and the density of water as a reference).

$$M = \frac{M_B}{1 - \rho_{\text{fluid}} / \rho_{\text{particle}}}$$

**Equation 3-2**

$$D = \sqrt[3]{\frac{6M}{\pi\rho_{\text{particle}}}}$$

**Equation 3-3**

## 3 Results and discussion

### 3.1 Silicone oil droplets in prefilled syringes

Expired prefilled syringes of etanercept and adalimumab were available for the study and analyzed in order to gain insight into relevant levels and size distributions of silicone oil droplets in marketed products as a worst case scenario. Four and six years after expiration, respectively, MFI determined for both products about  $4 \times 10^5$  particles/mL above  $1 \mu\text{m}$ . Based on the images generated by MFI, about 80% of the particles above  $5 \mu\text{m}$  in both products could be identified as silicone oil droplets using the “find similar” operation provided by the MVAS software. RMM determined  $3.2 \times 10^6$  particles/mL larger than  $0.5 \mu\text{m}$  for etanercept and  $2.0 \times 10^6$  particles/mL for adalimumab, of which 51% and 97%, respectively, could be attributed to silicone oil. Three and four years after expiration, RMM determined for both analyzed products lower concentrations of protein particles and of silicone oil droplets when compared to products four and six years after expiration, respectively (Table 3-1). This implies that total particle concentrations as well as the ratio between silicone oil droplets and protein particles can vary substantially between products, lots, and age of the product.

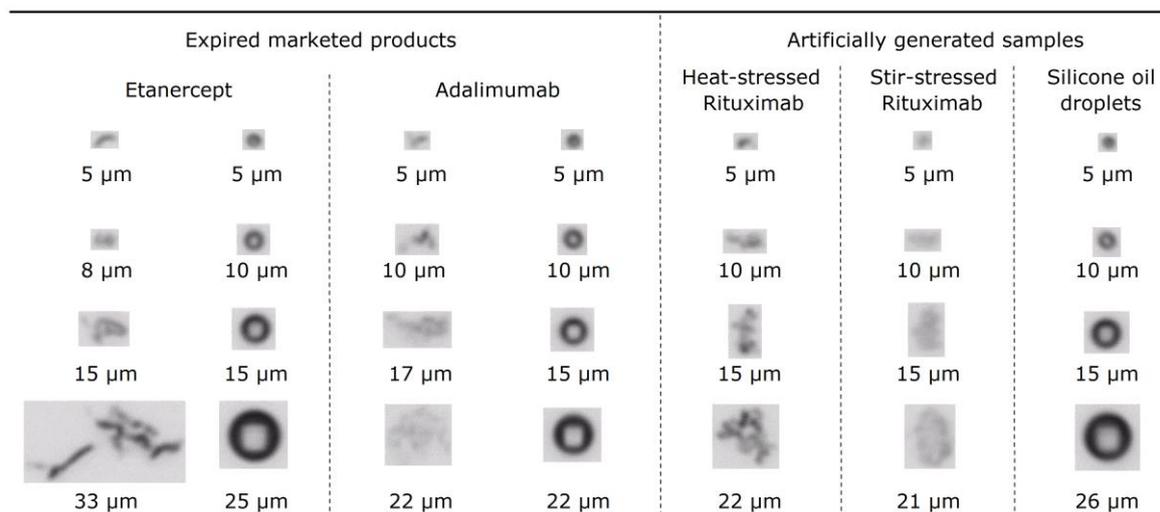
**Table 3-1: Total particle and silicone oil droplet concentrations of expired marketed products in prefilled syringes determined by RMM.**

Product	Total particle concentration per mL (> $0.5 \mu\text{m}$ )	Identified as silicone oil droplets per mL (> $0.5 \mu\text{m}$ )
Etanercept		
lot 32411, exp. 09/2009	$1.50 \times 10^6$	$1.46 \times 10^6$
lot 31576, exp. 12/2008	$3.25 \times 10^6$	$1.68 \times 10^6$
Adalimumab		
lot 430989A04, exp. 02/2008	$1.74 \times 10^6$	$1.61 \times 10^6$
lot 292209A05, exp. 10/2006	$2.01 \times 10^6$	$1.94 \times 10^6$

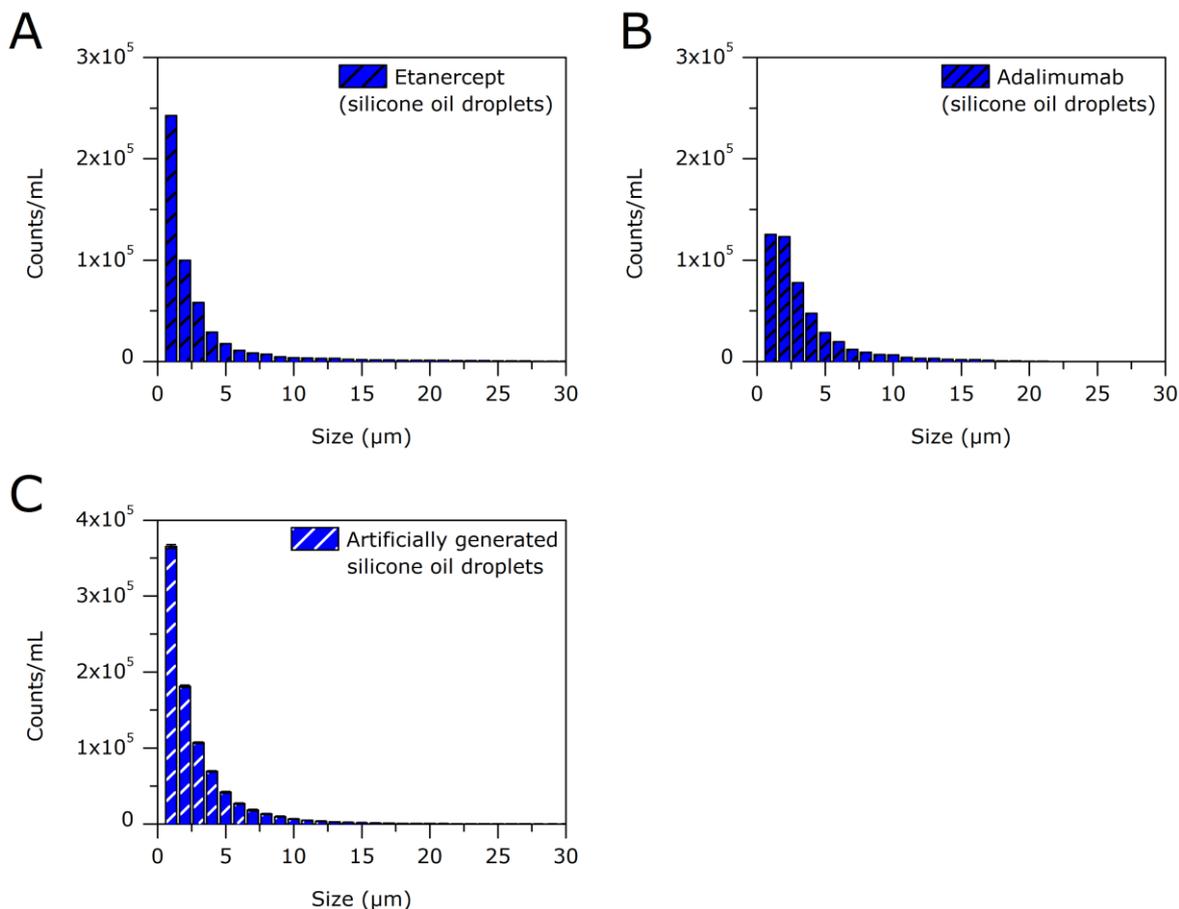
### 3.2 Determination of total particle concentrations (without discrimination)

For the evaluation of MFI and RMM, silicone oil droplets were artificially generated, which appeared similar to those found in etanercept and adalimumab prefilled syringes with respect to their shape, optical properties (Figure 3-3) and

size distribution (Figure 3-4). The concentrations used in our study (0.003% to 0.025% (w/v) silicone oil) provided droplet concentrations similar to those identified in the expired etanercept and adalimumab prefilled syringes and are in agreement with other studies suggesting the presence of up to 0.03% of silicone oil in prefilled syringes.<sup>38,39</sup>



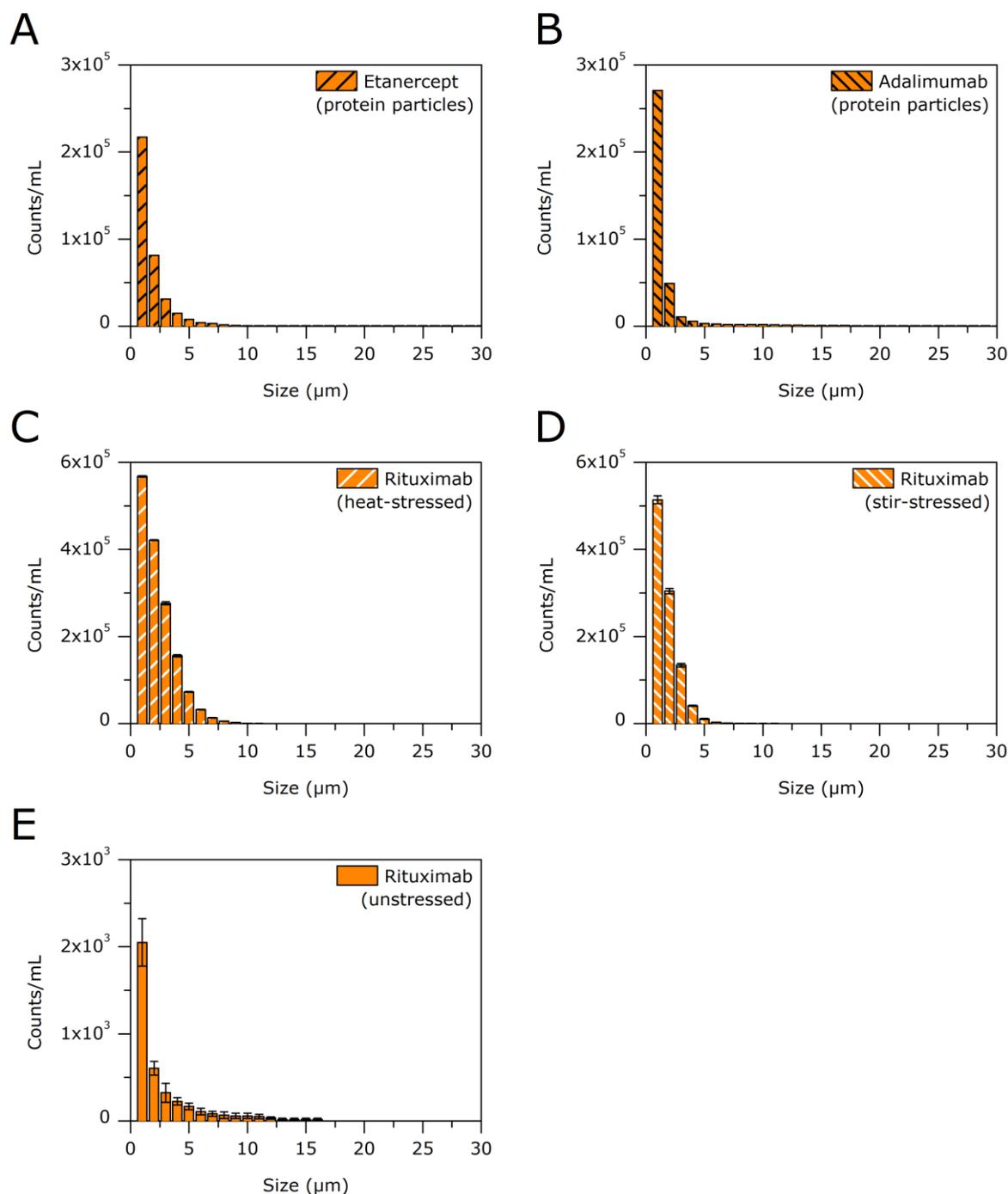
**Figure 3-3: Examples of MFI images of protein particles and silicone oil droplets detected in marketed products and artificially generated samples.**



**Figure 3-4: Cumulative size distributions of silicone oil droplets determined by MFI and identified by the “find similar” operation in (A) etanercept prefilled syringes, (B) adalimumab prefilled syringes, (C) a sample containing only artificially generated silicone oil droplets. Error bars represent standard deviations from triplicate measurements.**

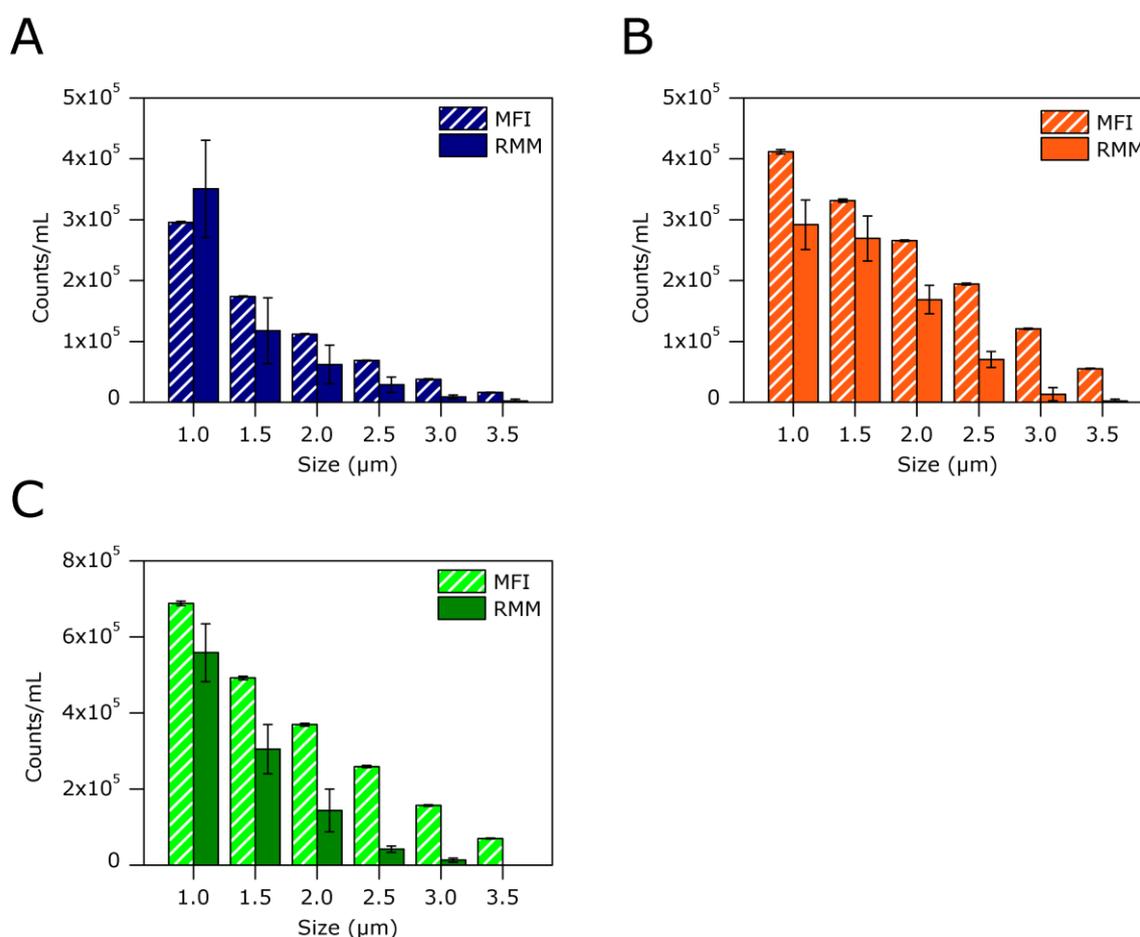
A heat-stress method was developed using rituximab as a model for the generation of particles with a similar appearance to protein particles in etanercept prefilled syringes. A stir-stress method was developed for the generation of particles similar to those in adalimumab prefilled syringes (Figure 3-3). All protein samples showed comparable particle size distributions with the smaller particles representing the largest fraction (Figure 3-5). Protein particles in concentrations from  $1 \times 10^5$  to  $5 \times 10^5$  particles/mL above 1 μm (according to MFI) were combined with silicone oil droplets in concentrations from  $1 \times 10^5$  to  $3 \times 10^5$  particles/mL above 1 μm (according to MFI). Using MFI and RMM, several samples with varying concentrations of protein particles and silicone oil droplets

were analyzed, both individually and as mixtures at various defined droplet/particle ratios.



**Figure 3-5: Cumulative size distributions of protein particles determined by MFI and identified by the “find similar” operation for silicone oil droplets (protein particles are identified as the inverse population) in (A) etanercept prefilled syringes, (B) adalimumab prefilled syringes, (C) heat-stressed rituximab, (D) stir-stressed rituximab, (E) unstressed rituximab. Error bars represent standard deviations from triplicate measurements.**

First, the particle concentrations for individual samples containing either only silicone oil droplets or only protein particles were determined by MFI and RMM. One combination is shown as a representative example in Figure 3-6 for the overlapping measurement size range of both techniques (1-4  $\mu\text{m}$ ). Overall, the results indicate that particle counts and size distributions by MFI and RMM are in general agreement. However, certain differences were observed depending on the type of sample and the ratio of protein particles and silicone oil droplets: For samples containing only silicone oil, RMM detected slightly more droplets of 1 to 4  $\mu\text{m}$  as compared to MFI, while MFI detected more droplets in the size range from 2 to 4  $\mu\text{m}$  (Figure 3-6A). This trend was reproducible for all silicone oil droplet samples, with an up to twofold higher silicone oil droplet count in the size range of 1 to 4  $\mu\text{m}$  detected by RMM as compared to MFI.



**Figure 3-6: Cumulative size distributions in the size range of 1-4  $\mu\text{m}$  of (A) a sample containing only silicone oil droplets, (B) a sample containing only protein particles (heat-stressed rituximab), and (C) the corresponding mixture (droplet/particle ratio 40:60 for particles  $> 1 \mu\text{m}$  based on MFI) as determined by MFI and RMM. Error bars represent standard deviations from triplicate measurements.**

This difference might be due to two major reasons:

(i) Silicone oil droplets of sizes up to  $50 \mu\text{m}$  were identified by MFI, which are much larger than the microchannel diameter of RMM ( $8 \mu\text{m}$ ). Those particles larger than  $8 \mu\text{m}$  represent only 4% of all silicone oil droplets in the sample detected by MFI by number; however, they contain 72% of the total mass of all silicone oil droplets in the sample detected by MFI (mass was calculated based on droplet counts at the respective diameter and the density of silicone oil of  $0.97 \text{ g/mL}$ ). These observations led us to the hypothesis that larger silicone oil droplets might be fragmented into smaller ones by shear forces inside the microchannels and capillaries of the RMM system. This would result in an



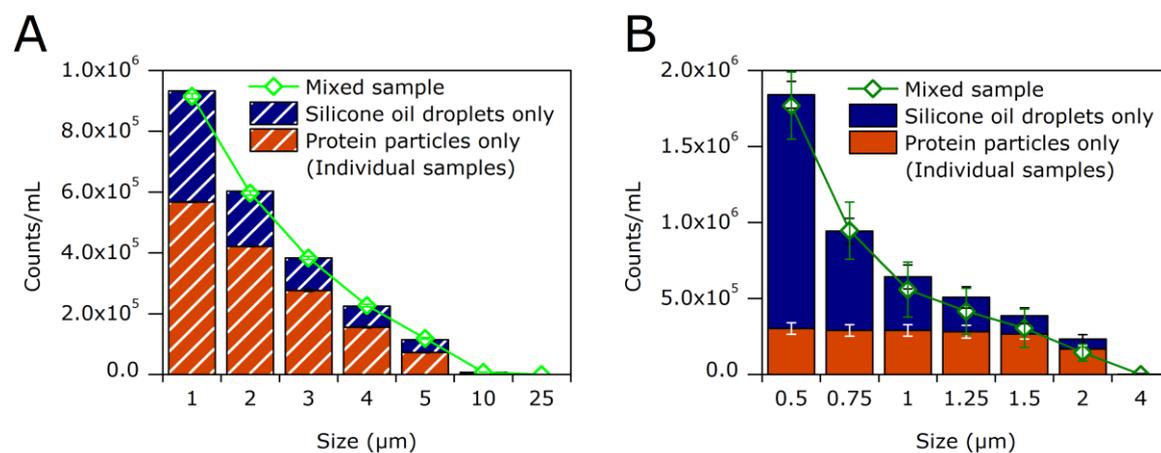


(ii) As a second reason, the micron-sized capillaries and channels of the RMM sensor are vulnerable to clogging by particles at or above the upper size limit of the system. Even though RMM offers several tools to remove stuck particles, clogging cannot always be avoided. Thus, large stuck particles could hinder other particles from reaching the sensor. This could explain why the concentration discrepancy between RMM and MFI is more pronounced at larger particle sizes, because smaller particles will pass a clogged site more easily, whereas larger particles, although still in the measurement range, are more likely to be excluded from the analysis. Altogether, this will result in lower apparent protein particle concentrations in RMM. A possible solution would be sample preparation for highly aggregated samples, e.g. filtration or centrifugation, which can however potentially change sample properties.

Total particle concentrations for mixed samples containing both silicone oil droplets and protein particles also revealed slight differences between MFI and RMM for the overlapping size range of 1 to 4  $\mu\text{m}$  (Figure 3-6C). For moderate ratios (silicone oil droplets/protein particles 40:60 based on MFI shown as a representative sample), RMM detected less particles than MFI, likely due to the underestimation of protein particles as described before. However, in mixed samples of higher silicone oil content (silicone oil droplets/protein particles 80:20 or 95:5 based on MFI) similar concentrations were determined by the two techniques. In those samples, the overestimation of silicone oil droplets by RMM was balanced out by the underestimation of protein particles by RMM leading to similar total particle counts in MFI and RMM. For all samples, RMM showed higher standard deviations than MFI. This is probably mainly due to the small analyzed volume in RMM (about 0.15  $\mu\text{L}$ ) as compared to MFI (about 35  $\mu\text{L}$ ).

It was further investigated whether the presence of both silicone oil droplets and protein particles within the same sample influenced the accuracy of MFI or RMM to determine total particle concentrations. For MFI, the concentration determined for mixed samples of silicone oil droplets and protein particles from heat-stressed rituximab matched very closely the sum of the concentrations determined for the corresponding individual samples (Figure 3-9A). For RMM, the concentration for the mixed sample reasonably matched the sum of the individual samples for the main size classes (Figure 3-9B). These observations were consistent for different

ratios and also for protein particles from stir-stressed rituximab mixed with silicone oil droplets. This justified the use of particle counts of individual samples as the theoretical concentrations for mixed samples.



**Figure 3-9: Cumulative size distributions in individual samples of silicone oil droplets and protein particles (heat-stressed rituximab) and the corresponding mixture analyzed by (A) MFI and (B) RMM. Error bars represent standard deviations from triplicate measurements.**

### 3.3 Discrimination between silicone oil droplets and protein particles

The discrimination between silicone oil droplets and protein particles by MFI and RMM is based on clearly different mechanisms (see above and Figure 3-8). The optical discrimination by MFI bears the potential risk of false classification due to optically similar silicone oil droplets and protein particles in the lower size range, especially near the detection limit. In contrast, the discrimination by RMM based on the physical parameter of particle buoyancy enables a clear discrimination with minimal risk of false classification. In this case, the difference in density between silicone oil droplets and protein particles is beneficial.

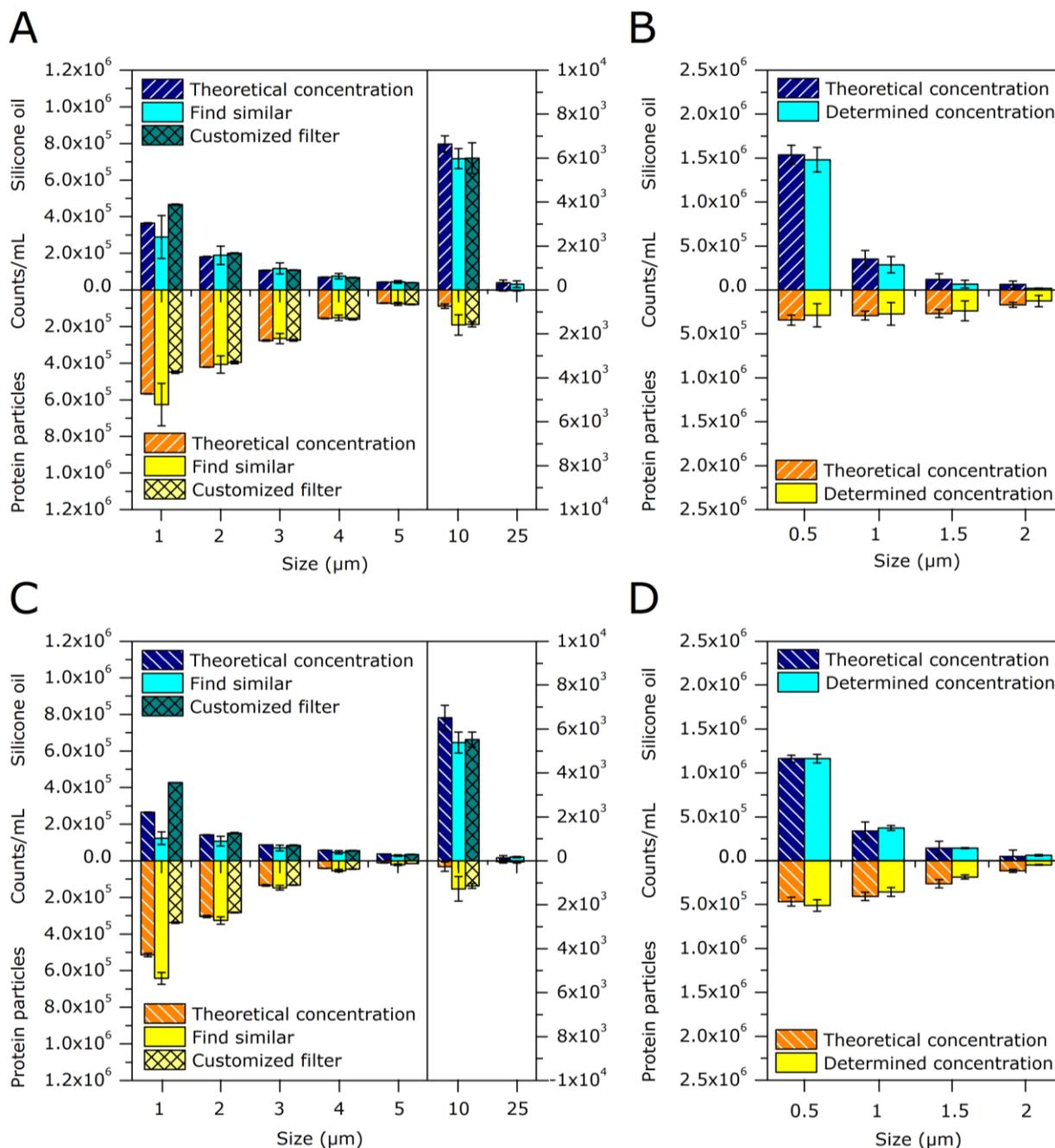
#### 3.3.1 Discrimination between droplets and particles by MFI

In the present paper, the performance of MFI was assessed using the built-in software solution "find similar" and a customized data filter developed specifically for this study. To evaluate the reliability of our customized filter, the following

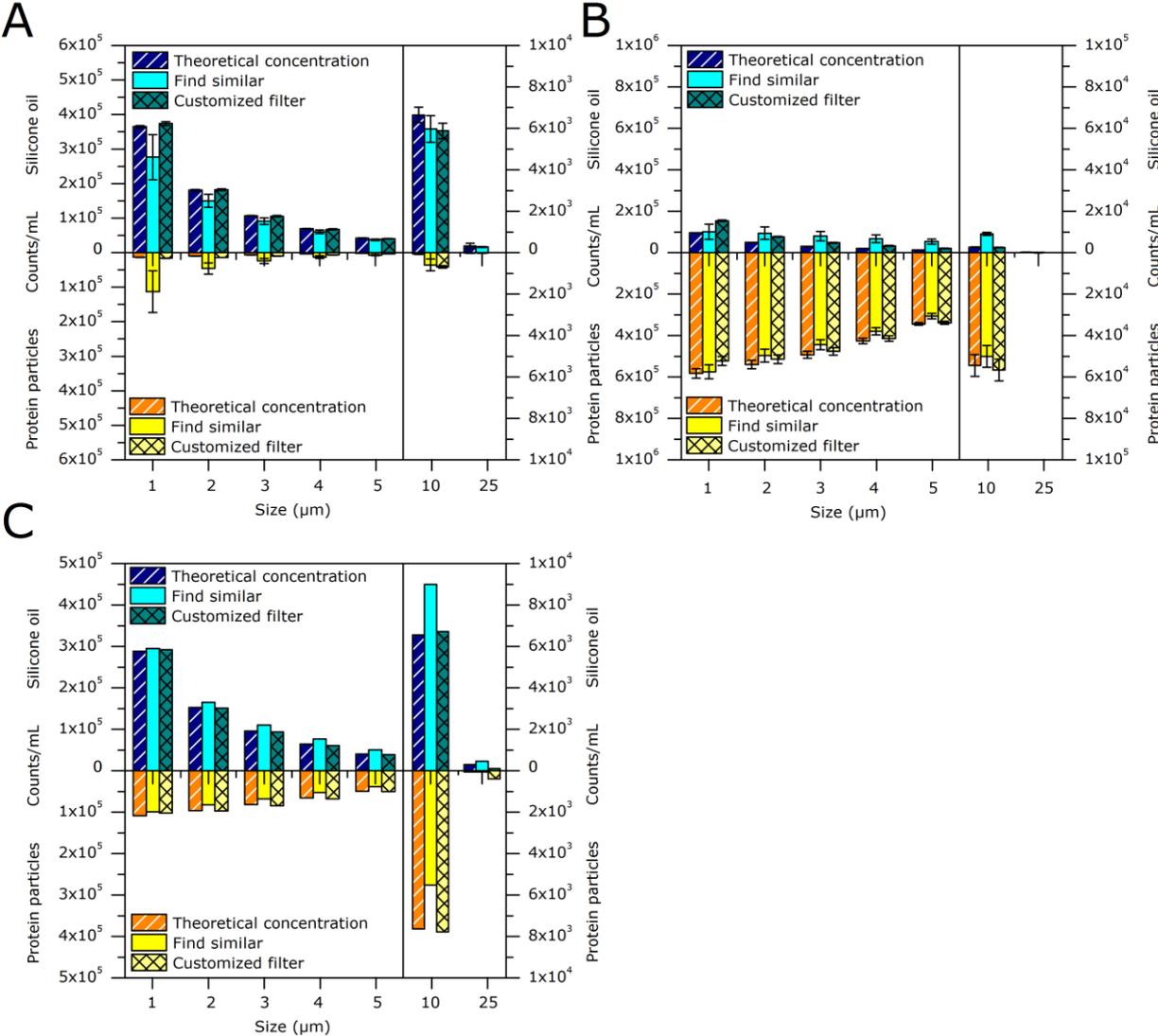
control experiments were performed: the filter was applied on samples containing only silicone oil droplets and the number of objects falsely marked as protein particles was determined and vice versa. Our customized filter marked less than 3% of the counts in the samples containing only silicone oil droplets ( $3 \times 10^5$  particles/mL  $> 1 \mu\text{m}$  based on MFI) falsely as protein particles ( $> 2 \mu\text{m}$ ) and less than 8% of the counts in the samples containing only protein particles ( $4 \times 10^5$  particles/mL  $> 1 \mu\text{m}$  based on MFI) falsely as silicone oil droplets ( $> 2 \mu\text{m}$ ). These controls illustrate the capability of our filter to properly discriminate protein particles and silicone oil droplets. The requirement that all four criteria of particle parameters need to be fulfilled at the same time is the main difference of our filter compared to the filter previously developed by Strehl *et al.*<sup>31</sup>, which used the product of four particle parameters as criterion for particle classification. In this case, extreme values in one parameter could shift the product to the side of one particle type although the other three parameters would classify it clearly as the other particle type. Thus, their filter led to errors of 10% to 12% ( $> 2 \mu\text{m}$ ) for silicone oil droplets classified falsely as protein particles; the error for protein particles classified falsely as silicone oil droplets depended strongly on the type of protein particles and varied between 2% and 42% in their study.<sup>31</sup> In contrast, our filter applies more strict criteria for silicone oil droplet identification as particles fulfilling only three out of four criteria are not marked as silicone oil droplets leading to lower errors as discussed above. However, for protein particles generated from a different monoclonal IgG (influximab) by heat stress or stir stress the customized filter marked up to 40% ( $> 2 \mu\text{m}$ ) falsely as silicone oil droplets. This was most likely due to the lower intensity (lower transparency) of particle images of this IgG, which makes a misclassification as silicone oil droplets of similarly low transparency more likely. This is in agreement with the literature, where large variations were also observed by Strehl *et al.*<sup>31</sup> when their filter was applied to different types of protein particles. The MVAS software filter could not be tested on these protein samples as it was based on manual selection of silicone oil droplet images which were not present in these pure protein samples.

The “find similar” operation of the MVAS software as well as the customized filter were both used to categorize particles from mixed samples into silicone oil droplets and non-silicone oil particles. Non-silicone oil particles were defined as

protein particles in our case. The obtained concentrations were compared to the theoretical concentrations based on the analysis of the individual samples, which were used to assess the accuracy of both methods (Figure 3-10A,C, Figure 3-11). For moderate droplet/particle number ratios from 30:70 to 70:30 based on MFI, both the selection by “find similar” and the customized filter were able to determine the correct concentrations within acceptable deviations for particles > 2  $\mu\text{m}$ . This was observed for samples containing silicone oil droplets and protein particles from heat-stressed rituximab (Figure 3-10A exemplarily shows the results for a sample with a droplet/particle ratio of 40:60 based on MFI). For stir-stressed rituximab (Figure 3-10C) the customized filter for MFI showed superior discrimination compared to the “find similar” method for particles > 2  $\mu\text{m}$ , even though the customized filter was designed based on heat-stressed rituximab particles. The even higher intensity of MFI particle images of stir-stressed rituximab compared to those of heat-stressed rituximab (Figure 3-3) likely contributes to this: since three out of four parameters of the customized filter are based on the particle intensity, it facilitates discrimination from the lower intensity silicone oil droplets. Furthermore, the customized filter was superior for samples with more extreme droplet/particle number ratios (see Figure 3-11A, B for representative examples) and for samples based on original, undiluted rituximab solution (Figure 3-11C).



**Figure 3-10: Results from MFI (A and C) or RMM (B and D) for the discrimination between silicone oil droplets and protein particles. Histograms comparing the theoretical concentrations (based on individual samples) and determined concentrations of silicone oil droplets and protein particles (A and B, heat-stressed rituximab; C and D, stirred-stressed rituximab) in mixed samples with moderate ratios (droplet-particle ratio 40:60 based on MFI). Error bars represent standard deviations from triplicate measurements.**



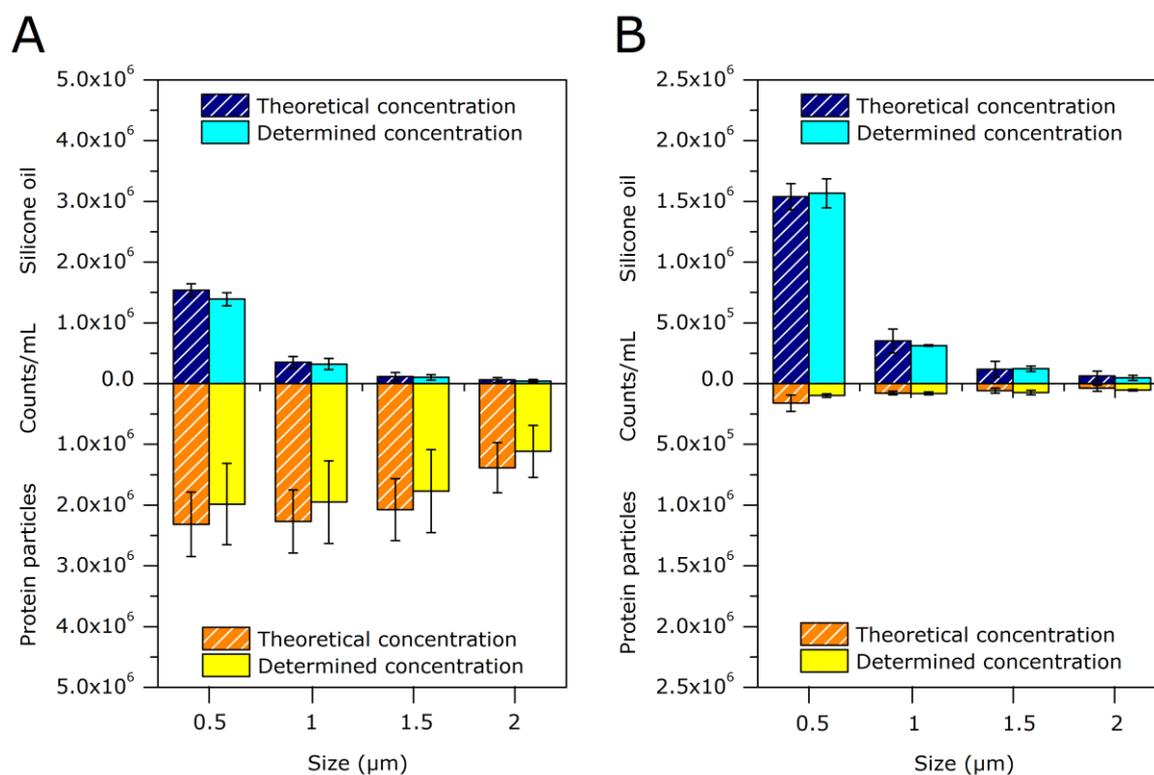
**Figure 3-11: MFI cumulative counts comparing theoretical concentrations (based on individual samples) and determined concentrations of silicone oil droplets and protein particles (heat-stressed rituximab) in droplet/particle ratios of (A) 95:5 and (B) 15:85 in samples containing 0.5 mg/mL rituximab as well as (C) 60:40 in a sample containing undiluted rituximab (10 mg/mL). Error bars (A and B) represent standard deviations from triplicate measurements.**

Thus, for particles between 2 µm and 25 µm, the development of a customized filter is useful for an accurate discrimination by MFI. For particles with a size below 2 µm, discrimination by an alternative method is recommended (e.g. RMM, as discussed later) as both “find similar” and the customized filter were not reliably capable of determining the correct concentration. For particles larger than 25 µm, due to usually low particle numbers in this size range, manual classification of the MFI images might be preferred over the built-in software

solution or a customized filter. Those particles can usually be identified easily by visual evaluation of the images.

### 3.3.2 Discrimination between droplets and particles by RMM

As described for MFI, RMM was evaluated with respect to an accurate discrimination between silicone oil droplets and protein particles in mixed samples (Figure 3-10B,D, Figure 3-12). For moderate particle/droplet ratios, RMM was consistently able to discriminate particles correctly with small deviations from the theoretical concentrations for heat-stressed (Figure 3-10B) and stir-stressed rituximab (Figure 3-10D). Large deviations of 20% or more from the theoretical concentration were only observed if the discrimination was based on less than 50 counted particles (corresponding in this case to total concentrations (droplets + particles)  $< 3 \times 10^5$  particles/mL) and thus statistical representation of the sample population was limited. This was for example the case for particles larger than  $2 \mu\text{m}$  (Figure 3-10B,D). Increasing the analyzed sample volume would compensate for the limited reliability of RMM to quantify low particle concentrations, as also reported by others.<sup>35</sup> However, it needs to be considered that very long measurement times associated with large analyzed volumes could also provoke changes in sample properties. In contrast, fairly high concentrations of protein particles  $> 2 \times 10^6$  particles/mL caused high standard deviations potentially due to the increased probability of coinciding particles and also blockage of the channel by particles (Figure 3-12A). However, extreme droplet/particle ratios with high amounts of silicone oil droplets provided moderate standard deviations and also fairly accurate determination of the theoretical concentration (Figure 3-12B exemplarily displays results for a droplet/particle ratio of 95:5 based on RMM). Those results provide evidence that RMM discrimination is reliable for particles below  $2 \mu\text{m}$ .



**Figure 3-12: RMM cumulative counts comparing theoretical concentrations (based on individual samples) and determined concentrations of silicone oil droplets and protein particles (heat-stressed rituximab) in droplet/particle ratios of (A) 40:60 and (B) 95:5. Error bars represent standard deviations from triplicate measurements.**

### 3.4 Comparison of results for MFI and RMM

For a final evaluation of MFI and RMM regarding the discrimination of silicone oil droplets and protein particles, results for the same sample were compared between the two techniques. For silicone oil droplets and heat-stressed rituximab (Figure 3-10A,B, droplet/particle ratio 40:60) as well as stir-stressed rituximab (Figure 3-10C,D, droplet/particle ratio 40:60), RMM detected a higher fraction of silicone oil droplets as compared to MFI for the sizes above 1 μm already in the individual samples. This originated foremost from the differences in total concentration determination as discussed earlier: RMM detected in general more silicone oil droplets than MFI, whereas MFI detected in general more protein particles than RMM (see also Figure 3-6). However, in this size range, RMM results for the mixed samples are considered more reliable as RMM differentiation was shown to be highly accurate (Figure 3-10B,D). MFI differentiation suffered from low image resolution in the lower size range leading to large deviations for

both the “find similar” operation and the customized filter (Figure 3-10A,C). With increasing particle size, the ratios between MFI and RMM in the individual samples converged and similar ratios for individual samples were obtained for particles  $> 2 \mu\text{m}$  (Figure 3-10A,B shows a droplet/particle ratio of 30:70 for particles  $> 2 \mu\text{m}$  in individual samples for both MFI and RMM). For mixed samples, the concentration obtained by MFI is suggested to be more reliable for sizes above  $2 \mu\text{m}$  as the discrimination between droplets and particles was highly accurate, especially when the customized filter was applied (Figure 3-10A,C). RMM analysis of objects with a size above  $2 \mu\text{m}$  was based on small numbers of counts, questioning the reliability of the determined concentrations (Figure 3-10B,D) in our study.

## 4 Recommendations and conclusions

Table 3-2 summarizes properties as well as pros and cons during the application of MFI and RMM which were identified in our study. For MFI, the customized filter was shown to provide correct results for moderate and extreme ratios between silicone oil droplets and protein particles. The filter was developed using heat-stressed rituximab particles, but was also found applicable for rituximab particles generated by stir stress and for samples containing rituximab solution in high concentrations (10 mg/mL). In contrast, the application for infliximab particles generated by either heat or stir stress resulted in large errors. These results emphasize the necessity of customizing the filter to each specific protein, the formulation, and the particle type / stress method of interest. Thus, the development of a customized filter for quality control of protein therapeutics in prefilled syringes with comparable manufacturing conditions can be considered reasonable. In contrast, the implementation during formulation development with varying conditions should be critically evaluated case by case. The separation by the MVAS software was acceptably accurate especially for moderate ratios of silicone oil droplets and protein particles. It could still be applied in those cases, when costs and time for the development of a customized filter would exceed the benefit of a more accurate discrimination. However, the differentiation by “find similar” showed clearly higher standard deviations as compared to the customized filter. This higher variation of the “find similar” operation originated most likely from the underlying sample and operator dependent manual selection of the particle images. For both MFI-based solutions it is important to consider that the separation is based on the identification of silicone oil droplets, whereas the remaining particles, identified only as “non-silicone oil particles”, are simply equated with protein particles by the operator.



RMM is a very robust technique for exactly this task. It needs to be considered that RMM can only discriminate one type of positively buoyant from one type of negatively buoyant particles. Thus, if a sample contains protein particles as well as other particles of higher density than the buffer, e.g. particles shed from filling pumps or rubber stoppers, RMM is not able to discriminate them. Here, methods such as SEM-EDS, FT-IR or Raman microscopy<sup>43</sup> could be used as orthogonal methods to further identify these "non-silicone oil" particles. Furthermore, complexes consisting of both protein and silicone oil can pose a challenge for the technique of RMM: The reported size of those complexes may be incorrect due to the simultaneous influence of both material densities on the density of the complex. As a worst case the complexes might be missed entirely as the higher density of protein is compensated by the lower density of silicone oil, eliminating a clear density difference between particle and formulation. Those complexes might be detectable by MFI (given that they are large enough) as shown for an IgG particle containing silicone oil.<sup>22</sup> In our study, only very few of those complexes were observed in MFI, because protein particles and silicone oil droplets were prepared separately to avoid interactions of protein and silicone oil during the particle formation process.

Taken together, the robust detection principle of RMM has brought significant benefit to the field of protein product characterization, especially for the discrimination of silicone oil droplets and protein particles. RMM differentiation is recommended for particles below 2  $\mu\text{m}$ , provided that sufficient particle quantities are detected. MFI differentiation is recommended above 2  $\mu\text{m}$ , preferably using a customized filter. In order to cover a size range as broad as possible, both techniques should be applied in parallel for a comprehensive analysis of samples potentially containing silicone oil droplets and protein particles in the size range from 500 nm to 70  $\mu\text{m}$ .

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# Chapter 4

## Flow imaging microscopy for protein particle analysis – a comparative evaluation of four different analytical instruments

### Abstract

Flow imaging microscopy was introduced as a technique for protein particle analysis a few years ago and has strongly gained in importance ever since. The aim of the present study was a comparative evaluation of four of the most relevant flow imaging microscopy systems for biopharmaceuticals on the market: MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV. The performance was critically assessed regarding particle quantification, characterization, image quality, differentiation of protein particles and silicone oil droplets, and handling of the systems. The FlowCAM systems, especially the FlowCAM VS1, showed high resolution images. The FlowCAM PV system provided the most precise quantification of particles of therapeutic monoclonal antibodies, also under impaired optical conditions by an increased refractive index of the formulation, and furthermore, the most accurate differentiation of protein particles and silicone oil droplets could be achieved with this instrument. The MFI systems provided excellent size and count accuracy (evaluated with polystyrene standards), especially the MFI5200 system. This instrument also showed very good performance for protein particles, also in case of an increased refractive index of the formulation. Both MFI systems were easier to use and appeared more standardized regarding measurement and data analysis as compared to the FlowCAM systems. Our study shows that the selection of the appropriate flow imaging microscopy system depends strongly on the main output parameters of interest and it is recommended to decide based on the intended application.

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*S. Zöllds\*, D. Weinbuch\*, M. Wiggerhorn, G. Winter, W. Friess, W. Jiskoot, A. Hawe: "Flow imaging microscopy for protein particle analysis – a comparative evaluation of four different analytical instruments"; The AAPS Journal (accepted); \*joint first authors*

# 1 Introduction

Protein aggregates and particles are important quality attributes of therapeutic protein formulations.<sup>1-3</sup> Especially micron sized aggregates (subvisible protein particles)<sup>4</sup> are considered as critical due to their potential risk of enhancing an immunogenic response.<sup>5</sup> Quantification of (not necessarily proteinaceous) subvisible particles larger than 10 µm and 25 µm in parenterals is required by the pharmacopoeias, and is commonly performed using light obscuration (LO) techniques.<sup>6,7</sup> For therapeutic protein products regulatory agencies increasingly ask for quantification and characterization of particles with a size below 10 µm by an orthogonal approach.<sup>8,9</sup> Furthermore, the availability of an increasing number of emerging techniques<sup>10,11</sup> extends the spectrum of particle analysis tools and enables a more detailed characterization of the particles counted. These factors inspired the development of a new educational chapter USP<1787> entitled "Measurement of Subvisible Particulate Matter in Therapeutic Protein Injections".<sup>12</sup> It is currently being discussed whether this chapter should include particle analysis starting already from 2 µm as well as the use of additional techniques, such as flow imaging microscopy. Flow imaging microscopy has already been used extensively in research and development<sup>13-19</sup> and more recently also for quality control/routine testing (own experiences).

Flow imaging microscopy uses a CCD camera with high magnification to capture images of the sample solution passing a thin flow cell. The flow cell is illuminated and particles with a different refractive index (RI) than the solution decrease the light intensity compared to the background and can be detected on the captured images.<sup>20,21</sup> Particle size and count information is then generated based on image analysis. Besides quantification, the digital particle images allow for subsequent morphological characterization including size, shape and optical parameters. This, however, requires sufficiently high image quality to draw reliable conclusions.<sup>21</sup> A prominent application example is the differentiation of silicone oil droplets and protein particles in prefilled syringes and cartridges. For this approach, flow imaging microscopy has been successfully applied in several studies.<sup>22-24</sup> In general, flow imaging microscopy tends to be more sensitive than LO for small transparent protein particles and therefore usually detects higher particle numbers.<sup>13,15,25</sup> An increased RI of the formulation, leading to a

decreased RI difference between particles and formulation, can impede a correct detection of protein particles by light-based techniques. Compared to LO, MFI was shown to be slightly more robust against such a decreased RI difference.<sup>13,26</sup>

There are several flow imaging microscopy instruments available on the market provided by different suppliers. Those are, for example, Sysmex Flow Particle Image Analyzer (FPIA) 3000 by Malvern Instruments (Worcestershire, UK), various Occhio Flowcell systems by Occhio (Angleur, Belgium), the MicroFlow Particle Sizing System by JM Canty (Buffalo, NY), several Micro-Flow Imaging (MFI) systems by Protein Simple (Santa Clara, CA), and various Flow Cytometer And Microscope (FlowCAM) systems by Fluid Imaging (Yarmouth, ME). In this study, MFI and FlowCAM systems with different settings were evaluated (Table 4-1). Both systems are often used for the analysis of subvisible particles in research and development and partly also for routine testing in a QC environment. A short general article about the handling of MFI and FlowCAM is available,<sup>27</sup> but no comprehensive report about a direct comparison of the four systems has been published until now.

Here we present the first study thoroughly challenging four of the most relevant flow imaging microscopy systems for biopharmaceuticals on the market: MFI4100 and MFI5200 as well as FlowCAM VS1 and FlowCAM PV. By that we want to provide a basis for the increasing use of such systems in QC and support industry and authorities in their efforts towards new standards in the field of subvisible particle characterization.





Infliximab solution at a concentration of 1 mg/mL was prepared by dilution of 10 mg/mL infliximab commercial product in 100 mM phosphate buffer (pH 7.2). The formulation was filtered through a 0.2 µm polyethersulfone syringe filter. Stir-stressed infliximab was prepared by incubating 8 mL of the 1 mg/mL infliximab solution in a 10R glass vial using a 18 mm Teflon®-coated stir bar at 250 rpm for 24 hours at room temperature on a magnetic stirrer (MR Hei-Standard, Heidolph, Schwabach, Germany).

For analysis of protein samples, stressed protein solution was diluted in the appropriate buffer (filtered through a 0.22 µm cellulose acetate/nitrate membrane filter, MF-Millipore®, Millipore), sucrose solution or water.

### 2.3 Preparation of silicone oil emulsion

Silicone oil was added to filtered formulation buffer in a particle-free 15 mL conical tube to a final concentration of 2% (w/v) to generate an emulsion without additives. After vortexing briefly, silicone oil droplet formation was induced by sonication in a water bath (Sonorex, Brandelin, Berlin, Germany) for 10 min. Fresh silicone oil emulsion (silicone oil droplet stock emulsion) was prepared on the day of the measurement and kept at room temperature.

### 2.4 Preparation of individual and mixed samples of silicone oil droplets and protein particles

Silicone oil droplet stock emulsion and/or protein particles stock suspension was diluted in unstressed protein solution or filtered formulation buffer for the preparation of mixed and individual samples. Mixed samples were prepared in a number ratio of 10:90 based on particle counts  $> 2\ \mu\text{m}$  determined by MFI4100. Individual samples were prepared to contain the same number of silicone oil droplets and protein particles, respectively, as in the mixed samples and are referred to as the theoretical concentration. All samples were prepared to a final protein concentration of 0.5 mg/mL rituximab. The samples were gently mixed with a pipette, kept at room temperature and measured on the day of preparation.



### 2.7.2 MFI5200

An MFI5200 system (ProteinSimple) equipped with a 100 µm flow cell and controlled by the MFI View System Software (MVSS) version 2-R2.6.1.20.1915 was used. The system was flushed with 10 mL purified water at maximum flow rate and flow cell cleanliness was checked visually between measurements. "Optimize illumination" prior to each measurement was done comparably to MFI4100. Samples of 0.5 mL with a pre-run volume of 0.2 mL were analyzed at a flow rate of 0.17 mL/min and a fixed camera rate (not adjustable by the user) leading to a sampling efficiency of about 80-85%. Samples were measured in triplicate and mean and standard deviation were calculated.

### 2.7.3 Particle data analysis MFI

For both systems, MFI View Analysis Suite (MVAS) version 1.2 was used for data analysis. Particles stuck to the flow cell wall were only counted once and edge particles were excluded from analysis. Particle size was evaluated as the diameter of a circle with the same projected area as the particle (designated as ECD, equivalent circular diameter, in the MFI software). For the discrimination of silicone oil droplets and protein particles, a minimum of 20 particles (MFI4100) or 50 particles (MFI5200) above 5 µm clearly recognizable as silicone oil droplets was selected for the "find similar" operation in the MVAS software.

## 2.8 FlowCAM analysis

### 2.8.1 FlowCAM VS1

A FlowCAM VS1 Benchtop B3 system (Fluid Imaging Technologies) was equipped with a 50 µm single-use cell, a 20x magnification lens and controlled by the VisualSpreadsheet software version 3.1.10. The system was flushed with 1 mL purified water at a flow rate of 0.5 mL/min and flow cell cleanliness was checked visually. 0.5 mL sample solution with a pre-run volume of 0.5 mL (primed manually into the flow cell) was analyzed with a flow rate of 0.07 mL/min and a camera rate of 20 frames/s leading to a sampling efficiency of about 5-8%. Only dark pixels were selected for particle size determination at the preset default threshold value of 20. Particle size was evaluated as the diameter of a circle with the same projected area as the particle (designated as ABD, area based

diameter, in the FlowCAM software). For the discrimination of silicone oil droplets and protein particles, a filter can be developed and the parameters can be saved in the software. However, to ensure comparability with the MFI systems and to represent the analysis of a single sample as good as possible, the selection of silicone oil droplets in this study was performed on a sample-by-sample basis. A minimum of 20 particles above 5  $\mu\text{m}$  clearly recognizable as silicone oil droplets was selected for the “find similar as selected” function. Samples were measured in triplicate and mean and standard deviation were calculated.

### 2.8.2 FlowCAM PV

A FlowCAM PV-100 Benchtop system (Fluid Imaging Technologies) was equipped with a 80  $\mu\text{m}$  multi-use cell, a 10x magnification lens and controlled by the VisualSpreadsheet software version 3.4.2. The system was flushed with 5x1 mL purified water by the flushing function in the software and flow cell cleanliness was accepted if less 10 particles were counted in 0.02 mL water in the “autoimage mode (no save)”. 0.5 mL sample solution with a pre-run volume of 0.2 mL (primed manually into the flow cell) was analyzed with a flow rate of 0.04 mL/min and a camera rate of 21 frames/s leading to a sampling efficiency of about 80-85%. Dark and bright pixels were selected for particle size determination at the preset default threshold value of 30. Particle size was evaluated as the diameter of a circle with the same projected area as the particle (designated as ABD, area based diameter, in the FlowCAM software). For the discrimination of silicone oil droplets and protein particles through the “find similar” operation, a minimum of 100 particles above 5  $\mu\text{m}$  clearly recognizable as silicone oil droplets was selected to generate a library. The complete particle population was filtered by the “find similar as library” function. The resulting particle population was sorted by filter score and particles with filter scores of 0 to 5 (with 0 describing images which the highest match to the images in the library) were defined as silicone oil droplets. This procedure was necessary as the software was not able to perform the same “find similar as selected function” as applied for the FlowCAM VS1 which was probably due to the clearly higher number of particles images captured by the FlowCAM PV. Samples were measured in triplicate and mean and standard deviation were calculated.

## 2.9 Performance evaluation

Critical performance parameters (e.g. image quality, size accuracy, and several other factors as described below) were ranked relatively within the evaluated systems. The system with the strongest performance for one specific parameter was scored as "4" (++++), the system with the weakest performance in this parameter was scored as "1" (+). In detail, the performance was quantified as follows: The image quality parameters were evaluated by eye. Polystyrene sizing and counting performance was judged with respect to the specifications by the manufacturer (NIST-traceable), linearity was evaluated based on the deviation from the theoretical concentration expected from the dilution factor and the linearity of the obtained concentrations (assessed by the  $R^2$  value). For the robustness towards RI influences, the relative decrease in the measured protein particle concentration in formulations with a higher RI was used for the ranking. The differentiation of silicone oil droplets and protein particles was evaluated based on the match with the theoretical concentration within the system (based on individual samples) and the standard deviation, defined as precision.

### 3 Results and discussion

#### 3.1 Count and size performance with polystyrene standards

The four systems MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV were first evaluated regarding their size and count performance with monodisperse certified polystyrene standards. All systems determined the correct concentration of a 5  $\mu\text{m}$  polystyrene count standard with  $3000 \pm 300$  particles/mL  $> 3 \mu\text{m}$  (Table 4-2).

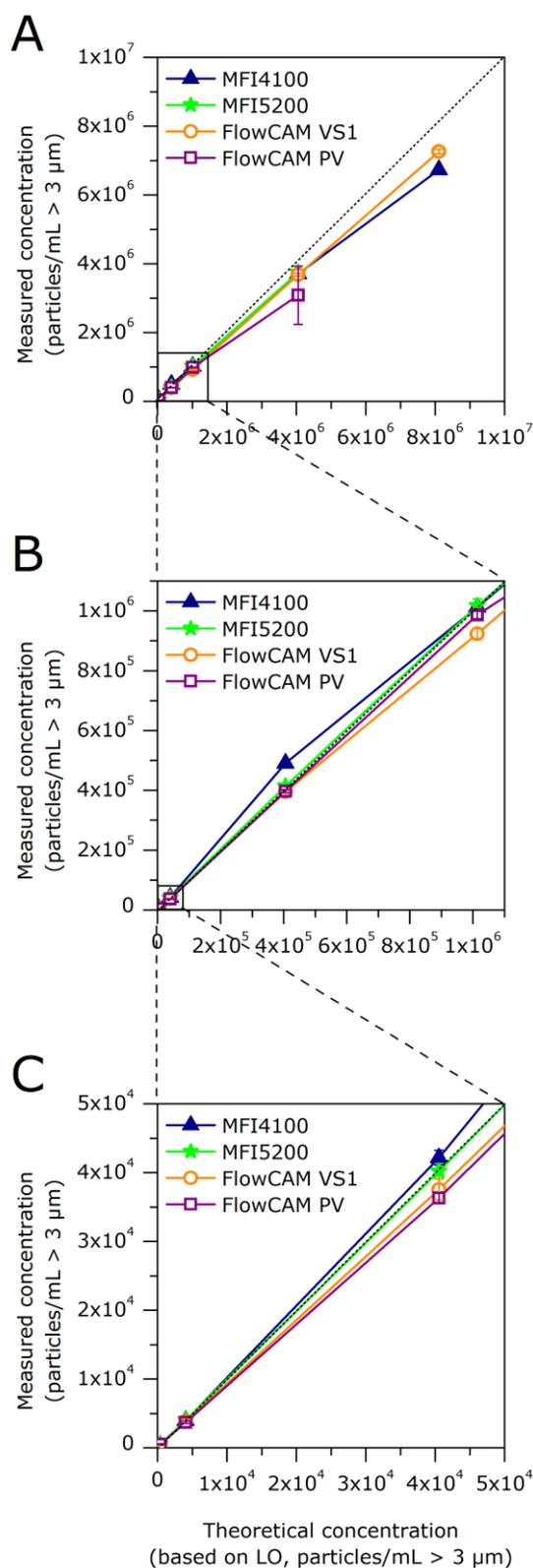
**Table 4-2: Results of polystyrene standard measurements with MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV.**

Standard type	Specification	MFI4100	MFI5200	FlowCAM VS1	FlowCAM PV
5 $\mu\text{m}$ count standard	$3000 \pm 300$ part./mL <sup>a</sup>	$2906 \pm 324$ part./mL <sup>c</sup>	$3203 \pm 116$ part./mL <sup>c</sup>	$2779 \pm 162$ part./mL <sup>c</sup>	$2974 \pm 184$ part./mL <sup>c</sup>
2 $\mu\text{m}$ size standard	$1.999 \pm 0.020 \mu\text{m}$ <sup>b</sup>	$1.74 \pm 0.28 \mu\text{m}$ <sup>d</sup>	$1.95 \pm 0.35 \mu\text{m}$ <sup>d</sup>	$3.20 \pm 1.39 \mu\text{m}$ <sup>d</sup>	$2.38 \pm 0.90 \mu\text{m}$ <sup>d</sup>
5 $\mu\text{m}$ size standard	$4.993 \pm 0.040 \mu\text{m}$ <sup>b</sup>	$5.10 \pm 0.80 \mu\text{m}$ <sup>d</sup>	$5.12 \pm 0.57 \mu\text{m}$ <sup>d</sup>	$5.94 \pm 1.61 \mu\text{m}$ <sup>d</sup>	$4.66 \pm 1.52 \mu\text{m}$ <sup>d</sup>
10 $\mu\text{m}$ size standard	$10.00 \pm 0.08 \mu\text{m}$ <sup>b</sup>	$10.56 \pm 1.22 \mu\text{m}$ <sup>d</sup>	$10.16 \pm 1.16 \mu\text{m}$ <sup>d</sup>	$10.71 \pm 2.41 \mu\text{m}$ <sup>d</sup>	$9.66 \pm 1.43 \mu\text{m}$ <sup>d</sup>

<sup>a</sup> based on light obscuration for particles  $> 3 \mu\text{m}$ ; <sup>b</sup> based on microscopy; <sup>c</sup> standard deviation from three measurements; <sup>d</sup> full peak width at half of the maximum height

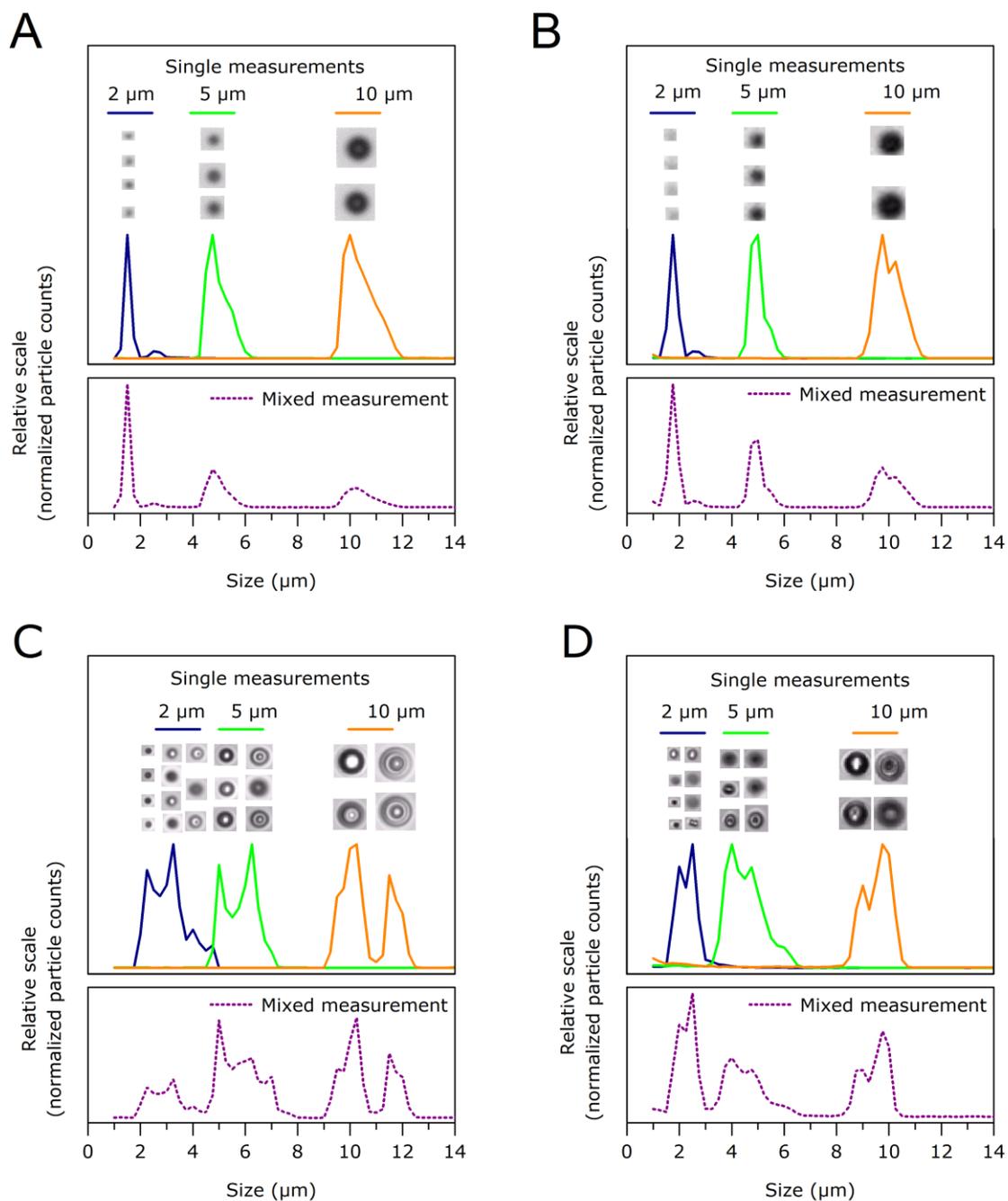
Concentration linearity was evaluated with different dilutions of 5  $\mu\text{m}$  polystyrene size standards over a wide range from about  $4 \times 10^2$  to  $8 \times 10^6$  particles/mL. The obtained concentrations for particles  $> 3 \mu\text{m}$  (as specified for the 5  $\mu\text{m}$  count standard) were compared to the theoretical concentration as determined by LO in the low concentration range (4056 particles/mL for the second highest dilution) and calculated for the higher concentrations (Figure 4-1). All systems showed good overall linearity, but underestimated the particle number at high concentrations (Figure 4-1A) probably due to coincidence of particles, meaning that two particles which are located very closely next to or behind each other are detected as one particle. For the highest concentration of theoretically  $8 \times 10^6$  particles/mL, a measurement was only possible with the MFI4100 and FlowCAM VS1. MFI5200 and FlowCAM PV were not able to handle such high particle

concentrations as the measurements were automatically aborted at  $1 \times 10^6$  and  $5 \times 10^5$  captured particles, respectively. This is due to a software setting limiting the number of captured particles to 500,000 per analysis to ensure proper data handling. The limit can be increased, but this would slow down data processing by the software. For the sample with a theoretical concentration of  $4 \times 10^6$  particles/mL, MFI4100, MFI5200, and FlowCAM VS1 underestimated the particle concentration by less than 10%, whereas the FlowCAM PV system detected 25% less particles than actually expected. In the medium concentration range of theoretically  $4 \times 10^3$  to  $1 \times 10^6$  particles/mL, all systems showed good results (Figure 4-1B,C). Whereas the FlowCAM systems slightly underestimated the concentration, the MFI4100 system overestimated the concentration in the case of theoretically  $4 \times 10^5$  particles/mL. The MFI5200 system constantly showed deviations from the theoretical concentration of less than 2%. For the lowest concentration of theoretically 406 particles/mL, MFI4100, MFI5200 and FlowCAM PV showed large deviations of 11-28% and only the FlowCAM VS1 system detected the theoretical concentration within 1% (Figure 4-1C). All systems showed large relative standard deviations in the low concentration range below  $4 \times 10^3$  particles/mL (8% for MFI5200, 18% and more for the other systems).



**Figure 4-1: Linearity of particle concentration measurements by MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV. 5 μm PS standards measured at various dilutions. The theoretical concentrations are based on the counts of the second highest dilution obtained by LO (result: 4056 particles/mL). (A) Full concentration range, (B) zoom into medium concentrations, (C) zoom into low concentrations. Error bars represent standard deviations from triplicate measurements.**

Size accuracy was evaluated with monodisperse polystyrene size standards of 2, 5, and 10  $\mu\text{m}$ . Overall, the MFI systems rendered images of poorer resolution, but better size accuracy as compared with the FlowCAM systems evaluated in this study (Table 4-2 and Figure 4-2). The MFI4100 system underestimated the size of the 2  $\mu\text{m}$  polystyrene standards due to resolution limitations for those small particles, but showed satisfying size accuracy for 5  $\mu\text{m}$  and 10  $\mu\text{m}$  as well as a narrow distribution for all sizes (Figure 4-2A). MFI5200 was the only system that determined all sizes accurately and with a high precision (Figure 4-2B). The images of size standards obtained by the MFI systems appeared rather blurry, but comparable in size and optical appearance, leading to the observed good size accuracy and precision. In contrast, the images obtained by the FlowCAM systems showed high resolution and sharpness, but also a large variability in size and optical appearance. Especially the FlowCAM VS1 system showed clear deviations from the correct size (Table 4-2) and also a broad size distribution with apparently more than one population per analyzed size standard (Figure 4-2C). This is particularly striking for the 10  $\mu\text{m}$  polystyrene standard, for which two apparent populations around 10  $\mu\text{m}$  and 12  $\mu\text{m}$  were detected. The 10  $\mu\text{m}$  peak particles appear to be captured in focus, whereas the 12  $\mu\text{m}$  peak particles appear out of focus as indicated by the concentric rings. Although the FlowCAM software VisualSpreadsheet is theoretically able to exclude out-of-focus particles, this was not performed as it would compromise the accuracy of the particle concentration and does therefore not represent a suitable option for real protein sample analysis. The FlowCAM PV rendered images of slightly lower resolution, but in return better size homogeneity leading to better size accuracy and precision (Figure 4-2D). For a mixed sample of 2, 5, and 10  $\mu\text{m}$  polystyrene size standards, the described differences in image quality and homogeneity led to a better separation between the sizes in the MFI systems as compared with the FlowCAM systems (Figure 4-2A-D, lower panels). The underlying reasons for the differing image quality and homogeneity are assumed to be (i) the magnification and (ii) the depth of focus (Table 4-1). Furthermore, the threshold value in the FlowCAM systems influences the size accuracy as there is always a trade-off between size accuracy and image fragmentation.

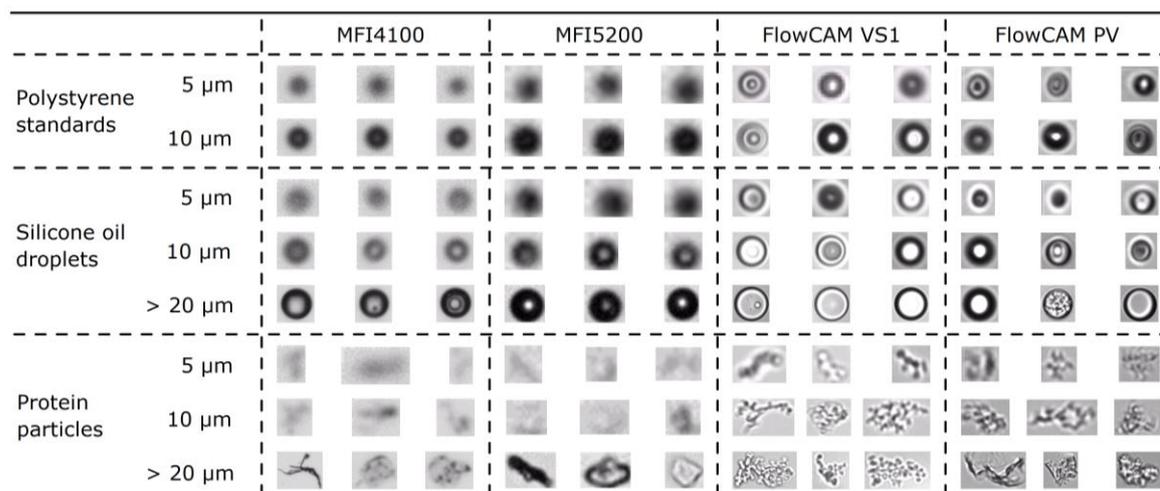


**Figure 4-2: Size accuracy and precision of 2  $\mu\text{m}$ , 5  $\mu\text{m}$  and 10  $\mu\text{m}$  PS size standards measured separately (upper panels) and as a mix (lower panels) by (A) MFI4100, (B) MFI5200, (C) FlowCAM VS1, and (D) FlowCAM PV. Representative images are shown above the corresponding peak of the size distribution.**

## 3.2 Image properties

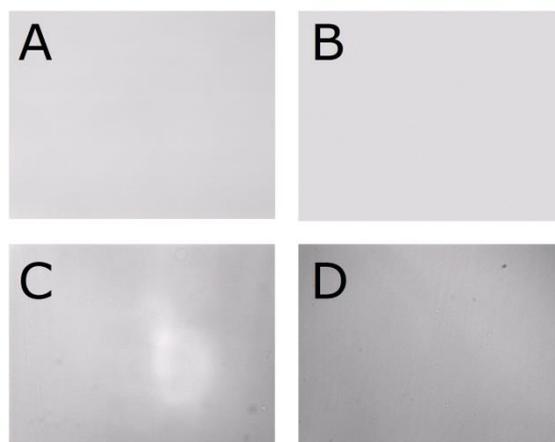
As discussed above, differences in the image properties and especially in the image homogeneity lead to divergences in size determination. Furthermore, the image quality is a crucial parameter for morphological analysis and for a reliable discrimination of different particle types, e.g. proteinaceous vs. non-proteinaceous particles. Therefore, we compared images of polystyrene standards, artificially generated silicone oil droplets, and protein particles (heat-stressed rituximab) (Figure 4-3). In general, images provided by the FlowCAM systems appeared sharper and of higher resolution than images captured by the MFI systems. This is mainly due to the smaller focus area and higher magnification of the FlowCAM optics. Thus, many morphological details were already visible on particles as small as 5  $\mu\text{m}$  in size, especially for the FlowCAM VS1 system. However, the small focus area caused particles of the same type to appear optically different, which could be well observed on images for polystyrene standards and silicone oil droplets. Dark particles with a bright halo as well as bright particles with a dark edge and several nuances in between were detected within one sample. For protein particles, images captured by the FlowCAM systems appeared more uniform regarding the optical contrast than for polystyrene standards and silicone oil droplets. The MFI4100 system provided comparable images of protein particles. In contrast the images captured by the MFI5200 system appeared more variable, presumably due to its larger view window which results in different illumination of particles depending on their location within the view window. For protein particles, this can lead to a high diversity in the optical appearance due to diffraction patterns within those heterogeneous particles.<sup>21</sup> However, it is difficult to judge which instrument displays the real heterogeneity of protein particles as this is not known. The difference in sharpness and resolution between MFI systems and FlowCAM systems was particularly obvious for protein particles with sizes of about 5  $\mu\text{m}$  and 10  $\mu\text{m}$ . Here, FlowCAM images provide more morphological details, whereas MFI images appear rather blurry. Furthermore, the MFI systems capture only pixels of the particle which are darker than the background. In contrast, the FlowCAM systems use a different background calibration procedure allowing the additional depiction of pixels brighter than the background which probably result from specific diffraction patterns.<sup>21</sup> This contributes to the enhanced visibility of

morphological details but also leads to the heterogeneity in FlowCAM images. Within the brands, the MFI4100 and FlowCAM VS1 captured better images than the MFI5200 and FlowCAM PV.



**Figure 4-3: Representative images of polystyrene standards, silicone oil droplets, and protein particles (heat-stressed rituximab) of different particle sizes scaled to the same image size.**

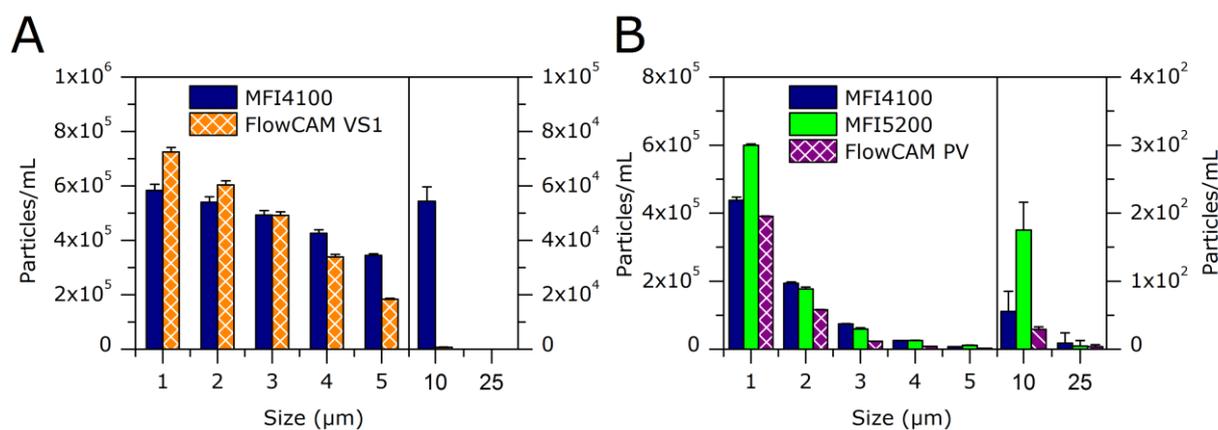
An additional cause of image variability in the FlowCAM systems for polystyrene standards and silicone oil droplets might be the illumination of the flow cell. While the background of an MFI flow cell appears uniformly grey (Figure 4-4A,B), the background of a FlowCAM flow cell seems to be less evenly illuminated, especially for the FlowCAM VS1 system (Figure 4-4C,D). This can affect the overall brightness of an image depending on where within the flow cell it was captured. According to the manufacturer, this feature is currently under development for the FlowCAM systems.



**Figure 4-4: Images of a clean flow cell (purged with water) in (A) MFI4100, (B) MFI5200, (C) FlowCAM VS1, and (D) FlowCAM PV.**

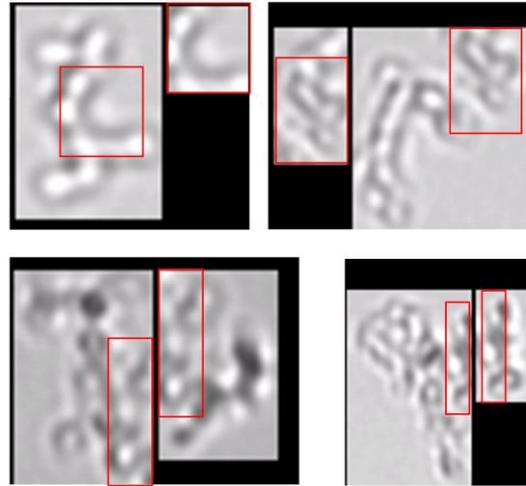
### 3.3 Quantification of protein particles

Because the captured particle images form the basis for particle analysis, a potential correlation between image quality and detected particle numbers was investigated. To this end, protein particles were generated by heating a rituximab formulation and analyzed by the four systems. Due to the time-shifted availability of the FlowCAM systems, the exact same sample could not be analyzed in parallel by all four systems. Instead, one sample was analyzed in parallel by the MFI4100 and FlowCAM VS1 (Figure 4-5A). Another sample, prepared later under the same conditions, was analyzed in parallel by the MFI5200 and FlowCAM PV as well as by MFI4100 for comparison (Figure 4-5B). Thus, the difference in the cumulative size distribution between Figure 4-5A and Figure 4-5B can be attributed to the variability in the sample preparation. System-dependent differences can only be evaluated within Figure 4-5A or within Figure 4-5B. Although the image resolution for particles below 2  $\mu\text{m}$  was poor and the official lower size limit of the FlowCAM systems is 2  $\mu\text{m}$ , counting of particles could be performed for particles  $> 1 \mu\text{m}$  with satisfying data quality for all systems. For the same sample, the FlowCAM VS1 system detected more particles below 3  $\mu\text{m}$  but fewer particles above 3  $\mu\text{m}$ , particularly above 10  $\mu\text{m}$ , as compared with the MFI4100 system (Figure 4-5A).



**Figure 4-5: Cumulative particle counts for protein particles of heat-stressed rituximab analyzed by (A) MFI4100 and FlowCAM VS1 and (B) MFI4100, MFI5200, and FlowCAM PV. Error bars represent standard deviations from triplicate measurements.**

A possible reason for this might be image fragmentation which was observed for the FlowCAM VS1 when using the setting “only dark particles” (Figure 4-6). It seems that bright parts of particles were detected as the particle boundary by the software. This effect was observed for particles larger than 10 μm. Although image fragmentation might also have occurred for smaller particles it could not be confirmed by optical evaluation of the images due to resolution limitations. Changing the settings to “dark & bright” might have decreased this effect but, as discussed earlier, failed to provide the correct size for polystyrene size standards and was therefore not chosen. This shows again that the user has to accept a certain trade-off between good size accuracy and robustness against image fragmentation for the FlowCAM systems which on the one hand brings along certain user-dependency and data variability. On the other hand, those many adjustable settings in the FlowCAM systems enable the handling of a specific problem. In contrast, the MFI systems require the trust of the user in the predefined settings which cannot be changed. For the other systems evaluated in this study image fragmentation was not observed for the same samples. However, for an IgG-containing sample from a different study image fragmentation was observed for the MFI4100 system (data not shown due to confidentiality).

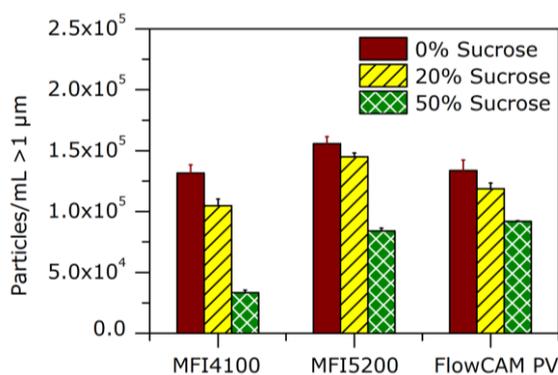


**Figure 4-6: Images of protein particles around 10  $\mu\text{m}$  (heat-stressed rituximab) captured by the FlowCAM VS1 system. Red boxes indicate overlapping or doubly imaged regions in two separate images due to image fragmentation.**

For the second sample analyzed, MFI5200 and FlowCAM PV detected similar size distributions with slightly less particles detected by the FlowCAM PV system (Figure 4-5B). Clearly more small particles larger than 1  $\mu\text{m}$  were detected by the MFI5200 system, pointing on the one hand towards a better sensitivity for small transparent particles, on the other hand potentially also towards undetected image fragmentation. For the FlowCAM PV system it needs to be considered that the official size range of this system starts only at 2  $\mu\text{m}$  and was extended consciously in this study. For total particle concentrations larger than 2  $\mu\text{m}$ , similar concentrations were detected by all three systems. The difference for particles larger than 10  $\mu\text{m}$  is probably due to the low total number in this size range causing higher standard deviations. In general, the MFI5200 and FlowCAM PV showed lower standard deviations for total particle counts larger than 1  $\mu\text{m}$  as compared with the MFI4100 and FlowCAM VS1, as could be expected from the differences in the analyzed volume.

It was shown earlier that light-based quantification of protein particles is influenced by the RI of both, particles and surrounding formulation and that this effect is partly system dependent.<sup>26</sup> Therefore, the robustness of MFI4100, MFI5200, and FlowCAM PV towards RI influences was determined by quantifying protein particles larger than 1  $\mu\text{m}$  (stir-stressed infliximab) in the same

concentration in formulations of increasing RI, adjusted by addition of sucrose (Figure 4-7). The FlowCAM VS1 system was not available at the time of these experiments. Particle concentrations obtained by MFI4100 were rather sensitive to an increase in RI of the formulation. In 20% sucrose (RI 1.36), 80% of the original particle concentration was still detected whereas in 50% sucrose (RI 1.42), only 25% could be detected. MFI5200 and FlowCAM PV were both more robust towards RI influences: in 20% sucrose, 93% and 89% of the original particle concentration, respectively, were still detected and in 50% sucrose the apparent concentration decreased only to 54% and 69% with MFI5200 and FlowCAM PV, respectively. The reason for the superior performance of MFI5200 and FlowCAM PV is potentially connected to optimized optical settings of these newer systems. Two different control experiments in a previous study have shown that the particle concentration was not affected directly by the high sucrose concentration, e.g. by dissolution or generation of particles.<sup>26</sup> Instead, the decreased RI difference between particles and surrounding formulation reduced the apparent particle concentration. The RI of a 20% sucrose solution (1.36) represents pharmaceutically relevant conditions, e.g. at high protein concentration or a combination of excipients such as sucrose and high protein concentration.<sup>26</sup>

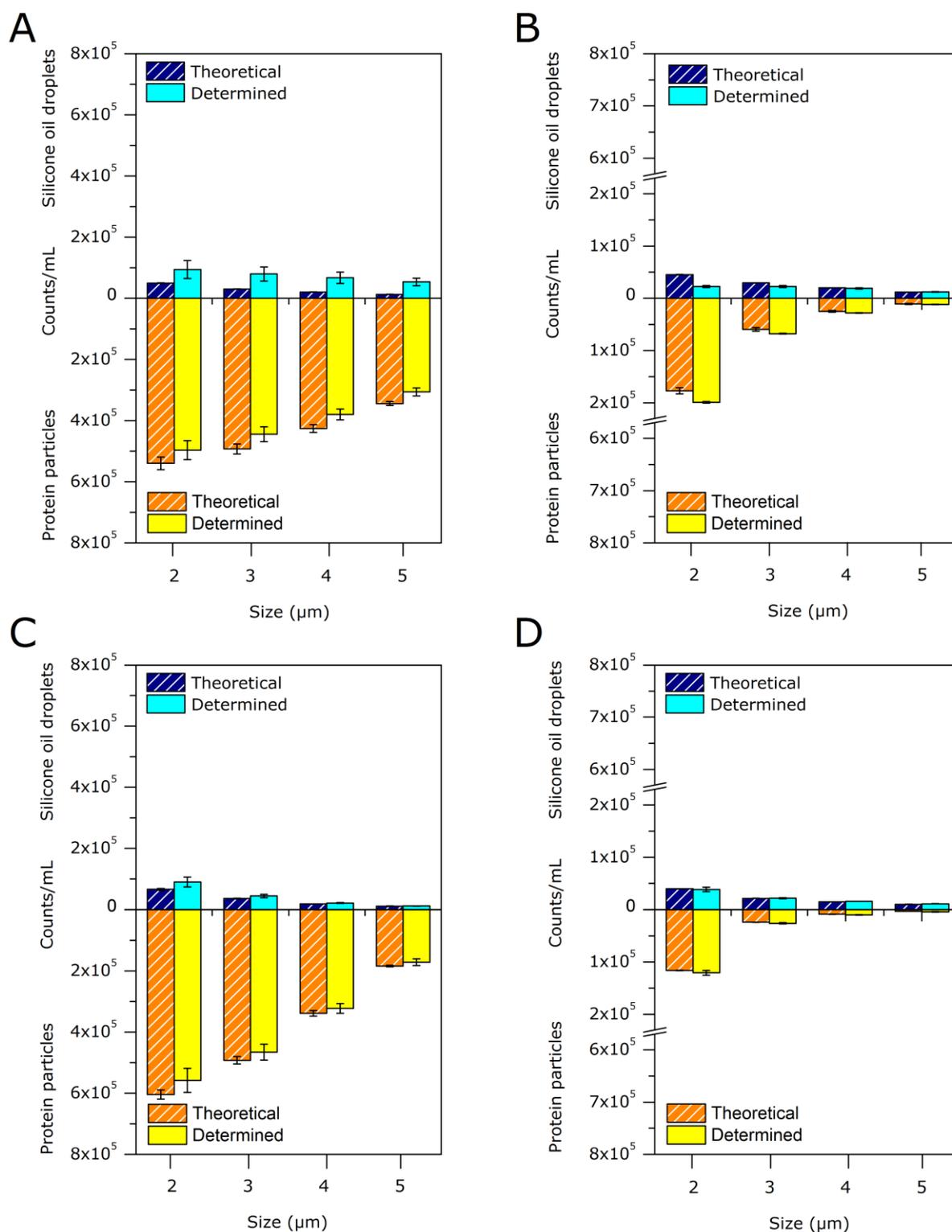


**Figure 4-7: Total particle counts for protein particles of stir-stressed infliximab for fixed particle concentrations in sucrose solutions of varying concentration and thus RI. Error bars represent standard deviations from triplicate measurements.**

### 3.4 Differentiation of silicone oil droplets and protein particles

A major advantage of flow imaging microscopy as compared with other analytical techniques for subvisible particles, e.g. LO or electrical sensing zone analysis, is the possibility to characterize particles based on images.<sup>10</sup> Parameters such as shape and transparency can be used to differentiate between different particle types.<sup>22,23</sup> In this context, the discrimination of silicone oil droplets and protein particles is especially relevant due to the increasing application of prefilled syringes. Similar to a previous study,<sup>22</sup> protein particles (heat-stressed rituximab) and silicone oil droplets were analyzed by MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV as individual samples (to obtain the theoretical concentration within the same system) and in controlled mixtures. The “find similar” algorithm in the respective software was used to differentiate between silicone oil droplets and protein particles. Due to the time-shifted availability of the FlowCAM systems, the exact same sample could not be analyzed in parallel by all four systems. Instead, one group of samples was analyzed in parallel by the MFI4100 and FlowCAM VS1 (Figure 4-8A,C). Another group of samples which was prepared later under the same conditions was analyzed in parallel by the MFI5200 and FlowCAM PV (Figure 4-8B,D). The concentration was adjusted in such a way that similar total particle counts larger than 1  $\mu\text{m}$  were obtained for both groups of samples with the MFI4100 as the bridging instrument. However, the relative size distribution for protein particles differed clearly between the two sample groups. Thus, the differentiation performance was evaluated within the systems, but not between the systems. The evaluation was based on the match of the detected concentration (in mixed samples) and the theoretical concentration (in individual samples) within each system. The theoretical concentration may differ from system to system and is only valid for the mixed samples analyzed by the same system. Although an optical discrimination of silicone oil droplets and protein particles based on the particle images, which is the basis for the “find similar” operation, was only reasonable for particles of 5  $\mu\text{m}$  and larger, the “find similar” function of the software was able to differentiate particles down to 2  $\mu\text{m}$ .

The FlowCAM PV system showed the best match with the theoretical concentration, thus the best differentiation of silicone oil droplets and protein particles (Figure 4-8D). The MFI5200 and FlowCAM PV (Figure 4-8B,D) showed a higher precision than the MFI4100 and FlowCAM VS1 (Figure 4-8A,C). However, the differences were rather small and results might depend on the specific sample properties. In conclusion, all systems proved to be suitable for the differentiation of silicone oil droplets and protein particles from 2 to 10  $\mu\text{m}$ . For particles below 2  $\mu\text{m}$ , alternative techniques such as resonant mass measurement (RMM) can be beneficial.<sup>22</sup> For particles larger 10  $\mu\text{m}$ , it is recommended independently of the system to differentiate particles by optical evaluation of the images rather than by applying the “find similar” function. This approach is feasible due to the clear images and usually low particle counts in this size range.



**Figure 4-8: Cumulative particle counts comparing theoretical concentrations (based on individual samples measured with the corresponding instrument) and determined concentrations (mixed samples) of artificially generated silicone oil droplets and protein particles (heat-stressed rituximab) in a droplet/particle ratio of 10:90 (based on particle counts  $> 2 \mu\text{m}$  with MFI4100). (A) MFI4100, (B) MFI5200, (C) FlowCAM VS1, (D) FlowCAM PV. Error bars represent standard deviations from triplicate measurements.**

### 3.5 Handling of the systems

Concerning the hardware, MFI systems only allow the adjustment of the sample volume. This ensures standardized, user-independent measurements and repeatable results, but requires full trust in the settings predefined by the manufacturer, which cannot be customized to specific needs or samples. In contrast, the FlowCAM systems allow changes in optical settings (e.g. threshold, shutter, and gain) or technical settings (flow rate, image capture rate) offering customization of the analysis to specific needs for experienced users, but impede comparability between samples analyzed by different operators, at different times or even by different instruments of the same type.

The exchange of a flow cell, which requires the adjustment of the focus as a critical parameter for image-based particle analysis, is straightforward and unambiguous for the MFI systems. For the FlowCAM systems, especially the FlowCAM VS1, this process was found to be cumbersome but this is currently being improved by the manufacturer. Furthermore, the MFI systems use a peristaltic pump enabling high flow rates and large volumes which is useful for an efficient cleaning step, but the flow rate needs to be calibrated regularly. The FlowCAM systems for small volumes (as applicable for protein samples) are typically equipped with a syringe pump, which does not require calibration by the user, but is restricted in volume and speed limited by the flow cell diameter. Thus, cleaning cycles with FlowCAM need to be performed several times with low volume and flow rate, especially in case of small syringe sizes.

Concerning the software, the MFI systems use different software types for the measurement (MFI View software for MFI4100, MVSS for MFI5200) and the data analysis (MVAS), whereas the FlowCAM systems apply the same software for both steps (VisualSpreadsheet). While the latter allows the analysis of the particle population, regarding size distribution and cropped images, already during the measurement as a real time analysis, this data becomes available only after the measurement for the MFI systems. However, the MVAS software includes an essential function to "remove stuck particles" (particles stuck to the flow cell wall which would otherwise be counted on every image they were captured on). This option is not yet available for VisualSpreadsheet but is

currently under development. In both software solutions, particle data can be exported in many different ways and the raw data of every single particle (e.g. shape or transparency values) is available. MVAS enables export of single particle images, whereas VisualSpreadsheet offers collages of particle images. Regarding the differentiation of silicone oil droplets and protein particles, the analysis of a single sample is simpler in MVAS, while VisualSpreadsheet enables the generation of libraries from selected particles, which can be used to build a filter for future samples. In addition, VisualSpreadsheet offers the possibility to sort the resulting population of similar particles by “filter score”, i.e. by similarity to the selected particles. Taken together, MFI systems are more standardized, whereas FlowCAM systems are designed for more flexibility for the user, concerning both hardware and software.

## 4 Conclusion

Our study showed that the selection of the appropriate flow imaging microscopy system depends strongly on the main output parameters of interest and the intended application. Each system shows its strengths and weaknesses in different aspects (Table 4-3). The four systems evaluated in this study can be categorized based on the technical data and the results obtained in this study into high-resolution systems (MFI4100 and FlowCAM VS1) and high-efficiency systems (MFI5200 and FlowCAM PV). The best images were obtained by the FlowCAM VS1 system, which was seen as the best system among the high-resolution instruments. The best performance regarding particle counting accuracy and precision was achieved by the MFI5200 system, which appeared to be the preferred system among the high-efficiency instruments. The MFI4100 and the FlowCAM PV system were observed as all-round systems which might be a good compromise between the other two systems that are more biased towards particle counting (MFI5200) or particle imaging (FlowCAM VS1).

**Table 4-3: Summarizing assessment of important analysis factors for MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV.**

Parameter	MFI4100	MFI5200	FlowCAM VS1	FlowCAM PV	
Image properties	Resolution	++	+	++++	+++
	Contrast within the particle	+	++	++++	+++
	Image consistency (standards)	++++	++++	+	+
Polystyrene size	Accuracy	+++	++++	+	++
	Precision	+++	++++	+	++
Polystyrene count	Accuracy	+++	++	+	++++
	Precision	+	++++	+++	++
	Linearity	++	++++	++++	+
Protein particle quantification	Precision	+	+++	+	++++
	Robustness against RI influences	++	+++	n.a.	++++
Differentiation of silicone oil droplets and protein particles	Match with the theoretical concentration	+	++	+++	++++
	Precision	+	+++	++	++++
Handling	Hardware	+++	++++	+	++
	Software for measurement	++++	+++	+	++
	Software for data analysis		++++		+++

++++ = strongest performance; +++ = second strongest performance; ++ = third strongest performance; + = weakest performance; all criteria were judged only relatively among the evaluated systems.

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# Chapter 5

## Material screening and investigation of particle density for the development of standardized protein-like particles

### **Abstract**

The aim of this study was the identification of suitable materials and preparation methods for the development of standardized protein-like particles. In the first part, a material screening based on optical particle properties was performed. Proteinaceous (human serum albumin (HSA)-starch particles, spray-dried HSA, gelatin particles, and zein) and non-proteinaceous materials (chitosan and polytetrafluoroethylene (PTFE)) were compared to HSA particles generated by heat stress as a representative model for protein particles in therapeutic formulations. The particle properties size, size distribution, shape, transparency, and stability were assessed by light obscuration (LO) and Micro-Flow Imaging (MFI). As a result, gelatin and PTFE particles reflected the most relevant optical properties (shape and transparency) of protein particles and were regarded as promising candidates for the development of standardized protein-like particles for light-based techniques. In the second part, the density of protein particles in aqueous formulations as a further crucial property was investigated. Two different methods based on resonant mass measurement (RMM) were developed to determine (i) the density of pure protein and (ii) the apparent density of protein particles including entrapped liquid. The first method provided a density around 1.4 g/mL for pure protein, which complied with theoretically calculated values. The second method was only applicable for particles showing a clear maximum in the size distribution and yielded an apparent density of around 1.1 g/mL for protein particles including entrapped liquid. Based on these parameters, PTFE particles were regarded as suitable standard material especially for light-based techniques, whereas gelatin particles could be used for both light-based and weight-based methods.

# 1 Introduction

The analysis of particles in therapeutic protein formulations requires calibration of the instruments with certified particle standards. Mostly polystyrene particle standards are used for this purpose, although these standards do not represent optical and morphological properties of protein particles.<sup>1</sup> Thus, novel standards are more and more claimed by academia and industry<sup>2-4</sup> which should be applied mainly for comparison and evaluation of results acquired by different techniques or instruments and if possible also for instrument calibration. These novel standards should reflect protein particle properties (size, size distribution, optical and morphological parameters), should be stable as an aqueous suspension, and should behave similar to protein particles in the commonly applied analytical techniques regarding measurement performance and data evaluation. This could involve the direct use of raw material powder or preparation of particles from raw materials. Proteinaceous and non-proteinaceous materials are theoretically suitable for both alternatives. Proteinaceous materials bring the benefit of high similarity, but might bear the risk of low stability, especially at ambient conditions in solution. Non-proteinaceous materials have the advantages of easier handling and potentially increased stability, but might face the problem of low conformity with protein particles as known for polystyrene standards.

Proteinaceous materials evaluated in this study include human serum albumin (HSA)-starch particles, which were originally developed as particles with an irregular and rough surface structure for mucosal delivery of vaccines,<sup>5</sup> spray-dried HSA and gelatin particles prepared by desolvation, both stabilized by a cross-linker, and the hydrophobic water insoluble protein zein as the raw material powder. Non-proteinaceous raw material powders screened for their suitability as standardized protein-like particles were the polysaccharide chitosan and the synthetic fluoropolymer polytetrafluoroethylene (PTFE). The application purpose of the novel standards are light obscuration (LO) and Micro-Flow Imaging (MFI) as the workhorses of protein particle analysis. Therefore, the focus was set on the similarity of particle properties relevant for those techniques: Size, size distribution, shape, and transparency for the novel materials were compared to those of HSA particles generated by heat stress as a

representative for particles of therapeutic proteins. Furthermore, the stability in an aqueous suspension was assessed.

The density of protein particles is a crucial parameter for protein particle analysis which has not been characterized well up to now.<sup>6</sup> Light-based techniques like LO and MFI determine the particle size based on the optical signal which is caused by the combination of protein parts and entrapped liquid within a particle. The novel technique of resonant mass measurement (RMM) detects the buoyant mass which is only influenced by protein parts within the particle and the size calculation depends on the particle density as an input parameter. Thus, the techniques consider the particle density in different ways and a deeper understanding of protein particle density is valuable for data evaluation and therefore also the development of protein-like standards. RMM was applied in this study to investigate (i) the density of pure protein and (ii) the density of protein particles including entrapped liquid and the results were compared to the density of the screened materials.

## 2 Materials and methods

### 2.1 Materials

5  $\mu\text{m}$  polystyrene particle size standards were purchased from Duke Scientific (through Thermo Scientific, Fremont, CA) and diluted in water for analysis. Dry borosilicate glass particle standards (5  $\mu\text{m}$ ) were purchased from Duke Scientific, suspended in water containing a minimum amount of isopropanol (according to the instructions by the manufacturer), sonicated for 1.5 h and vortexed directly before analysis. 5  $\mu\text{m}$  silica particle size standards were purchased from microparticles GmbH (Berlin, Germany) and diluted in water for analysis.

Rituximab (MabThera<sup>®</sup>, lot no. B6082) was provided by local hospitals, diluted to 1 mg/mL in 25 mM citrate buffer (pH 6.5) containing 154 mM NaCl and 0.07% polysorbate 80 and filtered (0.2  $\mu\text{m}$  polyethersulfone syringe filter, Sartorius, Göttingen, Germany) for further use. HSA was purchased from Sigma-Aldrich (Steinheim, Germany), formulated at 1 mg/mL in 50 mM citrate buffer (pH 4.8) or at 5% (m/v) in water and filtered (0.2  $\mu\text{m}$  cellulose acetate syringe filter, Minisart<sup>®</sup>, Sartorius Stedim Biotech, Aubagne, France) for further use. Gelatin from porcine skin (type A, medium gel strength, 170-190 g Bloom, for microbiology) was purchased from Sigma-Aldrich. Chitosan (poly-(D-glucosamine) deacetylated chitin, >75% deacetylated, coarse ground flakes and powder) was obtained from Sigma-Aldrich and suspended in water for analysis. Zein F 4000 was obtained as a gift from capol GmbH (Elmshorn, Germany), sieved through a 100  $\mu\text{m}$  mesh and suspended in 10% polysorbate 80 in water. PTFE (Microdispers 8000 from Polysciences Inc., Warrington, PA) was suspended at a concentration of 50 mg/mL in 0.5% polysorbate 80 using an Ultra Turrax dispersing system (T10 basic, IKA<sup>®</sup> Werke, Staufen, Germany) for 3 minutes. Larger agglomerates were removed by filtration through a coarse tea filter (dm, Karlsruhe, Germany).

Dimethylsulfoxide (DMSO, for synthesis) was purchased from Merck Schuchardt (Hohenbrunn, Germany). Rape oil was obtained from A&P (Kaiser's Tengemann, Mühlheim an der Ruhr, Germany). Glutaraldehyde (technical, 50% in water, 5.6 M) and hydrochloric acid were purchased from Sigma-Aldrich. White soluble



gelatin solution at room temperature with a precipitation time of 2 minutes. The supernatant was discarded and the deposit was redissolved in 25 mL water at 50 °C. The pH was adjusted to 3.9 with 1 M HCl. The second precipitation was initiated by adding 50 mL acetone at about 9-10 mL/min using a burette. After 10 minutes of stirring at 500-600 rpm, 500 µL glutaraldehyde (8%) were added as a cross-linker and the suspension was stirred for another 30 minutes. The particles were harvested by centrifugation at 10,000 g for 10 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 5 mL water and filtered through a coarse tea filter to remove large agglomerates.

For the evaluation of particle density, the preparation process was optimized to maximize the number of particles in the target size range of 2-8 µm. The cross-linking time with glutaraldehyde was extended to 40 minutes and the centrifugation speed for particle harvesting was decreased to 5,000 g. After filtration through a coarse tea filter to remove large agglomerates an additional purification step was introduced to minimize the number of particles below 2 µm: The pH of the filtrate was adjusted to pH 3 to provoke electrostatic repulsion between the particles prior to an additional centrifugation step (200 g, 20 minutes). The pellet was discarded and the supernatant was used.

HSA starch particles were produced by an emulsion-based process according to previous studies by Heritage *et al.*<sup>5</sup> 1 g starch was dissolved in 2 mL DMSO under stirring at 85 °C, cooled down to room temperature and subsequently 1 mL 10% (w/v) aqueous HSA solution was added drop-wise. This solution was emulsified drop-wise in 20 mL rape oil under stirring at 1250 rpm (Heidolph MR 3001K) and sonication. Afterwards this emulsion was added drop-wise to 400 mL acetone containing 0.5 mL polysorbate 80, again under stirring at 1250 rpm. The generated microparticles were then collected by filtration (0.22 µm Durapore® (PVDF) membrane filter, Millipore) under vacuum, washed with 1 L acetone, and dried on the filter by vacuum. The powder was sieved through a 100 µm mesh to exclude large agglomerates and the particles were stored under desiccation at 5±3 °C. 30 mg of the particles were suspended in 4 mL water for analysis.

### 2.3 Light obscuration (LO)

Subvisible particles in a size range between 1 and 200  $\mu\text{m}$  were analyzed by LO using a PAMAS SVSS-C (Partikelmess- und Analysesysteme GmbH, Rutesheim, Germany) equipped with an HCB-LD-25/25 sensor. Particle suspensions were diluted with the according buffer (filtered by a 0.22  $\mu\text{m}$  cellulose acetate/nitrate membrane filter, MF-Millipore®) or water in order to adhere to the concentration limit of the system of 120,000 particles/mL  $> 1 \mu\text{m}$ . Three measurements of a volume of 0.3 mL of each sample were performed with a pre-run volume of 0.5 mL at a fixed fill rate, emptying rate and rinse rate of 10 mL/min and the mean particle concentration per mL was reported by the system. Samples were measured in triplicates and mean and standard deviation were calculated.

### 2.4 Micro-Flow Imaging (MFI)

Subvisible particles in a size range between 1 and 70  $\mu\text{m}$  were analyzed by MFI using an MFI4100 (ProteinSimple, Santa Clara, CA) equipped with a high-resolution 100  $\mu\text{m}$  flow cell. Particle suspensions were diluted with the according buffer (filtered by a 0.22  $\mu\text{m}$  cellulose acetate/nitrate membrane filter) or water in order to adhere to the concentration limit of the system of 1,200,000 particles/mL  $> 0.75 \mu\text{m}$ . Samples were analyzed with a sample volume of 0.65 mL and a pre-run volume of 0.3 mL at a flow rate of 0.1 mL/min. Prior to each sample run the appropriate diluting buffer was flushed through the system to provide a clean flow cell and to perform "optimize illumination". Particles stuck to the flow cell wall were only counted once and edge particles were ignored for analysis. Samples were measured in triplicates and mean and standard deviation were calculated. Results were analyzed using the MFI view application software (version 1.2, ProteinSimple).

### 2.5 Resonant mass measurements (RMM)

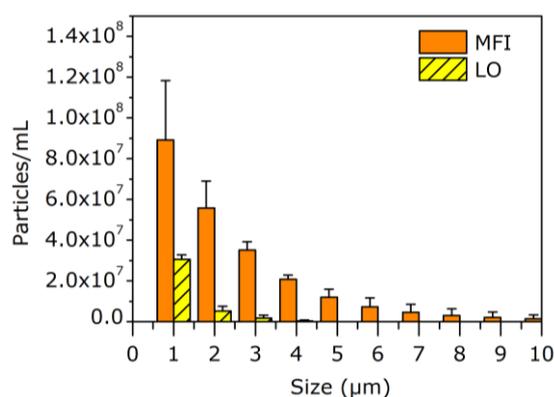
RMM was performed using the Archimedes particle metrology system (Affinity Biosensors, Santa Barbara, CA) equipped with a Micro sensor (size range 0.3  $\mu\text{m}$  to 4  $\mu\text{m}$ ) calibrated with 1  $\mu\text{m}$  polystyrene standards. Before each measurement, the system was filled with sample and the lower size limit of detection was determined three times in automatic mode. The mean value was set as a fixed

limit of detection for the measurement. The buffer density was determined for each sample based on the sensor frequency relative to the frequency and the density of water as a reference. The density of the solid part of a particle (pure protein in case of protein particles) was determined by quantifying the buoyant mass (sum of all particles in the sample) in aqueous buffer and two liquids of higher density (20% and 40% sucrose). The buoyant mass decreases with the decreasing density difference between particles and liquid and was extrapolated to a buoyant mass of zero which indicates a density match between particles and surrounding liquid. The density of the solution was then set as the density of the solid part of the particle (liquid parts within the particle do not contribute to the buoyant mass as they possess the same density as the surrounding liquid). The apparent density of protein particles including entrapped liquid was determined by adjusting the particle density input in the software from 1.37 g/mL (as used in another RMM study<sup>10</sup>) to smaller values until the size distributions determined by RMM and MFI for the same sample overlapped (defined by the same location of the maximum). Measurements were performed in triplicates and the sensor was filled with fresh sample for each measurement. The measured volume was 0.15  $\mu$ L and the overall sample volume for triplicate measurements was 600  $\mu$ L. Between triplicate measurements, the system was rinsed with water. Results were analyzed using the ParticleLab software (v1.8.570, Affinity Biosensors) with a size bin step of 250 nm.

### 3 Results and discussion

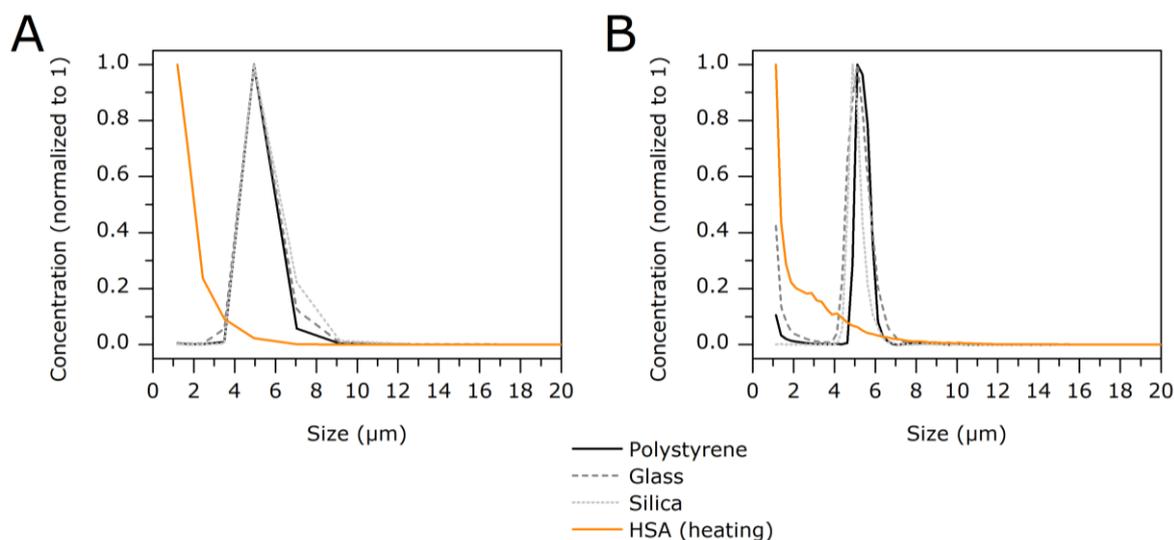
#### 3.1 Comparison of current standards to protein particles by LO and MFI

HSA particles generated by heat stress were analyzed as a representative for protein particles in general to determine typical properties of protein particles regarding size, size distribution, shape, and transparency in order to derive target specifications for novel standardized protein-like particles. The size distribution of HSA particles generated by heat stress appeared polydisperse with small particles representing the largest fraction (Figure 5-1). Furthermore, it revealed 3 to 10x higher particle concentration detected by MFI compared to LO which is in agreement with the literature.<sup>4,11,12</sup>



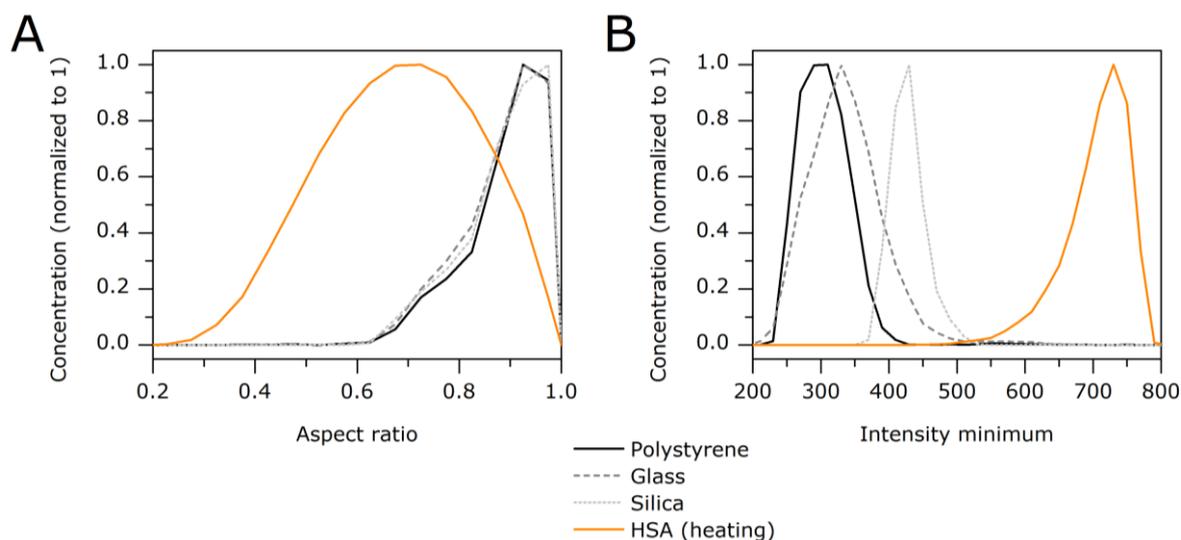
**Figure 5-1: Cumulative size distribution for HSA particles generated by heat stress as determined by MFI and LO. Error bars represent standard deviations from triplicate measurements.**

In contrast, 5 μm size standards made of polystyrene, glass, and silica showed a monodisperse size distribution (Figure 5-2) and identical concentrations in LO and MFI (data not shown).



**Figure 5-2: Differential size distribution of 5  $\mu\text{m}$  polystyrene, glass, and silica particle size standards and HSA particles generated by heat stress determined by (A) LO and (B) MFI.**

The optical particle properties shape and transparency are especially important for standards for the light-based techniques LO and MFI. The aspect ratio (between 1 for an absolutely spherical particle and 0 for a needle with an infinite length) of HSA (heating) particles was broadly distributed between 0.3 and 1.0 whereas the particle standards showed higher aspect ratios mainly above 0.8 (Figure 5-3A). The transparency was evaluated by the directly proportional intensity minimum which describes the darkest pixel on a particle image.<sup>13</sup> As the intensity depends on the particle size<sup>14</sup> the 2-6  $\mu\text{m}$  range was used for the evaluation of the different materials (see Chapter 6 for further details). Particle standards displayed low intensity values (polystyrene standards approx. 300) corresponding to low transparency. In contrast, HSA (heating) particles are highly transparent with intensity values of about 700, which is close to the maximum intensity values of the instrument of about 850 typically (Figure 5-3B). These clear differences in shape and transparency between the current standards and protein particles are also reflected in the MFI images (Table 5-1). The results demonstrate that current standards do not represent protein particles adequately and justify a material screening in order to identify better materials for novel standardized protein-like particles.



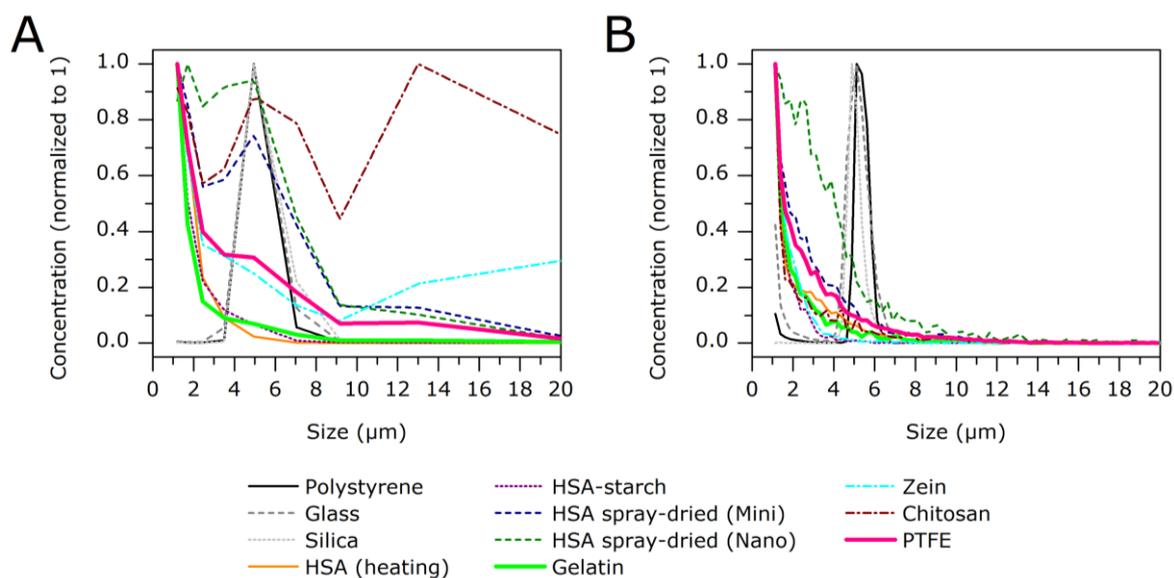
**Figure 5-3: Histogram of (A) aspect ratio of all particles and (B) intensity minimum of particles in the size range of 2-6  $\mu\text{m}$  for 5  $\mu\text{m}$  particle size standards and HSA particles generated by heat stress determined by MFI.**

**Table 5-1: Representative MFI images of 5  $\mu\text{m}$  particle size standards and protein particles.**

Material	Polystyrene	Glass	Silica	HSA (heating)
Representative MFI images				

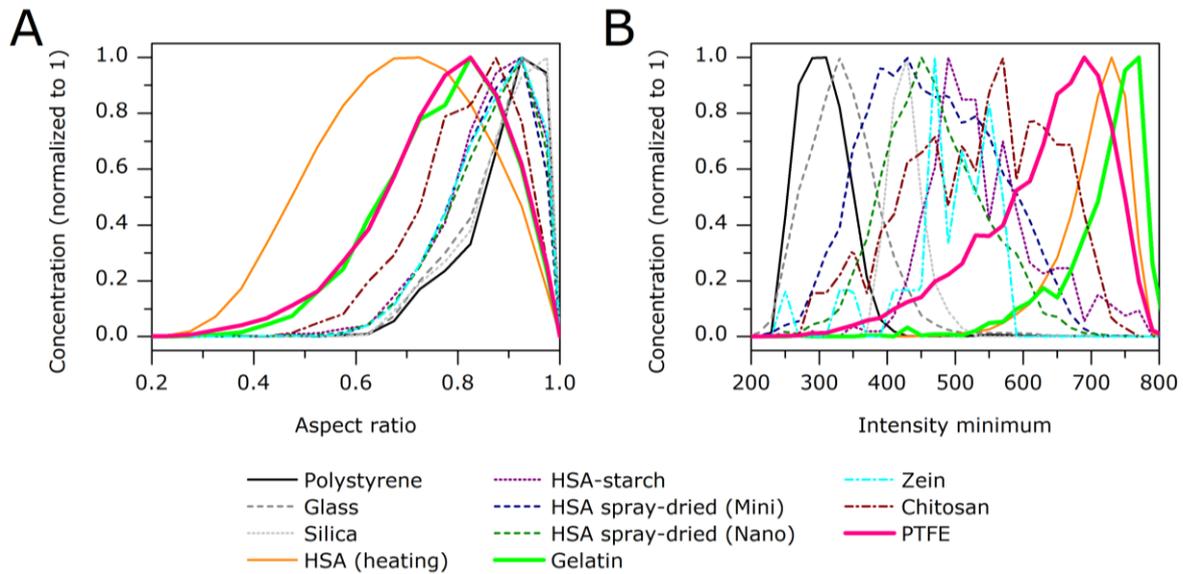
### 3.2 Evaluation of novel materials

HSA starch particles, spray-dried HSA, gelatin particles and raw powder of zein, chitosan, and PTFE were evaluated as candidates for standardized protein-like particles. In LO, only HSA-starch, gelatin, and PTFE particles displayed a similar size distribution as HSA (heating) particles, whereas spray-dried HSA and in particular chitosan and zein particles contained substantial fractions of larger particles (Figure 5-4A). These larger particles were hardly detected by MFI, presumably as they were stuck at the flow cell inlet. Overall, in MFI the size distributions of the different materials appeared rather similar (Figure 5-4B).



**Figure 5-4: Differential size distribution (normalized) of 5 μm particle size standards and evaluated materials determined by (A) LO and (B) MFI.**

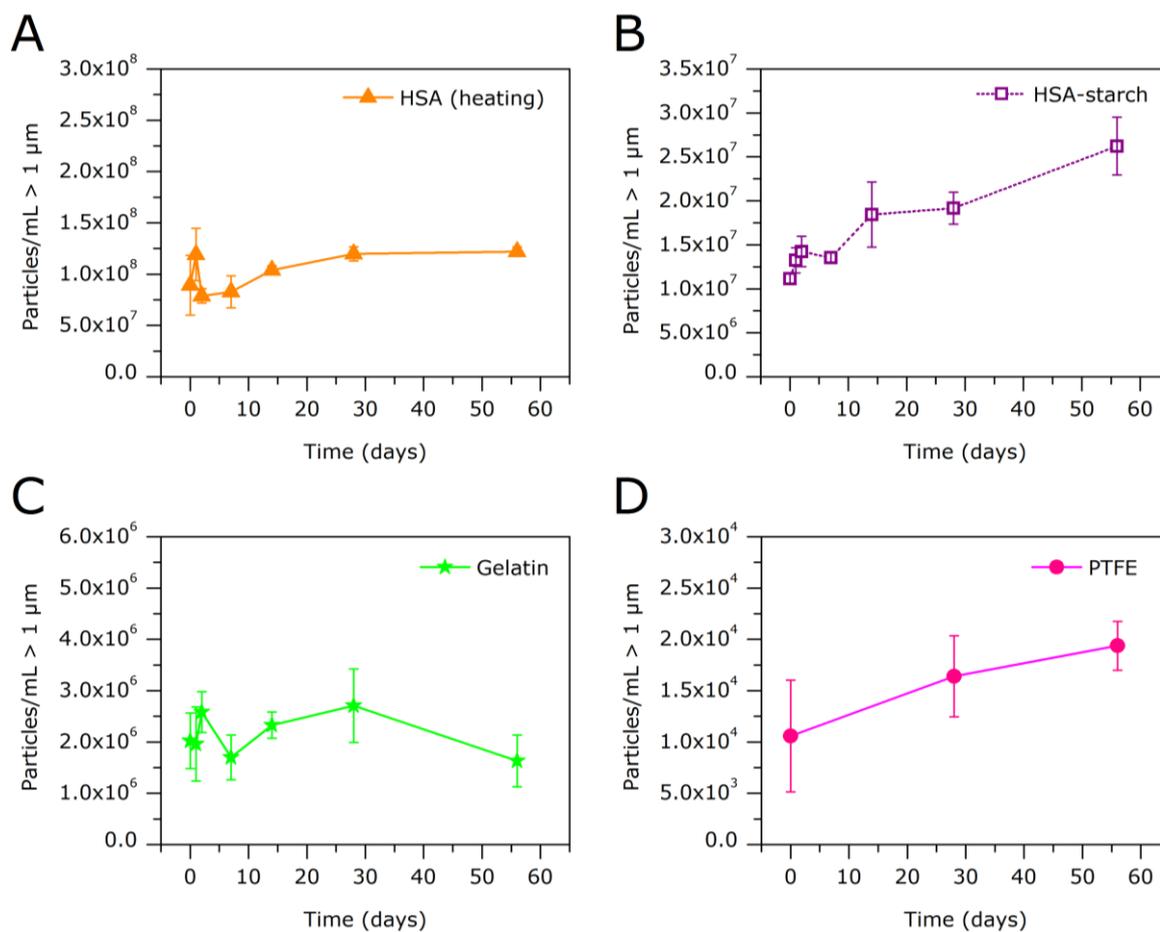
With aspect ratios between 0.4 and 0.9 all evaluated particles appeared more spherical than HSA (heating) particles, but less spherical than the current particle standards (Figure 5-5A). Gelatin and PTFE particles showed higher similarity to HSA (heating) particles as compared to the other materials. The transparency as a crucial parameter was only matched by gelatin and PTFE particles whereas all other materials were clearly less transparent (Figure 5-5B). Spray-dried HSA particles were even more similar to the particle standards than to HSA (heating) particles. HSA-starch, chitosan, and zein particles showed a broad distribution in transparency with most particles in medium transparency region around 500.



**Figure 5-5: Histogram of (A) aspect ratio of all particles and (B) intensity minimum of particles in the size range of 2-6  $\mu\text{m}$  for 5  $\mu\text{m}$  particle size standards and evaluated materials determined by MFI.**

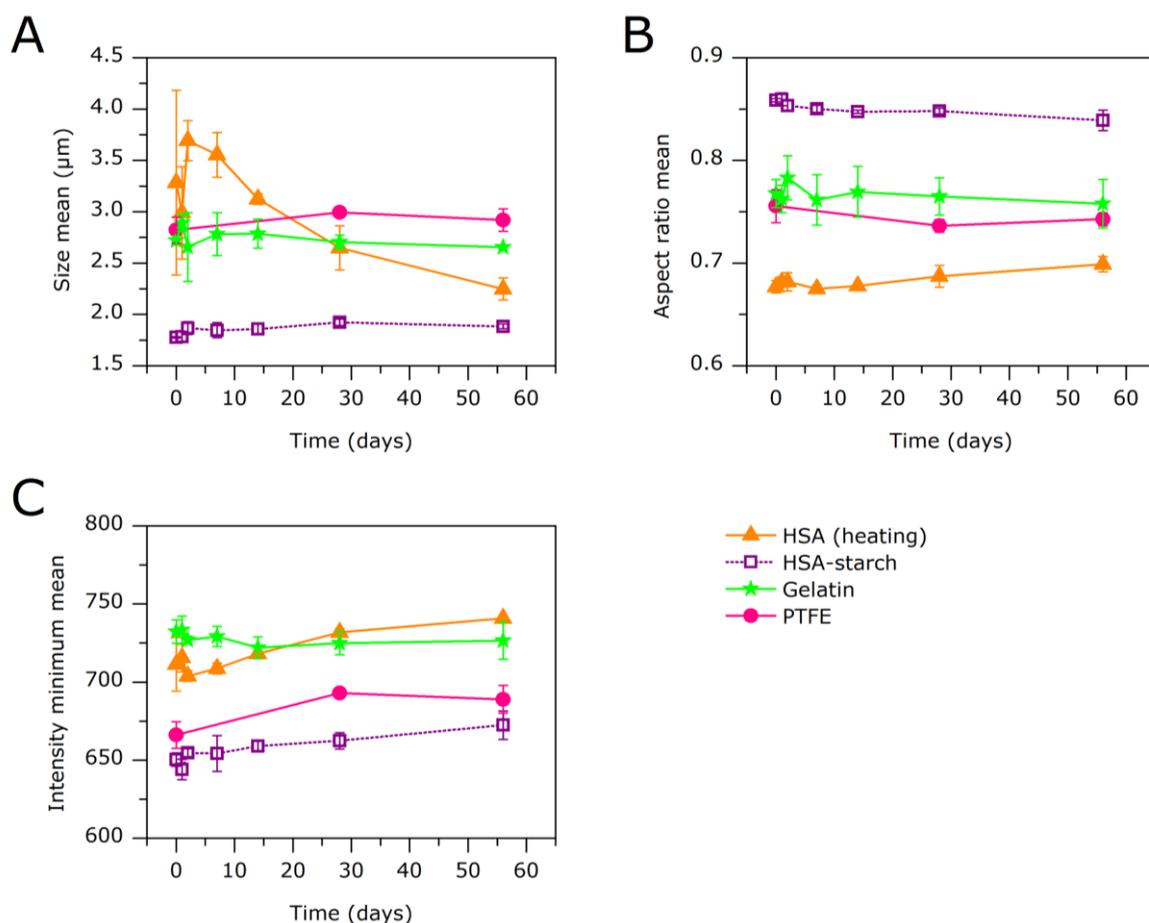
The comparison of all evaluated parameters renders HSA starch, gelatin, and PTFE particles as most similar to HSA (heating) particles (Table 5-2). Of those three, only gelatin and PTFE particles displayed an aspect ratio mean over all particles below 0.8.





**Figure 5-6: Particle concentration over time as determined by MFI for (A) HSA particles generated by heat stress, (B) HSA-starch particles, (C) gelatin particles, and (D) PTFE particles. Error bars represent standard deviations from triplicate samples.**

The limited stability of HSA (heating) particles became obvious in the strong decrease in mean particle size in contrast to consistent values for HSA-starch, gelatin, and PTFE particles over storage time (Figure 5-7A). The aspect ratio varied only slightly for all materials (Figure 5-7B) and the intensity minimum mean increased slightly for all materials except gelatin particles (Figure 5-7C). Taken together, gelatin particles possessed the most constant particle properties in this stability study followed by PTFE particles. HSA-starch particles were not considered suitable due to the clear increase in concentration pointing towards particle instability.



**Figure 5-7: Particle properties (A) size mean, (B) aspect ratio mean, and (C) intensity minimum mean over time. Error bars represent standard deviations from triplicate samples.**

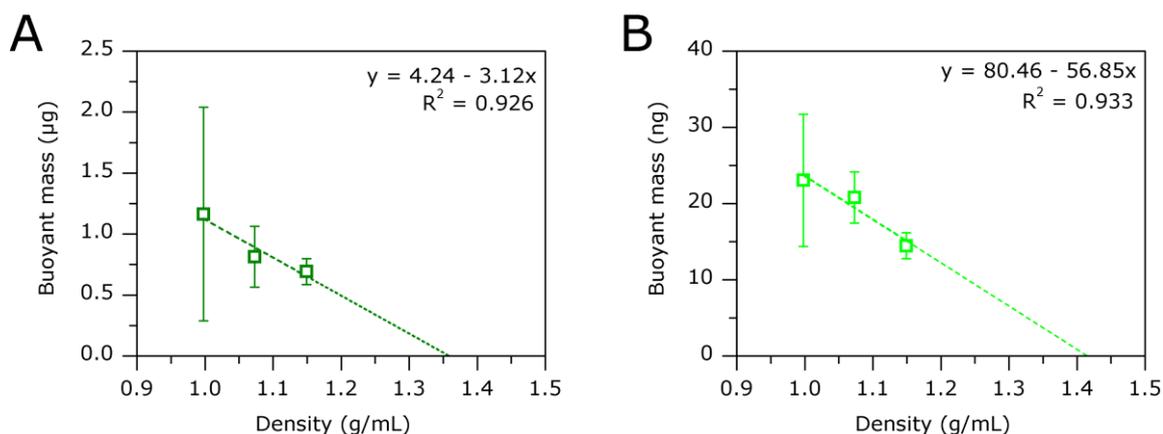
### 3.3 Investigation of protein particle density

In addition to particle size and optical properties evaluated above, the particle density is an important criterion for particle analysis which is not yet well understood:<sup>6</sup> On the one hand, a high density difference between medium and particles could influence the result by particle sedimentation or floating during the measurement. On the other hand, the non-optical technique of RMM requires knowledge of the particle density for correct size calculation. Thus, the density of protein particles is a critical property for the development of standardized protein-like particles, especially for RMM. In general, protein particles are irregularly structured and may contain substantial amounts of liquid between the solid protein parts.<sup>6</sup> Therefore, two different types of density can be defined: (i) the density of only the protein part and (ii) the apparent mean density of the

complete particle including protein parts and entrapped liquid. Accordingly, methods to determine both types of density were developed in this study.

The density of only the protein part was determined based on the RMM principle.<sup>10,15</sup> The buoyant mass measured by the system is converted to the particle size based on the density difference between particle and surrounding liquid. This means that if the system is not able to detect particles, the two densities must be identical making the particles "weightless". However, an exact density match is difficult to achieve due to density variation of the particles and a viscosity limit of the system which constraints the addition of excipients for density adjustment. Alternatively, the particles can be analyzed in solutions of increasing density thereby decreasing the buoyant mass. From this data, the density at which the particles would theoretically become "weightless" can be extrapolated. As only the solid part of a particle contributes to its buoyant mass (the liquid within the particle has the same density as the surrounding liquid), only the density of the solid part is the decisive factor. This principle was used to determine the pure protein density of rituximab particles generated by heat stress and gelatin particles prepared by a two-step desolvation method.

Rituximab particles and gelatin particles were analyzed in aqueous buffer without or with 20% and 40% sucrose. Due to the broad size distribution of the particles, the sum of the buoyant mass of all particles was used for the calculation rather than the mean buoyant mass. The sum of the buoyant mass in the respective solutions was extrapolated to a buoyant mass of zero indicating a density match of particles and surrounding liquid (Figure 5-8). This method provided a density of about 1.36 g/mL and 1.42 g/mL for the solid part of rituximab (Figure 5-8A) and gelatin particles (Figure 5-8B), respectively. The results are in the range of calculated values of 1.38-1.39 g/mL for a 150 kDa antibody<sup>16</sup> and 1.39-1.42 g/mL<sup>16</sup> or 1.41-1.44 g/mL<sup>17</sup> for a 40-50 kDa protein like gelatin, based on van der Waals radii and hydrodynamic volume of atomic groups (based on crystal structures of small molecules with the same atomic groups).

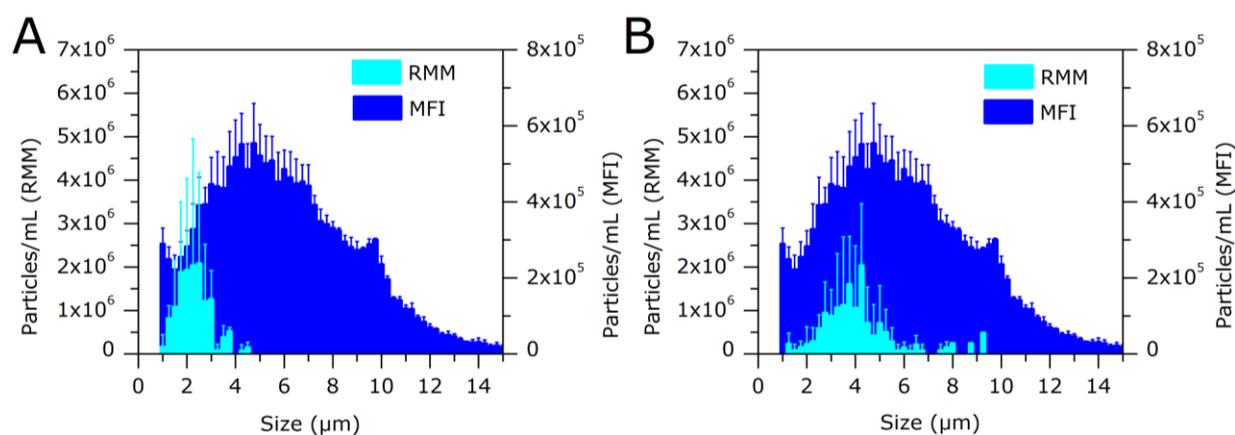


**Figure 5-8: Sum of the buoyant mass over all particles determined in sucrose solutions of different density and extrapolated to the density match of particles and solution for (A) rituximab particles generated by heat stress and (B) gelatin particles. Error bars represent standard deviations from triplicate measurements.**

The second type of particle density, the density of protein particles including entrapped liquid, is important for correct size determination by RMM and cross-correlation of data obtained from RMM and optical techniques. MFI and LO as light-based techniques do not consider the particle density in the size calculation. Instead, the border of the particle's image (MFI) or shadow (LO) including both solid parts and liquid parts is defined as the border of the particle. The particle size is then calculated as the equivalent circular diameter, i.e. the diameter of a circle with the same area as the particle. Thus the same particle size can only be detected by MFI/LO and RMM if the density of protein particles including liquid parts is used for the calculation in RMM. When adjusting the density value in the RMM software until the same particle size is detected in MFI/LO and RMM, i.e. the size distributions overlap, this very density reflects the density of the protein particles including liquid parts. This method requires a clear maximum in the size distribution as a reference point.

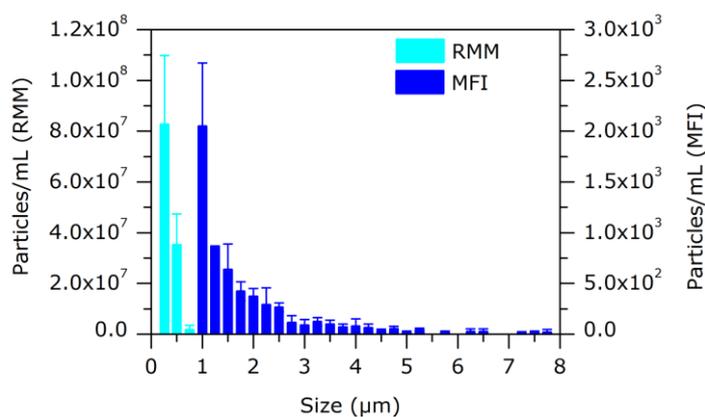
Rituximab particles generated by heat stress showed an interesting size distribution in MFI and RMM with a clear maximum (Figure 5-9) which is not typical for protein.<sup>4,14,18,19</sup> Usually small particles represent the largest fraction in a protein particle size distribution as seen for example for HSA particles generated by heat stress (Figure 5-1) or other therapeutic proteins (see also Chapter 2, Chapter 3, Chapter 4, and Chapter 6). In RMM, the maximum for

rituximab particles was located at about 2.5  $\mu\text{m}$  with an input density of 1.37 g/mL as used in another study for RMM<sup>10</sup> (Figure 5-9A). In this case, the particle size represents the size of a protein particle without liquid parts. In MFI, the maximum was located at about 4.5  $\mu\text{m}$  which displays the particle size including liquid parts (Figure 5-9A). The input density for size calculation in RMM was then adjusted until the size distributions from both techniques overlapped (assessed by the location of the maximum, Figure 5-9B). This suggested a particle density including liquid parts of 1.07 g/mL  $\pm$  0.05 g/mL and indicated that a protein particle could consist of approx. 70-95% of aqueous liquid and only 5-30% of protein (calculated with 1.02 g/mL as the lowest possible and 1.12 g/mL as the highest possible density including liquid parts, 1.0 g/mL for aqueous liquid and 1.36 g/mL for pure protein as determined above).



**Figure 5-9: Differential size distribution of rituximab particles generated by heat stress determined by MFI and RMM using an input density of (A) 1.37 g/mL and (B) 1.07 g/mL for RMM. Error bars represent standard deviations from triplicate measurements.**

For gelatin particles, density determination by this method was not possible due to a size distribution without a clear maximum of the size distribution in the  $\mu\text{m}$  range (Figure 5-10).



**Figure 5-10: Differential size distribution of gelatin particles determined by MFI and RMM using an input density of 1.37 g/mL. Error bars represent standard deviations from triplicate measurements.**

Thus, particle density is important for the development of standardized protein-like particles to be used not only in light-based methods. The density of polystyrene standards (1.05 g/mL) is very close to the density of protein particles including entrapped liquid whereas glass and silica standards show clearly higher density values (2.5 g/mL and 1.8-2.0 g/mL). Thus, polystyrene standards can be considered suitable for RMM if an input density of 1.07 g/mL is used. If the density of pure protein of around 1.4 g/mL (calculated or determined) is used, gelatin particles might be better as they showed a similar density of pure protein (calculated and determined).

## 4 Conclusion

This study identified gelatin particles, prepared by a two-step desolvation method with subsequent cross-linking by glutaraldehyde, and PTFE particles (raw material powder), as optically similar to particles of therapeutic proteins. Especially the particle properties shape and transparency were represented well – in contrast to polystyrene standards. This justifies the further investigation of gelatin and PTFE particles as promising candidates for the development of standardized protein-like particles especially for light-based techniques (see Chapter 6 for an application of PTFE particles). Two methods for determination of protein particle density based on RMM were developed in this study. They revealed a density of pure protein of around 1.4 g/mL, which was congruent with theoretical values, and furthermore a density of protein particles with entrapped liquid of 1.07 g/mL. Taken together, both optical properties and density are crucial for the development of novel standardized protein-like particles. PTFE particles showed useful properties especially for light-based techniques whereas gelatin particles might be suitable for both light-based and weight-based methods.

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# Chapter 6

## How subvisible particles become invisible – relevance of the refractive index for protein particle analysis

### **Abstract**

The aim of the present study was to quantitatively assess the relevance of transparency and refractive index on protein particle analysis by the light-based techniques light obscuration (LO) and Micro-Flow Imaging (MFI). A novel method for determining the refractive index of protein particles was developed and provided a refractive index of 1.41 for protein particles from two different proteins. An increased refractive index of the formulation by high protein concentration and/or sugars at pharmaceutically relevant levels was shown to lead to a significant underestimation of the subvisible particle concentration determined by LO and MFI. A refractive index match even caused particles to become “invisible” for the system, i.e. not detectable anymore by LO and MFI. To determine the influence of formulation refractive index on particle measurements, we suggest the use of polytetrafluoroethylene (PTFE) particles to test a specific formulation for refractive index effects. In case of refractive index influences, we recommend also using a light-independent technique such as resonant mass measurement (Archimedes) for subvisible particle analysis in protein formulations.

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# 1 Introduction

Protein aggregates and particles are an important instability product in therapeutic protein formulations, which need to be quantified and characterized due to quality requirements, potential loss of activity and the potential risk of immunogenicity.<sup>1-4</sup> For many years pharmacopeias have required the analysis of subvisible particles, i.e. particles below 100  $\mu\text{m}$ , also designated as micron aggregates,<sup>5</sup> in size classes larger than 10  $\mu\text{m}$  and 25  $\mu\text{m}$ .<sup>6,7</sup> However, in the last few years, there has been a trend to monitor particles in the size range below 10  $\mu\text{m}$ . This trend is due to regulatory interest in particle data for sizes below 10  $\mu\text{m}$  as part of the analytical characterization of a new product and post marketing commitment.<sup>8</sup> This resulted amongst others in the compilation of the new educational chapter USP<1787> which deals with the analysis of subvisible particles, specifically in protein formulations, also below 10  $\mu\text{m}$  and will be available soon.<sup>9</sup>

Light-based techniques like light obscuration (LO) and flow imaging techniques, e.g. Micro-Flow Imaging (MFI), are commonly used for subvisible particle analysis.<sup>10-15</sup> Light obscuration is the current compendial method but both LO and MFI will be included in the new educational chapter USP<1787>.<sup>9</sup> Alternative techniques which do not rely on the interaction of particles with light are electrical sensing zone (ESZ, Coulter counter)<sup>16</sup> or resonant mass measurements (RMM, Archimedes).<sup>17</sup> However, ESZ requires large sample volumes and sufficient buffer conductivity which is often not feasible for protein formulations<sup>14</sup> and experience using RMM is limited.<sup>18,19</sup> Particle techniques are generally calibrated with polystyrene particle standards which have optical and morphological properties clearly different from those of protein particles.<sup>12</sup> Thus, researchers in the field of particle analysis from industry, regulatory agencies, and academia have emphasized the need of "proteinaceous subvisible particle standards",<sup>20</sup> "alternative particle standards with more protein-like morphology",<sup>10</sup> or "relevant protein particulate standards".<sup>1</sup>

One of the major differences between polystyrene particle standards and protein particles is the transparency<sup>12,21-23</sup> which is in turn connected to the refractive index (RI) of the particles.<sup>24</sup> The RI is a dimensionless unit which describes the

refraction of light by a specific material.<sup>25</sup> However, the RI of protein particles has not been determined so far and is only estimated to be in the range from 1.33 to 1.4<sup>12</sup> or 1.4 to 1.6.<sup>11</sup> The influence of the RI on light-based techniques for particle analysis has been qualitatively studied by analyzing glass particle standards in ethylene glycol<sup>12</sup> and protein particles in highly-concentrated protein solutions.<sup>11</sup> Consequently, there is a need for methods for RI determination of protein particles<sup>24,26</sup> as well as for the quantitative evaluation of RI effects on protein particle analysis.

Our aim was to quantitatively assess the relevance of the optical properties transparency and RI for protein particle analysis. Therefore, we set out to develop a method for RI determination of protein particles based on the immersion principle (minimized light scattering and maximized light transmission at the RI match).<sup>27</sup> The influence of the RI difference of particles and surrounding formulation on the measured particle concentration and size by LO and MFI was investigated at pharmaceutically relevant test conditions and in marketed pharmaceutical products.

## 2 Materials and methods

### 2.1 Materials

Infliximab (Remicade<sup>®</sup>, lots no. 7GD9301402, 7FD8701601, 7RMKA81402, pooled), rituximab (MabThera<sup>®</sup>, lot no. B6073, exp. 12/2013), adalimumab (Humira<sup>®</sup>, lot no. 292209A05, exp. 10/2006) and etanercept (Enbrel<sup>®</sup>, lot no. 31576, exp. 12/2008) were provided by local hospitals. Infliximab solution (IgG A) at a concentration of 1 mg/mL was prepared by dilution of 10 mg/mL infliximab in 100 mM phosphate buffer (pH 7.2). Rituximab solution (IgG B) at a concentration of 1 mg/mL was prepared by dilution of 10 mg/mL rituximab in 25 mM citrate buffer (pH 6.5) containing 154 mM NaCl and 0.07% polysorbate 80. Adalimumab solution at a concentration of 5 mg/mL was prepared by dilution of 50 mg/mL adalimumab in 15 mM phosphate/citrate buffer (pH 5.2) containing 105 mM NaCl, 1.2% mannitol and 0.1% polysorbate 80. Etanercept solutions at concentrations of 1, 2, and 5 mg/mL were prepared by dilution of 50 mg/mL etanercept in 25 mM phosphate buffer (pH 6.3) containing 100 mM NaCl, 25 mM arginine hydrochloride and 1% sucrose. Human serum albumin (HSA) was purchased from Sigma-Aldrich (Steinheim, Germany) and formulated at 1 mg/mL in a 50 mM citrate buffer (pH 4.8). All protein formulations were filtered using a 0.2  $\mu$ m cellulose acetate syringe filter (Minisart<sup>®</sup>, Sartorius Stedim Biotech, Aubagne, France) for further use.

Particles were generated by (i) stir stress, (ii) freeze-thaw stress or (iii) heat stress. For (i), 8 mL of the formulation was stirred in a 10R glass vial with a 18 mm Teflon<sup>®</sup>-coated stir bar at 250 rpm at room temperature on a magnetic stirrer (Heidolph MR Hei-Standard, Schwabach, Germany) for 24 hours (IgG A (stirring)). For (ii), 1 mL of the formulation in a 1.5 mL low protein binding reaction tube (Eppendorf, Hamburg, Germany) was subjected to 7 freeze-thawing cycles of 30 minutes in a -80  $^{\circ}$ C freezer and 10 minutes in a 25  $^{\circ}$ C water bath (IgG A (freeze-thawing)). For (iii), 0.5 mL of the formulation was heated for 30 minutes at 60  $^{\circ}$ C (IgG A (heating)) or 1.5 mL of the formulation was heated for 30 minutes at 71  $^{\circ}$ C (IgG B (heating)) in a 1.5 mL low protein binding reaction tube in a thermomixer (Eppendorf, Hamburg, Germany) or 50 mL of the

formulation was heated in a 50 mL tube (Greiner bio-one, Frickenhausen, Germany) for 30 minutes at 70 °C in a water bath (HSA (heating)).

Polystyrene and glass particle standards were purchased from Duke Scientific (through Thermo Scientific, Fremont, CA), silica particle standards from Microparticles GmbH (Berlin, Germany) and PTFE microparticles (Microdispers 8000) from Polysciences Inc. (Warrington, PA). Polystyrene, glass, and silica particle standards were suspended in water. PTFE particles were suspended at a concentration of 50 mg/mL in 0.5% polysorbate 80 using an Ultra Turrax dispersing system (T10 basic, IKA® Werke, Staufen, Germany) for 3 minutes. Larger agglomerates were removed by filtration through a coarse tea filter (dm, Karlsruhe, Germany).

Sucrose solutions were prepared by dilution (w/w) of a 70% (w/w) solution (prepared by dissolving sucrose in water under stirring and heating to 60 °C in a closed container). All solutions were filtered using a 0.2 µm cellulose acetate syringe filter and air bubbles were removed by centrifugation for 5 minutes at 10,400 rpm (Centrifuge 5810R, Eppendorf, Hamburg, Germany) prior to use.

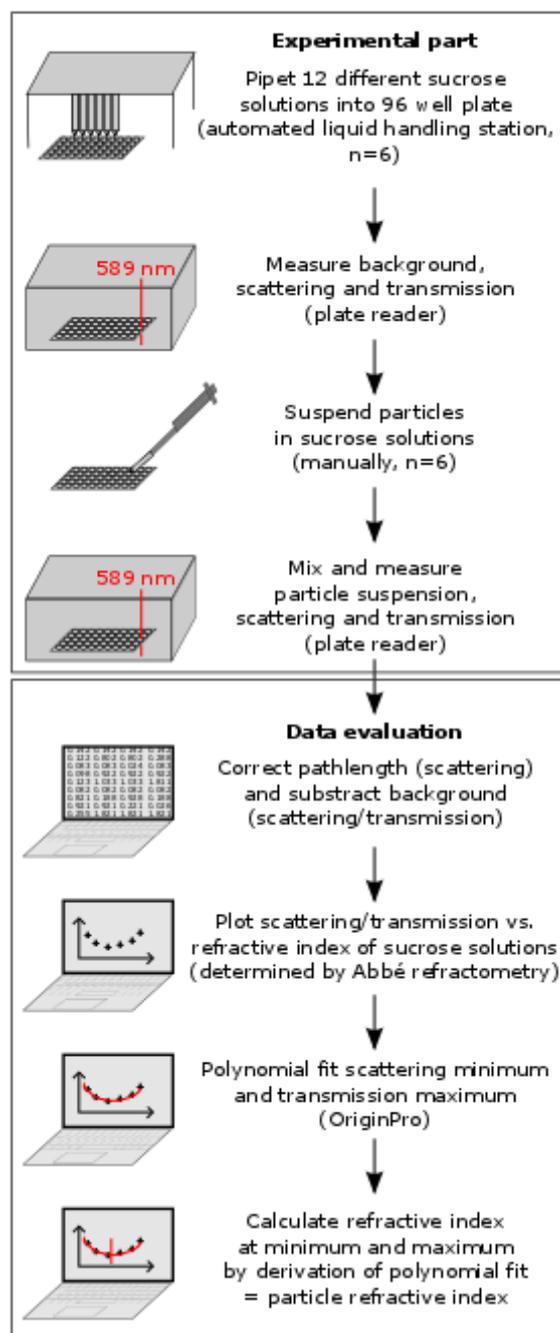
Sucrose, citric acid monohydrate, sodium hydroxide, di-sodium hydrogenphosphate dihydrate and sodium dihydrogenphosphate dihydrate were purchased from Merck KGaA (Darmstadt, Germany). Sodium chloride, sodium citrate dihydrate and polysorbate 80 were from VWR (Darmstadt, Germany). The water used in this study was highly purified water (Advantage A10 purification system, Millipore, Newark, NJ).

## 2.2 Refractive index determination

Refractive indices of sucrose and HSA solutions as well as Humira® and Enbrel® formulations were determined using an Abbé refractometer (Carl Zeiss AG, Oberkochen, Germany). Measurements were performed in triplicates at a wavelength of 589 nm at room temperature.

For particle RI determination, the protein particle suspensions were concentrated by centrifugation and resuspension of the pellet for 5 minutes at 10,400 rpm to a final concentration between  $1 \times 10^8$  particles/mL and  $5 \times 10^8$  particles/mL > 1 µm as

controlled by LO (corresponding to a minimal protein concentration of about 70 µg/mL within the particles based on a minimum particle size of 1 µm and a density of protein particles of 1.32 g/mL). An overview of the procedure for particle RI determination, based on immersion, is given in Figure 6-1. Twelve different sucrose solutions, in a concentration range depending on the expected RI of the particles, e.g. from 5% to 60% in 5% steps, were pipetted into a 96 well plate (Corning Inc., Corning, NY) by an automated liquid handling station (Microlab Star<sup>®</sup>, Hamilton Robotics, Reno, NV) in surface dispense mode (n=6, 190 µL per well with parameters optimized for highly viscous solutions). A background measurement of the sucrose solutions was performed using a Safire<sup>2</sup> plate reader (Tecan Group AG, Männedorf, Switzerland) with optimized measurement parameters. Light scattered by the sample was determined in "absorbance mode" (= scattering) and light transmitted through the sample was determined in "fluorescence mode" (= transmission), both at a wavelength of 589 nm. A pathlength correction was performed for the absorbance mode to account for the varying pathlength due to the different viscosity of the sucrose solutions according to the manufacturer's recommendation.<sup>28</sup>



**Figure 6-1: Schematic overview of the developed method for RI determination of (protein) particles based on the immersion principle.**

For particle measurements, 10  $\mu\text{L}$  concentrated particle suspension were added manually to each well (total volume 200  $\mu\text{L}$ ) and scattering and transmission were determined as described before. Mean and standard deviation for each sixuplicate were calculated, outliers were excluded from further calculation (Grubbs test,  $\alpha = 0.05$ ) and means of scattering and transmission were plotted

against the RI of the sucrose solutions (determined by Abbé refractometry). Scattering minimum and transmission maximum (= RI match points between sucrose solution and particles) were determined by a polynomial fit (OriginPro software, version 8.5) and the particle RI was calculated as the mean from three independent experiments.

### 2.3 Light obscuration (LO)

Subvisible particles in a size range between 1 and 200  $\mu\text{m}$  were analyzed by LO using a PAMAS SVSS-C (Partikelmess- und Analysesysteme GmbH, Rutesheim, Germany) equipped with an HCB-LD-25/25 sensor. Particle suspensions were diluted with the appropriate buffer (filtered by a 0.22  $\mu\text{m}$  cellulose acetate/nitrate membrane filter, MF-Millipore<sup>®</sup>, Millipore) or water in order to adhere to the concentration limit of the system of 120,000 particles/mL > 1  $\mu\text{m}$ . Three measurements of a volume of 0.3 mL of each sample were performed with a pre-run volume of 0.5 mL at a fixed fill rate, emptying rate and rinse rate of 10 mL/min and the mean particle concentration per mL was reported by the system. Samples were measured in triplicates and mean and standard deviation were calculated.

### 2.4 Micro-Flow Imaging (MFI)

Subvisible particles in a size range between 1 and 70  $\mu\text{m}$  were analyzed by MFI using a MFI4100 (ProteinSimple, Santa Clara, CA) equipped with a high-resolution 100  $\mu\text{m}$  flow cell. Particle suspensions were diluted with the appropriate buffer (filtered by a 0.22  $\mu\text{m}$  cellulose acetate/nitrate membrane filter) or water with the same dilution factor as for LO. Samples were analyzed with a sample volume of 0.65 mL and a pre-run volume of 0.3 mL at a flow rate of 0.1 mL/min. Prior to each sample run the respective diluting agent was flushed through the system to provide a clean flow cell. The “optimize illumination” procedure was performed with an appropriate diluent of the respective sample, e.g. formulation buffer, to ensure correct thresholding of the MFI system. Particles stuck to the flow cell wall were only counted once and edge particles were excluded for analysis. Samples were measured in triplicates and mean and standard deviation were calculated. Results were analyzed using the MFI view application software version (MVAS) 1.2. For samples in prefilled syringes,















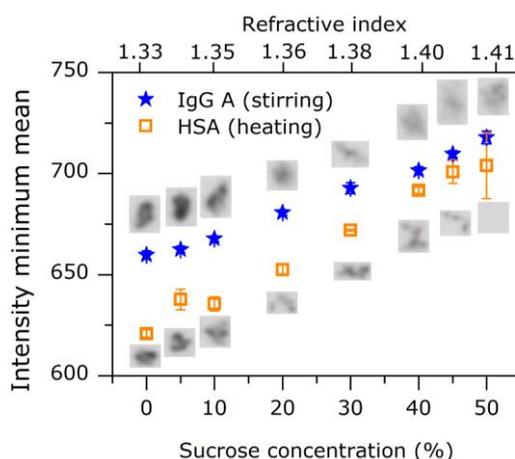






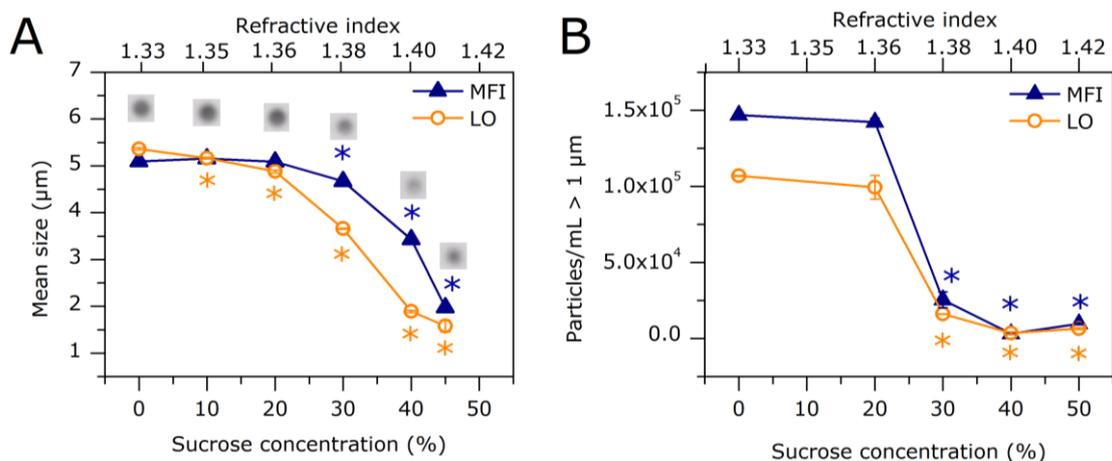
different measurement principle and set-up including a lower wavelength LED (470 nm) in MFI compared to a higher wavelength laser (670 nm) in LO; and (2) the “optimize illumination” process in MFI which allows the system to set the sensitivity according to the optical properties of the respective liquid.<sup>12,23</sup> Strikingly, again, in both techniques, protein particles became “invisible” at the RI match of 1.41 similar to silica particles.

The transparency of protein particles shown as the intensity minimum (mean of the complete particle population of 2-6  $\mu\text{m}$ ) generated from MFI images increased linearly ( $R^2 > 0.99$ ) with increasing sucrose concentration (Figure 6-6). This can also be seen in the MFI images (Figure 6-6) and explains the decrease in MFI particle counts with increasing RI of the formulation. In LO, “shadows” of the particles are projected on a light-sensitive area at the detector and the particle size is calculated proportional to the area of the shadow. Therefore, it can be assumed that increased light transmission caused by decreased RI differences, as described above in the context of the RI determination method, led to the decrease in LO counts. Similarly to the RI determination method it can be expected that particles “reappear” when the RI of the formulation is further increased exceeding the RI match point. However, those experiments were not performed due to the viscosity limit for LO and MFI.



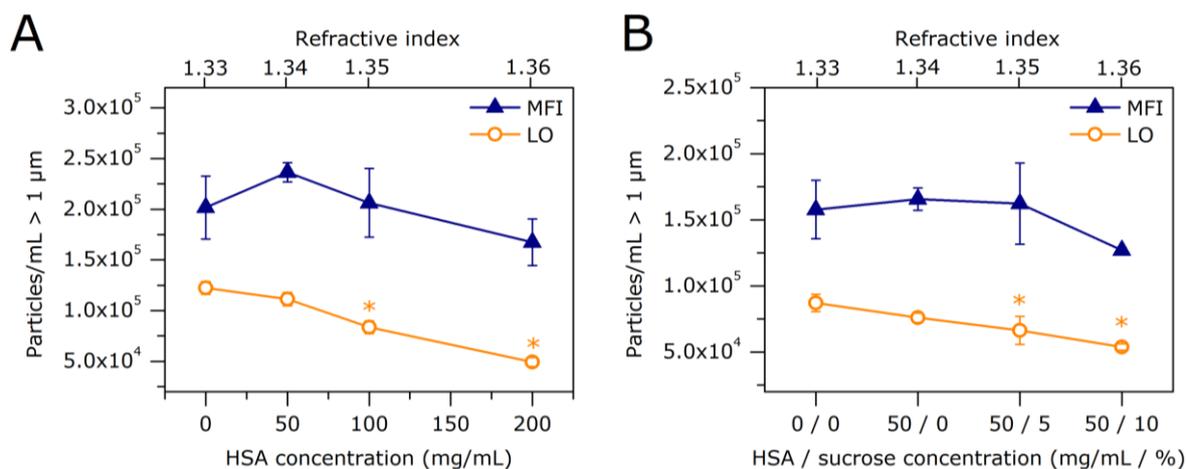
**Figure 6-6: Increase in intensity minimum values from MFI (shown as the mean of the complete particle population of 2-6  $\mu\text{m}$ ) depending on the sucrose concentration of the solution and representative images of 10  $\mu\text{m}$  particles from MFI. The additional x-axis shows the RI of the respective solutions. Error bars represent standard deviations from triplicate samples (dilutions were prepared in triplicates).**

We propose the following mechanism for the phenomenon of subvisible particles becoming “invisible”: With increasing RI of the formulation, the particles get more difficult to detect by light-based techniques as the particle contour becomes blurred (also observed in MFI images of 5  $\mu\text{m}$  silica particles, Figure 6-7A). For particles of uniform size and shape, this leads to a constant decrease in the apparent particle size for both LO and MFI as observed for 5  $\mu\text{m}$  silica particle standards (Figure 6-7A). Nevertheless, in the case of 5  $\mu\text{m}$  silica particle standards, the particles are still counted accurately as the decrease in size does not reach the detection limit of the systems until 40% sucrose with LO and 45% sucrose with MFI. Those particles only become invisible at the RI match. In the case of 2  $\mu\text{m}$  silica particle standards, the particles become “invisible” at lower RI as the particle size decreases below the detection limit before the RI of particles and formulation match (Figure 6-7B). In the case of protein particles, particles become partly “invisible” at lower sucrose concentrations due to the polydisperse size distribution and the presence of smaller particles (Figure 6-2). These small particles “shrink” below the detection limit of the systems already at only slightly increased RI values, whereas larger particles are still detected and only become “invisible” when the refractive indices match. Additionally, the stronger RI influence on protein particles compared to silica particles is probably also due to other factors such as the irregular shape and surface structure, higher surface roughness and the lower compactness of protein particles which hamper the detection by light-based systems.<sup>21</sup>



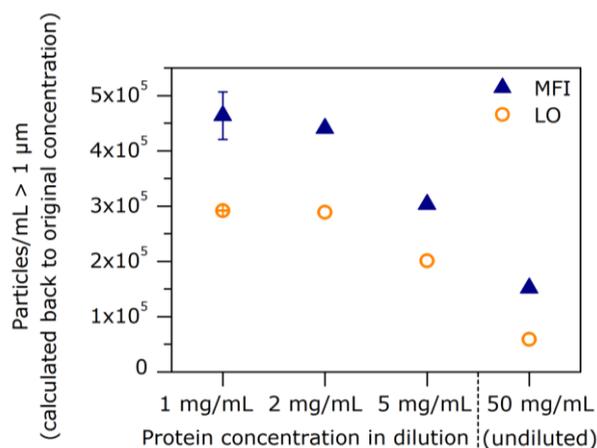
**Figure 6-7: (A) Mean particle size of 5 μm silica particle standards determined by LO and MFI in sucrose solutions of varying concentration. Stars (\*) indicate data points of significantly smaller particle size compared to the initial size (ANOVA,  $p < 0.05$ ). Representative images from MFI show the softening contours of the imaged particles with increasing RI. (B) Particle counts of 2 μm silica particle standards determined by LO and MFI for a fixed particle concentration in sucrose solutions of varying concentration. Stars (\*) indicate data points differing significantly from the initial concentration (ANOVA,  $p < 0.05$ ). The additional x-axis shows the RI of the respective solutions.**

Another formulation parameter that can affect RI is protein concentration. Pharmaceutically relevant protein concentrations cover a range from below 1 mg/mL up to about 200 mg/mL or even higher. Thus, the influence of RI on protein particle analysis was further evaluated with high protein concentration. Significant differences in the concentration of HSA particles (heating) were observed by LO at 100 mg/mL HSA concentration (Figure 6-8A) or 50 mg/mL HSA combined with 5% sucrose (Figure 6-8B). Both conditions represent common formulation conditions.



**Figure 6-8: Particle counts of HSA particles (heating) at a fixed concentration determined by LO and MFI in formulations of (A) HSA and (B) HSA and sucrose. The additional x-axis shows the RI of the respective solutions. Stars (\*) indicate data points differing significantly from the initial concentration (ANOVA,  $p < 0.05$ ). Error bars represent standard deviations from triplicate samples (dilutions were prepared in triplicates).**

A high protein concentration is pharmaceutically relevant especially for subcutaneous administration, for example Simponi<sup>®</sup> (golimumab) and Cimzia<sup>®</sup> (certolizumab) are formulated at 100 mg/mL and 200 mg/mL, respectively.<sup>38,39</sup> Also formulations with lower protein concentration but with excipients increasing the RI are represented by our model solutions such as Humira<sup>®</sup> (50 mg/mL adalimumab and 1.2% mannitol)<sup>40</sup> and Enbrel<sup>®</sup> (50 mg/mL etanercept and 1% sucrose).<sup>41</sup> For both Humira<sup>®</sup> and Enbrel<sup>®</sup> an RI of 1.35 was determined by Abbé refractometry which corresponds to the RI of formulations containing 100 mg/mL HSA or 50 mg/mL HSA with 5% sucrose. For example, the original etanercept formulation was analyzed undiluted and in several dilutions in the formulation buffer by LO and MFI (Figure 6-9). Clearly more particles (calculated back to the original concentration) were detected in diluted formulations of lower protein concentration for both LO and MFI. These results indicate that mainly LO, but also MFI might not detect the real particle load of a sample, but might instead underestimate subvisible particle numbers due to a low RI difference between particles and formulation.



**Figure 6-9: Particle counts in expired prefilled syringes of Enbrel® (etanercept) measured undiluted (50 mg/mL) and diluted in the appropriate formulation buffer by LO and MFI. The error bar for 1 mg/mL represents the standard deviation from a duplicate sample (dilution was prepared in duplicate). Formulations at 2 mg/mL, 5 mg/mL, and 50 mg/mL were analyzed only n=1 due to limited material availability.**

To understand whether the obtained data are prone to error due to detection problems caused by the formulation RI we suggest two possible ways to cope with the influence of high formulation RI when using light-based techniques:

- (i) use of PTFE particles for the evaluation of the “invisible particle effect” in LO,
- (ii) use of alternative (light-independent) measurement principles.

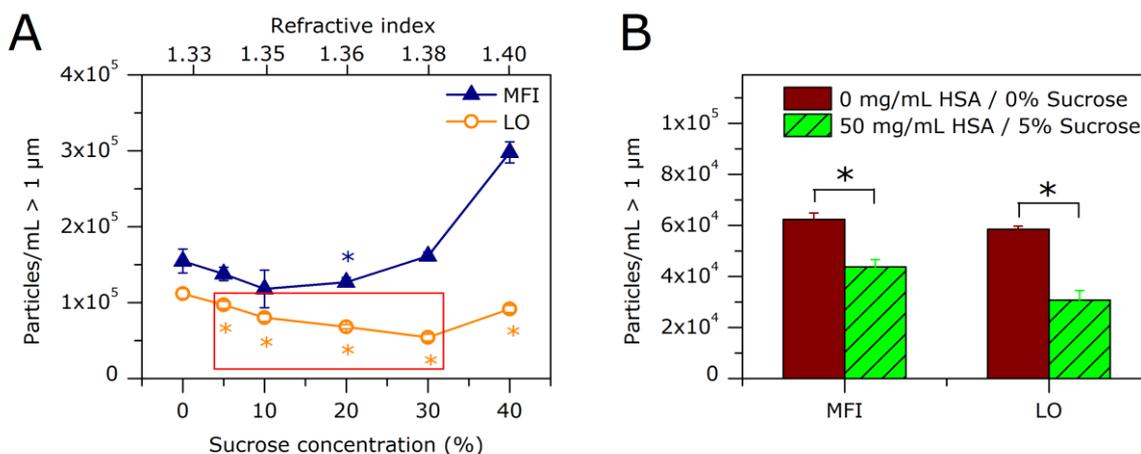
### 3.4 PTFE particles for the evaluation of the “invisible particles effect” in LO

To address the question whether the RI of the formulation potentially influences the outcome of light-based subvisible particle detection methods, the formulation of interest can be tested for the “invisible particles effect” using PTFE particles. These polymeric particles in suspension show similar optical properties as protein particles (Figure 6-3) and can be spiked as a highly-concentrated suspension into the formulation of interest.

As shown in Figure 6-10A, the measured (“apparent”) concentration of PTFE particles by LO and MFI was influenced by the RI of the surrounding formulation. Decreases in PTFE particle concentration by LO were already observed at only 5% sucrose. The relative apparent particle concentrations determined by LO compared to the real concentration in the sample were very similar for protein

particles (e.g. 87%, 82%, and 58% for HSA particles and 97%, 89%, and 60% for IgG particles in 5%, 10%, and 20% sucrose, Figure 6-5C,D) and PTFE particles (88%, 72%, and 61%, Figure 6-10A). The apparent increase of the PTFE particle concentration in LO at 40% sucrose is probably due to the lower and more polydisperse RI of PTFE (RI 1.35-1.38<sup>30,31</sup>) as compared to protein (RI 1.41). As the RI of 40% sucrose (RI 1.40) exceeds the RI of the particles, detection is facilitated when compared to 30% sucrose solution (RI 1.38) and particles "reappear", due to the increase of light scattering after the RI match point (Figure 6-4). With MFI, the PTFE concentration was also affected by the RI of the sucrose solution as particle counts decreased until 10-20% sucrose. The apparent increase in PTFE particle concentration after the RI match was observed at lower sucrose concentrations compared to LO. The measured particle concentration at 40% sucrose was even higher than the initial concentration in water, presumably because of the better optical contrast of PTFE in 40% sucrose as compared to PTFE in water which is due to the low RI of PTFE.

The utility of using PTFE particles was confirmed by LO and MFI analysis of a PTFE particle suspension in a model protein formulation containing 50 mg/mL HSA and 5% sucrose (Figure 6-10B). PTFE particles were even more sensitive towards the "invisible particles effect" than protein particles. The relative apparent particle concentration by LO in the formulation containing 50 mg/mL HSA and 5% sucrose compared to the concentration in water was clearly lower for PTFE particles (53%, Figure 6-10B) as compared to HSA particles (76%, Figure 6-8B). With MFI, the apparent concentration of PTFE particles decreased to 70% in 50 mg/mL HSA and 5% sucrose whereas there was no significant effect for HSA particles. Overall, PTFE particles are recommended to test formulations for the "invisible particles effect" by LO, for an RI range up to 1.38. This range covers protein formulations with protein concentrations up to at least 150 mg/mL IgG (RI 1.37<sup>11</sup>) or 200 mg/mL HSA and 10% sucrose (RI 1.38, own results).



**Figure 6-10: (A) Particle counts of PTFE particles at a fixed concentration determined by LO and MFI in sucrose solutions of varying RI. The red box indicates the range in which PTFE particles could be used to test specific formulations for the “invisible particles effect”. The additional x-axis shows the RI of the respective solutions. Stars (\*) indicate data points differing significantly from the initial concentration (ANOVA,  $p < 0.05$ ). (B) Particle counts of PTFE determined by LO and MFI in a solution containing HSA and sucrose in pharmaceutically relevant concentrations for an application test of PTFE particles as model particles. Stars (\*) indicate significant differences between data points (ANOVA,  $p < 0.05$ ). Error bars represent standard deviations from triplicate samples (dilutions were prepared in triplicates).**

A group from the National Institute of Standards and Technology (NIST) also aimed to develop more proteinaceous particle standards and identified ethylenetetrafluoroethylene (ETFE) as a proper material.<sup>26</sup> This supports the general suitability of fluorinated polymers as protein particle-like materials and encourages searching for potentially even better fitting candidates within this class of materials.

In order to examine the formulation of interest for the “invisible particles effect” by means of PTFE particles, we suggest the following procedure: spike the same amount of a highly-concentrated PTFE particle suspension into water and into the formulation of interest, to achieve a final concentration similar to that expected in the protein formulation to be analyzed. Determine the apparent particle concentration of PTFE particles in both solutions by LO. A significant difference points towards a potential “invisible particles effect” for the analysis of protein particles in the formulation of interest. In this case, we recommend including orthogonal analytical techniques, preferably techniques with light-independent underlying principles such as ESZ or RMM (see below).

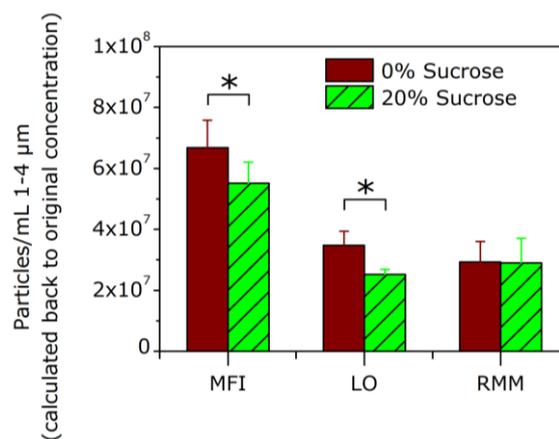
PTFE particles might furthermore serve as a protein particle surrogate material for the comparison of different analytical techniques or instruments. This could help to explain and bridge differing results for particle concentrations obtained from different instruments.

### 3.5 Orthogonal techniques for protein particle analysis to cope with RI influences

Difficulties with subvisible particle analysis due to RI influences can be addressed by using techniques with other underlying measurement principles. An example of a light-independent particle counting technique is the ESZ method (e.g. Coulter counter) which was originally developed for cell counting, but has recently also been applied for the analysis of protein particles.<sup>11,36,42-44</sup> Drawbacks of ESZ are the large required sample volume and that the sample needs to be suspended in a conductive solution if the sample buffer does not have sufficient conductivity.

Another non-optical technique for particle counting and sizing that has recently become commercially available is the Archimedes system. In this technique, the principle of RMM is applied using a suspended microchannel resonator (SMR) or microcantilever, which resonates mechanically and changes its frequency depending on the buoyant mass of particles passing the channel.<sup>17-19</sup> The buoyant mass is converted to absolute mass and then to particle size based on the density of both particle and fluid. We evaluated RMM as an orthogonal technique to LO and MFI for IgG A particles (stirring) in phosphate buffer containing 0% or 20% sucrose and compared apparent particle concentrations in the overlapping size range of 1-4 µm (Figure 6-11). The IgG particle concentration in phosphate buffer determined by RMM was in a similar range as determined by LO. In 20% sucrose, significantly less particles were detected by LO and MFI as compared to phosphate buffer (similar as in Figure 6-5D). In contrast, no significant difference was found for the same conditions by RMM. This emphasizes the suitability of light-independent techniques for the analysis of particles in therapeutic protein formulations and it can be recommended that they be included in an analytical package. This is especially important for formulations containing high protein concentration and/or excipients that

increase the RI of the formulation such as sugars. Otherwise, by applying only light-based methods particle counts in therapeutic protein formulations may be significantly underestimated.



**Figure 6-11: Particle counts of IgG A particles (stirring) at a fixed concentration determined by LO, MFI, and RMM in pure phosphate buffer (0% sucrose) and phosphate buffer containing 20% sucrose in the overlapping size range between the three systems. Results were calculated back to the original concentration as samples were analyzed in different dilutions due to different concentration limits of the systems. Stars (\*) indicate significant differences between data points (ANOVA,  $p < 0.05$ ). Error bars represent standard deviations from triplicate samples (dilutions were prepared in triplicates) for MFI and LO and for a triplicate measurement for RMM.**

## 4 Conclusion

Our study showed that transparency, which is related to the RI of both particles and formulation, is an important parameter for protein particle analysis by light-based techniques. A fast batch method for RI determination of protein particles developed in this study provided an RI of 1.41 for protein particles generated by heat as well as mechanical stress. We envision the use of the method for research purposes in the development phase to get an insight into the RI of the particles of a certain protein and to judge the suitability of light-based methods for detecting subvisible particles suspended in a specific formulation. The RI difference between protein particles and surrounding formulation has a strong influence on the performance of LO and MFI. At pharmaceutically relevant sucrose concentrations (5%) and protein concentrations (100 mg/mL), the particle concentrations were clearly underestimated by LO and MFI. An RI match even caused particles to become “invisible” for the system, i.e. not detectable anymore by LO and MFI. Therefore, increased attention is required in the evaluation of subvisible particle analysis in formulations of high protein concentration and/or sugars. To address the influence of the RI, we recommend two alternatives: (1) Use of PTFE particles as model particles to test specific formulations for an RI influence in LO, because these particles have similar optical properties to protein particles. (2) Include light-independent techniques, e.g. RMM (Archimedes), in the analytical package as RMM was not affected at all by increased RI in the formulation. These procedures should help to avoid significant underestimation of the particle concentration in therapeutic protein formulations due to RI influences; which is critical for both development and release.

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# Chapter 7

## Summary of the thesis

The overall goal of this thesis was to identify and evaluate critical factors for protein particle analysis and to apply this knowledge for the development of novel standardized protein-like particles. Thorough analysis of particles in therapeutic protein formulations is crucial due to regulatory requirements, the potential immunogenicity of protein aggregates and particles, and the need for quality and stability control of the product.

Chapter 1 provides a comprehensive overview of analytical methods for the detection and characterization of particles in therapeutic protein formulations. The extensive portfolio of available methods does not only offer more flexibility and cross-validation of results, but also brings along the difficulty how to handle and to interpret differing results from several analytical techniques or instruments. In this chapter, the underlying theory, output parameters, benefits, shortcomings, and illustrative examples for each technique are described. In this context, the necessity of method evaluation before data analysis is outlined which requires the development of novel more proteinaceous particle standards.

In Chapter 2, novel techniques or instruments (Sysmex FPIA-3000 and Occhio FC200S+ (flow imaging microscopy), Coulter counter Multisizer 4 (electrical sensing zone (ESZ)), Archimedes (resonant mass measurement (RMM)), rapID (image directed Raman spectroscopy)) were evaluated regarding their performance for (protein) particle counting, sizing, or characterization. Results from flow imaging microscopy differed strongly depending on the applied settings and the used system. More established flow imaging techniques such as Micro-Flow Imaging (MFI) or FlowCAM were regarded preferable in this case. The non-optical particle techniques ESZ and RMM presented useful additions to the pool of techniques as they provided good size and count accuracy when compared to the established techniques dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA). Particle identification by rapID proved to be an interesting approach, but with the need for further improvements. Chapter 2 showed that

novel techniques for particle analysis can be useful, but their strengths, weaknesses, and output parameters need to be evaluated thoroughly for the intended application.

A prominent application in the field of particle analysis is the differentiation of protein particles from silicone oil droplets. The latter may especially be introduced into products filled in prefilled syringes which are siliconized for lubrication. Chapter 3 describes this critical differentiation by MFI and RMM for samples of artificially generated silicone oil droplets and protein particles in controllable defined mixtures. MFI was identified as reliable for particles with a size above 2  $\mu\text{m}$  and with moderate droplet/particle ratios (70:30 – 30:70) when using the built-in software algorithm for the identification of similar particle images. The performance could be improved, especially for more extreme ratios (95:5 – 15:85), by a customized filter which was developed specifically for this study based on particle transparency and shape. RMM was considered as highly accurate for particles from 0.5 to 2  $\mu\text{m}$  if the total droplet/particle concentration was in a statistically sufficient range. As a conclusion from this chapter, MFI and RMM should be applied as orthogonal techniques in combination to achieve an accurate and reliable differentiation of protein particles and silicone oil droplets.

Flow imaging microscopy is of increasing importance due to extended particle characterization possibilities beyond sizing and counting. Therefore, regulatory agencies tend to include the technique into the pharmacopeias, in addition to the conventional technique of light obscuration (LO). However, results from flow imaging microscopy may vary substantially depending on the used instrument as observed in Chapter 2. In Chapter 4, four of the pharmaceutically most relevant flow imaging microscopy systems (MFI4100, MFI5200, FlowCAM VS1, and FlowCAM PV) were subjected to a detailed evaluation of particle quantification, characterization, image quality, differentiation of protein particles and silicone oil droplets, and handling of the systems. The FlowCAM systems provided higher image quality and were more flexible with respect to adjustment of settings, whereas the MFI systems appeared more useful for standardized applications. In detail, the FlowCAM VS1 was considered as the best choice for high resolution images, the FlowCAM PV for an accurate quantification and differentiation of protein particles and silicone oil droplets. The MFI systems showed their strength

in size and count accuracy, the MFI5200 was especially suitable for protein particle analysis under impaired optical conditions by an increased refractive index of the formulation. The results from this chapter indicate again that the choice of the appropriate instrument depends strongly on the output parameters of interest.

Based on the knowledge on critical particle properties in different analytical techniques acquired in the previous chapters, a material screening for the development of novel standardized protein-like particles for light-based techniques was performed in Chapter 5. In the screening, proteinaceous (human serum albumin (HSA)-starch particles, spray-dried HSA, gelatin particles, and zein) and non-proteinaceous materials (chitosan and polytetrafluoroethylene (PTFE)) were assessed regarding their optical similarity to particles of therapeutic proteins (represented by HSA particles generated by heat stress). Based on numerous particle properties (size, size distribution, shape, transparency, and stability) gelatin and PTFE particles were considered the most promising materials for light-based applications. The density of protein particles, as a crucial particle parameter for weight-based techniques like RMM, has not been characterized well up to now. Thus, two novel methods based on RMM for density determination of pure protein and protein particles including entrapped liquid were developed. The methods provided a density of about 1.4 g/mL for pure protein (rituximab and gelatin), in congruence with theoretically calculated values of 1.38-1.44 g/mL. For protein particles including entrapped liquid an apparent density of about 1.07 g/mL was obtained for rituximab particles generated by heat stress. This chapter indicated that both gelatin particles and PTFE might be valuable in the development of standardized protein-like particles depending on the application purpose: gelatin particles might be suitable for both light-based and weight-based techniques whereas PTFE particles could be used for light-based techniques.

As many analytical techniques for protein particles are based on the interaction of the particles with light, the particle transparency plays a crucial role for accurate particle quantification on the one hand and the development of suitable standardized protein-like particles on the other hand. Chapter 6 is focused on the relevance of the refractive index (RI), which is closely related to transparency,

for particle analysis. As the RI of protein particles has been unknown until now, a novel method for RI determination of protein particles was developed. This method provided an RI of 1.41 for particles from two different therapeutic proteins (HSA and IgG). The relevance of the RI was then investigated by increasing the RI of the surrounding formulation until particles became “invisible”, i.e. not detectable anymore by light-based systems (in this case LO and MFI) at the RI match. As an increased RI is of practical significance at high protein concentration and/or the use of excipients such as sugars, potential solution strategies were also investigated in this chapter. As a result, PTFE particles, as identified in the material screening in Chapter 5, turned out to be suitable to test a specific formulation for RI effects. Furthermore, light-independent techniques such as RMM can be beneficial in case of RI influences.

Taken together, this study provides new insight into the analysis of particles in therapeutic protein formulations. It illustrates that it is crucial to not only comprehensively understand the techniques’ principle and limitations, but to also evaluate data from different techniques carefully in order to draw reliable conclusions. In this regard, potential candidates for the development of novel standardized protein-like particles identified in this study are very valuable and can help to improve protein particle analysis in the future.

# Publications and presentations associated with this thesis

## Review articles

**S. Zölls**, R. Tantipolphan, M. Wiggenhorn, G. Winter, W. Jiskoot, W. Friess, A. Hawe: "Particles in therapeutic protein formulations, part 1: overview of analytical methods"; J Pharm Sci 101(3):914-935 (2012)

## Book chapters

A. Hawe, **S. Zölls**, A. Freitag, J. F. Carpenter: "Subvisible and visible particle analysis in biopharmaceutical research and development", in Biophysical characterization of proteins in developing biopharmaceuticals, Elsevier, editors: D. Houde, S. Berkowitz (submitted)

## Research articles

**S. Zölls**, M. Gregoritza, R. Tantipolphan, M. Wiggenhorn, G. Winter, W. Friess, A. Hawe: "How subvisible particles become invisible – relevance of the refractive index for protein particle analysis"; J Pharm Sci 102(5):1434-1446 (2013)

D. Weinbuch\*, **S. Zölls\***, M. Wiggenhorn, W. Friess, G. Winter, W. Jiskoot, A. Hawe: "Micro-Flow Imaging and resonant mass measurement (Archimedes) – complementary methods to quantitatively differentiate protein particles and silicone oil droplets"; J Pharm Sci 102(7):2152-2165 (2013); \*joint first authors

**S. Zölls\***, D. Weinbuch\*, M. Wiggenhorn, G. Winter, W. Friess, W. Jiskoot, A. Hawe: "Flow imaging microscopy for protein particle analysis – a comparative evaluation of four different analytical instruments"; The AAPS Journal (accepted); \*joint first authors

## Oral presentations

**S. Zölls**: "Micro-Flow Imaging for protein particles – getting more than just numbers". Protein Simple User Meeting, Basel, CH, July 3-4, 2012

## Poster presentations

**S. Zölls**, D. Weinbuch, M. Wiggendorfer, G. Winter, W. Friess, W. Jiskoot, A. Hawe: "Comparative evaluation of four different flow imaging microscopy instruments for protein particle analysis"; National Biotech Conference, San Diego, CA, May 20-22, 2013

**S. Zölls**, D. Weinbuch, M. Wiggendorfer, G. Winter, W. Friess, W. Jiskoot, A. Hawe: "Micro-Flow Imaging and resonant mass measurement (Archimedes) for the differentiation of silicone oil droplets and protein particles"; PepTalk, Palm Springs, CA, January 21-25, 2013; and National Biotech Conference, San Diego, CA, May 20-22, 2013

**S. Zölls**, D. Weinbuch, W. Friess, A. Hawe: "Differentiation of silicone oil droplets and protein particles by MFI and RMM"; Controlled Release Society (CRS) German Local Chapter Meeting, Ludwigshafen, Germany, March 21-22, 2013

**S. Zölls**, R. Tantipolphan, M. Wiggendorfer, G. Winter, W. Friess, A. Hawe: "Evaluation of Archimedes and Coulter counter for the analysis of (protein) particles"; National Biotech Conference, San Diego, CA, May 21-22, 2012

**S. Zölls**, M. Gregoritzka, R. Tantipolphan, M. Wiggendorfer, G. Winter, W. Friess, A. Hawe: "How subvisible particles get invisible - Relevance of refractive index for protein particle analysis"; PEGS Protein engineering summit, Boston, MA, April 30 – May 3, 2012; and National Biotech Conference, San Diego, CA, May 21-22, 2012

**S. Zölls**, R. Tantipolphan, M. Wiggendorfer, G. Winter, W. Friess, A. Hawe: "Comparative analysis of subvisible particles induced by freeze-thawing, stirring and heating of an IgG antibody"; Colorado Protein Stability Conference, Breckenridge, CO, July 19-21, 2011; and National Biotech Conference, San Francisco, CA, May 16-18, 2011

**S. Mickisch**, R. Tantipolphan, M. Wiggendorfer, W. Friess, G. Winter, A. Hawe: "Subvisible particles in a monoclonal antibody formulation analyzed by nanoparticle tracking analysis and Micro-Flow Imaging"; National Biotech Conference, San Francisco, CA, May 16-19, 2010; and Workshop on protein aggregation and immunogenicity, Breckenridge, CO, July 20-22, 2010

# Curriculum vitae

## SARAH ZÖLLS, GEB. MICKISCH

### PERSONAL DETAILS

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Date of birth: 23.04.1984  
 Place of birth: Starnberg  
 Nationality: German

### EDUCATION

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05/2010 - 06/2013	PhD studies Coriolis Pharma, Martinsried Supervisor: Dr. Andrea Hawe in collaboration with Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics, Ludwig-Maximilians-Universität München Supervisor: Prof. Dr. Wolfgang Frieß
10/2009 - 04/2010	Research project Max Planck Institute of Biochemistry Supervisor: Prof. Dr. F.-Ulrich Hartl
10/2007 - 09/2009	Studies of Pharmaceutical Sciences (M.Sc.) Ludwig-Maximilians-Universität München
09/2007 - 04/2008	Research stay Oncology Research Institute, Greenville, SC, USA Supervisor: Prof. Dr. Xianzhong Yu
10/2004 - 08/2007	Studies of Pharmaceutical Sciences (B.Sc.) Ludwig-Maximilians-Universität München
10/2003 - 07/2004	Studies of Pharmacy Ludwig-Maximilians-Universität München
09/1994 - 07/2003	Highschool, graduation with "Abitur" Gymnasium Starnberg