

**Analysis of sub-visible particles in complex injectable formulations** Sediq, A.S.

### Citation

Sediq, A. S. (2017, May 9). *Analysis of sub-visible particles in complex injectable formulations*. Retrieved from https://hdl.handle.net/1887/49076

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Author: Sediq, A.S. Title: Analysis of sub-visible particles in complex injectable formulations Issue Date: 2017-05-09



# A flow imaging microscopy based method using mass-to-volume ratio to derive the porosity of PLGA microparticles



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Manuscript in preparation

## Abstract

The release of drugs from poly(lactic-co-glycolic acid) (PLGA) microparticles depends to a large extent on the porosity of the particles. Therefore, porosity determination of PLGA microparticles is extremely important during pharmaceutical product development. Currently, mercury intrusion porosimetry (MIP) is widely used despite its disadvantages, such as the need for a large amount of sample (several hundreds of milligrams) and residual toxic waste. Here, we present a method based on estimation of the volume of a known mass (a few milligrams) of particles using Micro-Flow Imaging (MFI) to determine microparticle batch porosity. Factors that are critical for the accuracy of this method (*i.e.*, particle concentration, density of the suspending fluid and post sample rinsing) were identified and measures were taken to minimize potential errors. The validity of the optimized method was confirmed by using non-porous polymethylmethacrylate microparticles. Finally, the method was employed for the analysis of seven different PLGA microparticle batches with various porosities (4.0 - 51.9%) and drug loadings (0 - 38%). Obtained porosity values were in excellent agreement with the MIP derived porosities. Altogether, the developed MFI based method is an excellent tool for deriving the total volume of a known mass of particles and therewith the porosity of PLGA microparticles.

# Introduction

Particulate controlled release systems, such as poly(lactic-co-glycolic acid) (PLGA)-based microparticles, are established tools for increasing the therapeutic efficacy of small molecules and peptides by maintaining drug concentrations within target ranges<sup>1</sup>. This property also decreases side effects caused by peak concentrations and repeated administrations. The formulation design<sup>2</sup> as well as the production method<sup>3</sup> of drug-containing PLGAmicroparticles are determinative for the characteristics of the particles and hence their performance. Therefore, it is crucially important to have analytical methods available to measure the characteristics of PLGA microparticles. One of these characteristics is porosity, which is a measure of the volumetric void fraction of the particle. This parameter has been shown to greatly influence the burst release of the drug from PLGA microparticles<sup>4</sup>. Additionally, by enhancing the effective surface area pores can influence the extended release of the drug from the microparticles through several mechanisms<sup>5</sup>.

Several analytical methods exist for determination of the porosity of microparticles, such as mercury intrusion porosimetry (MIP), gas expansion method, scanning electron microscopy (SEM)<sup>6</sup>. MIP is currently one of the most commonly employed methods for porosity determination because of its robustness and ability to provide in-depth porosity information. The popularity of MIP has led to the development of standardized protocols, reference materials and automatized equipment for this method<sup>7</sup>. However, the method has several disadvantages: it requires extensive expertise and model based calculation processes, the use of mercury generates toxic waste, and relatively large amounts (typically hundreds of milligrams) of powder are needed to perform the analysis which especially is a problem in the early stages of formulation and process development. Given the abovementioned disadvantages of MIP, there is a need for an economical, robust and straightforward analytical method for porosity determination.

We hypothesized that flow imaging microscopy could be used for that purpose, because it accurately measures the number and size of microparticles<sup>8,9</sup>, requires relatively small amounts and does not generate toxic waste. This technique uses imaging of particles in suspension to derive the particle concentration, morphology and size distribution. In the previous chapter, we successfully determined PLGA microparticle porosity by tracking particle sedimentation velocity using a flow imaging microscope. In this study, we developed a new methodology, using flow imaging microscopy, to determine the porosity of PLGA microparticles by sizing and counting all microparticles in a suspension with a known mass of microparticles. This allows for determination of the total volume of the particles and, together with the known mass, one can calculate the density and therewith the porosity. The developed method was used for porosity determination of 7 different PLGA microparticle batches using only a few milligrams of powder.

# **Materials and Methods**

### Materials

Cesium chloride (CsCl) and polysorbate 80 were obtained from Sigma (Sigma-Aldrich, Steinheim, Germany). Phosphate buffered saline (PBS; 8.2g/LNaCl, 3.1g/LNa, HPO, .12H, O, 0.3 g/L NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O, pH 7.4) was purchased from Braun (B. Braun Melsungen AG, Germany) and filtered with a syringe driven 0.22-µm polyethersulfone filter unit (Millex GP, Millipore, Carrigtwohill, Ireland). Ultrapure water (18.2 MΩ.cm) was dispensed from a Purelab Ultra water purification system (ELGA LabWater, Marlow, UK). Solutions of PBS containing 0.01% (w/v) polysorbate 80 (PBS-T) were prepared to facilitate wetting of the particles. Dry polymethylmethacrylate polymer beads (PMMA; average size 14.7 ± 1.3 µm; non-porous with a density of 1.19 g/mL) were purchased from Polysciences Europe GmbH (Eppelheim, Germany). Seven batches of PLGA microparticles were kindly provided by Dr. Reddy's (IPDO Leiden, the Netherlands). One of these batches contained no active pharmaceutical ingredient (API) and the other six were loaded with different amounts of API. A summary of these batches including their drug loading and porosity information (based on MIP) are shown in Table 1. The residual water and organic solvent contents of each PLGA microparticle batch were lower than 0.5% and not taken into account for the calculations.

I	I	
Batch	MIP derived porosity (%)	Drug loading (w/w %)
A	4.0	0
В	13.6	1
С	21.6	11
D	24.4	35
E	26.1	38
F	29.1	33
G	51.9	10

Table 1: Properties of the microparticle batches used in this study.

### Sample preparation for MFI measurements

For all the samples in individual studies about 1 mg of powder, accurately weighed using a microbalance (Sartorius model SE2, Goettingen, Germany) with a nominal resolution of  $0.1 \,\mu$ g, was used. Immediately after addition of the dry PMMA beads or PLGA microparticles to the suspending fluid (depending on desired concentration) the suspension was homogenized by vortexing. Thereafter, the suspension was sonicated for 20 minutes and left at ambient conditions for at least 24 hours. The suspension was vortexed again prior to the measurement.

#### Testing influential parameters with PMMA beads

As part of method development multiple factors were tested, namely the density of the suspending fluid, the microparticle concentration and post-sample rinsing during the measurement (explained in detail in the next section). Different concentrations of CsCl were added to PBS-T in order to vary the fluid density. Fluid densities of 1.15 g/mL and 1.27 g/mL were acquired with 0.63 g/mL and 1.25 g/mL CsCl, respectively. PMMA bead concentrations ranging from 0.1 mg/mL to 2.0 mg/mL were used for investigation of the effect of concentration on the accuracy of the method.

#### MFI measurements

Samples were analyzed by using a Micro Flow Imaging 5200 instrument (MFI; Protein Simple, Santa Clara, CA, USA) with MFI View System Software (MVSS) Version 2. The system was flushed with 3 mL of particle-free suspending fluid at 5 mL/min prior to each measurement. Flow cell cleanness was checked visually. The background was calibrated by performing the 'optimize illumination' procedure using particle-free suspension fluid. The MFI analysis was started without any discarded purge volume prior to the start of fluid imaging. For the study on the effect of concentration and density of the suspension, no post-sample rinsing was applied. The volume of the analyzed suspensions depended on the concentration of the sample, ranging from 0.5 mL to 10 mL. We strived to achieve at least 50,000 particle counts for the sake of statistical power of the measurement.

A 5-mL pipette tip was placed at the sample introduction inlet of the MFI, into which the suspension was poured for analysis. The whole suspension of each sample was analyzed. The MFI measurements that included a post-sample rinsing procedure were accompanied by the following additional steps. Just before the end of the sample measurement, 0.5 mL of particle-free suspending fluid was used to rinse the sample container and was poured over the whole inner surface into the 5-mL pipette tip while continuing to measure with MFI. For the optimization of the MFI analysis (see results section), the potential effect of adsorbed particles at the container, pipette tip and inner tubing surfaces on the porosity calculations was investigated and the required extent of rinsing during the analysis was determined. The number of rinsing steps used to measure the PLGA microparticle batches was chosen based on the results from these optimization studies. No software filters were applied during the runs. After the runs, MFI View Analysis Suite (MVAS) version 1.4 was used to analyze the data recorded by MVSS and to remove stuck, edge and slow moving particles. In a preliminary study a simple filter was developed based on the Intensity parameter to exclude all contaminants (such as dust, fibers, etc.) from the analysis. For all samples only particles with a mean intensity value equal to or lower than 300 were included (total intensity range = 0 - 1023; with 0 corresponding to a black pixel and 1023 to a white pixel). The final size distribution of each sample was extracted, where the particle size was expressed as equivalent circular diameter (ECD), and used for further calculations.

#### Porosity calculations

Porosity ( $\varphi$ ) is basically the ratio of the pore volume (V<sub>pores</sub> in mL) to the total volume of a given (known) amount of suspended particles (V<sub>particles</sub> in mL) in a certain sample volume. The total particle volume was calculated from the final measured size distribution (size over counts) by MFI. For this purpose each size bin (from 1 µm and larger, with steps of 0.125 µm) was converted into a volume bin, by assuming a spherical shape. Thereafter, the sum of the multiplication of each bin volume with its corresponding total particle count was obtained. V<sub>particles</sub> consists of the total pore volume (V<sub>pores</sub> in mL) and the skeletal volume (V<sub>solids</sub> in mL) or the volume of the solid components in the entire particle population within a given sample volume. Porosity is then calculated based on the following equation:

$$\varphi = \frac{V_{pores}}{V_{particles}} \times 100\% = 1 - \frac{V_{solids}}{V_{particles}} \times 100\%$$
 Eq. 1

 $V_{solids}$  can be calculated from the mass of powder (m) that was used for the suspension and the density ( $\rho_{solids}$ ) of the solid components:

$$V_{solids} = \frac{m}{\rho_{solids}}$$
 Eq. 2

In case of PLGA microparticles the density can be calculated from the drug loading and the reported densities of the PLGA and API accordingly:

$$\rho_{solids} = f_{API} \times \rho_{API} + f_{PLGA} \times \rho_{PLGA} = f_{API} \times \rho_{API} + \rho_{PLGA}(1 - f_{API})$$
 Eq. 3

Here  $f_{API}$  (hence drug loading) and  $f_{PLGA}$  are the weight fractions of the API and the PLGA, respectively, in the solid content (derived from the drug loading). For the API and PLGA a density of 1.30 g/mL (as stated by the manufacturer of the microparticles) and 1.34 g/mL<sup>10</sup>, respectively, were used. By incorporating Eq. 3 into Eq. 2 and subsequently incorporating the resulting equation into Eq. 1, one can derive Eq. 4 to calculate the porosity of PLGA microspheres:

$$\varphi = 1 - \frac{m}{(f_{API} \times \rho_{API} + \rho_{PLGA}(1 - f_{API})) \times V_{particles}} \times 100\%$$
 Eq. 4

### Results

### Density of the suspending fluid

To optimize the sample preparation, we have investigated the influence of the density of the suspending fluid on the homogeneity of the PMMA bead suspension (in terms of particle concentration) during measurement. Homogeneity of the suspension is desired in order to minimize locally high particle concentrations that would lead to an increase of non-isolated particles. Non-isolated particles include true aggregates in the raw PMMA material, aggregates formed due to high bead concentration and optically overlapping particles during the measurement, all of which could compromise the accuracy of the method. The PMMA beads were suspended in PBS-T alone and in PBS-T containing two different concentrations of CsCl. For each suspension the total particle volume was measured with MFI. Thereafter, the analyzed particle volume relative to total particle volume at specific time points during the MFI measurement was calculated and the resulting graphs are presented in Figure 1. The error bars show the deviation caused by the size distribution of particles at different time points. Note that the size distribution becomes wider with the presence or increase in number of non-isolated particles. These graphics show in rough terms how changes in the density of the suspending fluid would influence the particle homogeneity in the sample liquid. All three graphs show low particle counts at 0 min due to initial dilution of the sample with the preceding particle-free suspending fluid used for the 'optimize illumination' step. At about 1 min, particle counts appeared to stabilize and all three graphs present a plateau stage. Suspending fluid containing no CsCl showed a slightly descending plateau stage, up to 3 minutes of measurement (Fig. 1A). Thereafter, the particle count decreased much faster. The standard deviation in the particle volume decreased at later stages of the plateau phase and thereafter. This indicates that percentage of non-isolated particles was not constant throughout the entire measurement. On the contrary, the suspending fluid with 0.63 g/mL CsCl having a density of 1.15 g/mL showed a much longer and more stable plateau stage (Fig. 1B). Here, the particle count started decreasing only after 4.5 min. During this measurement the standard deviation was relatively constant over a larger part of the plateau stage, indicating a constant percentage of non-isolated particles. Further increase of the fluid density to 1.27 g/mL (1.25 g/mL CsCl) resulted in relatively lower counts (Fig. 1C) during the first 3 minutes of the measurement compared to the other two suspensions. Thereafter, a progressive increase in analyzed particle volume was observed. A slight increase in the standard deviation of the particle volume was observed by the end of the measurement, indicating that the percentage of non-isolated particles increased towards the end of the measurement.

Observations seen in this set of experiments indicate that a homogeneous particle concentration over the entire (or large part of) MFI measurement can be achieved using a



Figure 1: Percentage of the total particle volume over the measured time in samples of 0.3 mg/mL PMMA beads in different suspending fluids: A) PBS and 0.01% polysorbate 80 (density 1.03 g/mL); B) PBS, 0.01% polysorbate 80 and 0.63 g/mL CsCl (density 1.15 g/mL); and C) PBS, 0.01% polysorbate 80 and 1.25 g/mL CsCl (density 1.27 g/mL).

fluid density that matches the particle density. In our study we found a fluid density of 1.15 g/mL to be the best choice with respect to homogeneity during the measurement of PMMA beads and therefore used this as suspending liquid in following studies. In this suspending liquid the density difference between the particles and the fluid was about 3% (relative to the PMMA density).

#### Effect of particle concentration

During the second part of the study on influential factor, we focused on the particle concentration. Increasing particle concentration would theoretically result in increasing numbers of detected non-isolated, due to optically overlapping particles during the measurement. These non-isolated particles mostly do not have a spherical shape and therefore it is expected that the accuracy in total particle volume calculation would decrease with increasing number and volume of non-isolated particles. For this part, increasing concentrations of PMMA beads were prepared in duplicates using the suspending fluid with the density of 1.15 g/mL. After MFI measurement, the percentage of non-isolated particles (volume and number based) was calculated.



Figure 2: The effect of PMMA bead concentration on the accuracy of total particle volume calculation. A: Contribution of non-isolated (sum of aggregated and optically overlapping) particles to the total particle counts (black circles) and total particle volume (red squares) in suspensions with different PMMA concentration, as measured with MFI. Each data point shows the average and the upper and lower value of the duplicate measurement of suspensions. Examples of MFI images of a single and a few different non-isolated particles are displayed below the graph. B: Total particle volume of each sample displayed in graph A as function of particle concentration. The theoretical values derived from the sample concentration and known PMMA density (black circles) and measured total particle volumes with MFI (red squares) are compared.

Figure 2A shows that an increasing bead concentration resulted in more non-isolated particles, as expected. This in turn led to an increase in the number and volume of the non-isolated particles relative to the total counts and total particle volume, respectively. At the highest bead concentration tested (2 mg/ml) almost 70% of the total particle volume belongs to non-isolated particles. Using the density of the PMMA particles and the PMMA sample concentrations, the total particle volume per sample ( $V_{particles}$ ) was calculated. In Figure 2B these theoretical values are compared with the experimentally determined total particle volume for each tested sample. It is seen that the measured particle volume started to significantly underestimate  $V_{particles}$  at PMMA concentrations of 1 mg/mL and higher. For each following study with the PMMA beads a concentration of 0.1 mg/mL was used.

#### Post-sample rinsing during MFI measurement

The last potentially influential factor that was studied concerned the measurement procedure. Here an attempt was made to minimize loss of sample, which would lead to erroneous total particle volume measurements. Loss of sample could be due to adsorption of particles (and liquid containing particles) to the container, pipette tip and instrument tubing. After the entire suspension of 0.1 mg/mL PMMA beads was measured (after 22 minutes), 5 post-sample rinsing steps of 0.5 mL each were applied, as described in the

*Methods* section. As seen from the results in Figure 3A, over the whole measurement including the rinsing steps PMMA beads of about 15  $\mu$ m were detected.

Although fewer particles were detected when the rinsing fluid passed through the flow cell and was being measured (see drop in particle volume after 22 minutes), still substantial amounts of particles were included in the measurement when rinsing was applied. Figure 3B shows that less than 80% of the total particle volume found in suspension had passed the camera view at the moment when the first 0.5 mL of rinsing fluid was added. Only after 3 rinsing steps were applied, almost all particles found in the suspension had passed the camera view.



Figure 3: The effect of post-sample rinsing steps during the MFI measurement on analysis of adsorbed particles (and liquid containing particles) to the tubing and pipette tip. A: Particle size (mean and standard deviation; red circles) and total particle volume (mean and standard deviation; black squares) at different time points. The latter is achieved through multiplication of the particle count and single particle volume at given time points. Single particle volume is calculated from the average particle size and formula for volume of a sphere. B: Cumulative particle volume at different time points during the measurement expressed in percent, with 100% being the total particle volume measured in the experiment. The red arrows indicate the time points at which 0.5 mL of particle free suspending fluid was used to rinse the tubing, pipette tip and subsequently measured by MFI, as described in Methods section.

Therefore, for the following studies we have applied 3 post-sample rinsing steps with 0.5 mL of the particle free suspension in order to measure the particles adsorbed to the tubing and pipette tip.

#### Porosity measurements of PMMA beads (method validation)

For the validation of the presented method for porosity determination we used PMMA beads. Based on the previous experiments, the following conditions were used: the beads were suspended at a PMMA concentration of 0.1 mg/mL in a suspending fluid of PBS-T with 0.63 g/mL CsCl (and a density of 1.15 g/mL), the sample volume was 5 mL, and 3 0.5-mL

post-sample rinsing steps were applied.

The measurements resulted in a measured density of  $1.17 \pm 0.03$  g/mL and a derived porosity of  $2 \pm 2\%$  for the studied PMMA beads. These values are in excellent agreement with the given information by the manufacturer (density of 1.19 g/mL, 0% porosity).

Porosity measurements of PLGA microparticles (method application)



Figure 4: Examples of MFI pictures of individual particles in batch A and G of the studied PLGA microparticles. Batches B-F had particles looking similar to the ones from batch A. The scale bar at the right bottom depicts 100 µm.

The developed method was applied for determination of the porosity of 7 different batches of PLGA microparticles. Based on the MIP measurements these batches differed in porosity (see Table 1). For each PLGA microparticle batch the particle concentration was optimized according to the previous section. Briefly, the optimal concentration was chosen such that the percentage of non-isolated particles was lowest. In order to keep the measurement duration not more than 40 minutes per sample the suspension volume was limited to 5 mL. The optimal fluid density was based on the expected density of each PLGA microparticle batch when suspended in the liquid (calculated from the loading and porosity value derived with MIP). Given the almost similar density of the API and the PLGA raw material, for all the suspensions the same fluid density was used. The chosen fluid density differed less than 3% from each (expected) PLGA microparticle density. A summary of these suspension conditions and measured average and standard deviation of the particle sizes are given in Table 2. All the batches contained predominantly spherical particles, except for batch G, which showed more unevenly shaped particles (Figure 4). In Figure 5, the average and standard deviation of the calculated porosity (n = 3) values are plotted against the values obtained by MIP, illustrating that the results from the developed flow imaging microscopy based method are in excellent agreement with the ones found by MIP. The line shows the linear regression (goodness of fit:  $R^2 = 0.9854$ ), with the dashed lines representing the 95% confidence interval (CI) range. The 95% CI range of the slope was 1.006 – 1.334, which indicates high similarity between the MFI derived and MIP derived porosity.



Figure 5: Porosity results gained from the MFI measurements against MIP derived porosity values for seven different PLGA microparticle batches. For each batch the mean and standard deviation of three measured samples are shown. The linear relation between MIP and flow imaging microscopy porosity is denoted as the solid line, with the 95% confidence of interval of the linear relation between the dashed lines.

**Table 2:** Summary of the conditions used for the studies with the 7 PLGA microparticle batches and the size and morphological parameters obtained by MFI.

Batch	Suspending fluid density used (g/mL)	Concentration used (mg/mL)	Average ± standard deviation of particle size (µm)	Intensity (mean ± standard deviation; a.u.)	Circularity (mean ± standard deviation)	Aspect ratio (mean ± standard deviation)
Α	1.3	0.2	44.1 ± 12.5	188 ± 266	0.90 ± 0.11	0.95 ± 0.12
в	1.3	0.2	46.1 ± 12.6	191 ± 268	0.89 ± 0.10	0.94 ± 0.10
С	1.3	0.3	42.9 ± 11.9	200 ± 269	0.89 ± 0.11	0.95 ± 0.15
D	1.3	0.3	43.4 ± 11.6	197 ± 268	0.88 ± 0.10	0.93 ± 0.11
E	1.3	0.2	48.7 ± 14.9	186 ± 265	0.88 ± 0.10	0.92 ± 0.11
F	1.3	0.2	46.1 ± 13.4	193 ± 265	0.87 ± 0.15	0.92 ± 0.10
G	1.3	0.2	48.9 ± 14.4	215 ± 260	0.88 ± 0.18	0.81 ± 0.19*

\*Significantly different from the other batch(es) (one-way Anova (p<0.0001) followed by post-hoc Tukey's multiple comparison test (p<0.05))

### Discussion

The porosity of PLGA microparticles has been recognized as an important parameter for the release kinetics of the encapsulated drug<sup>4,11</sup>. Currently, MIP is the most employed method for porosity measurements. Considering the limitations of the MIP method, there is a need for a more straightforward method. In this investigation we have developed a flow imaging microscopy based method using PMMA microparticle beads and applied the developed method for porosity determination of a number of PLGA microparticle batches. Our method depends on the precision and accuracy of two parameters that are included in the calculations, namely the mass of the particles used for the suspension preparation and the total particle volume as measured by MFI. The error in the weighing was minimized by the use of a microbalance for the masses of about 1 mg used for our measurements (translating into a theoretical weighing error of about 0.01%). However, other factors related to sample preparation and measurement also contribute to the overall error in the measurement and determination of the total particle volume.

The calculation of particle volume is based on the size distribution of the sample. For high

accuracy of the latter parameter, a flow imaging microscopy technique with high sizing and counting accuracy is essential. The sizing accuracy of MFI was shown to be high for small polystyrene beads  $(2 - 10 \,\mu\text{m})^{12}$ . In a separate study we found that the sizing accuracy larger  $(30 - 70 \,\mu\text{m})$  NIST-traceable polystyrene beads was also high (see Supplementary Table 1). Zölls and coworkers found high counting accuracy, which was suggested to be a result of high sample efficiency of the MFI 5200 system (about 85%)<sup>12</sup>. The counting accuracy depends on the concentration and size of particles in the measured sample. Therefore, the manufacturer of the MFI provides concentration limitations for different particle sizes up to 10  $\mu$ m, with respect to the counting accuracy of the system, with a recommended maximum concentration of 20,000 10- $\mu$ m particles per mL<sup>13</sup>. In our study we have shown that this recommendation is also valid when total particle volume determination is the ultimate goal of a measurement. We found that the total particle volume calculation (from the average particle size and concentration) was accurate up to a (15- $\mu$ m sized) PMMA bead concentration of 0.5 mg/mL, which corresponds to about 24,000 particles per mL.

One caveat with respect to volume determination of microparticles is that any significant swelling of the particles would lead to overestimation of the original size and total particle volume, which would consequently result in underestimation of the porosity. However, it has been shown that, for PLGA particles, detectable swelling appears at the later stages of the degradation, that is after several days when the molecular weight of the PLGA matrix gets lower than 20 kDa<sup>14</sup>. Including studies on optimizing the condition and replicate measurements the achievement of porosity value as presented in our investigation does not cost more than a couple of hours.

MFI derives the size of a particle from the measured particle area, by calculating the diameter of a circle with an equivalent projected surface area. In case of PLGA microparticles the nearly spherical shape reduces the chance of inaccurate sizing. However, optically overlapping particles and aggregates (possessing mostly non-spherical shapes) are more subjected to inaccurate sizing. The amount of non-isolated particles detected in the suspension was found to mainly depend on the particle concentration. More particles per volume unit of the liquid obviously lead to a higher chance that multiple particles would coincidentally overlap in the field of view. In some cases particles that are too close to each other in the field of view (but still obviously physically separated), are erroneously detected as one agglomerate (*e.g.*, see rightmost particle image in Figure 2). Probably this is caused by a combination of light diffraction from two particles close to each other and a very sensitive detection threshold used by the MFI software. This threshold together with the so-called minimum distance-to-the-neighbor setting in MFI (by default 3  $\mu$ m), results in detection of the pixels between these particles as part of the agglomerated particle<sup>13</sup>. Naturally, the detected non-isolated particles may also be real aggregates, meaning that

the microparticles are physically bound to each other.

Measures can be taken to reduce the chances of optically occluded particles and presence of real particle aggregates in the PLGA microparticle batches. For the latter surfactants present in the suspending agent, vortexing steps and sonication should in principle reduce physical aggregation. For PLGA microparticles it is well known that the tendency of microparticles to aggregate on drying is related to the extent of the particle hydration and the residual ethyl acetate in wet microparticles<sup>15</sup>. According to the manufacturer, the residual solvent content in the different batches of the microparticles were measured to be of insignificant value (below 0.1%).

Reduction of the particle concentration in order to reduce the chance of overlapping particles in the field of view has its limitations. Namely that a very large volume of suspension must be measured to make sure that a high number of particles is counted. Therefore, it is necessary to define a threshold for the amount of non-isolated particles (relative to the total) that still delivers accurate particle volume determination. The threshold for this amount of non-isolated particles we had set at 30%. This threshold was based on our study with the PMMA beads, where even up to 30% (volume-based) non-isolated particles accurate total particle volumes were achieved (see Figure 2B). At the end the volume percentage of non-isolated particles for the studies with PLGA microparticle was not more than 12%.

These theoretical facts combined with our observations, brings us to the conclusion that, in order to reduce the error introduced by the non-isolated particles, low microparticle concentrations should be used for the MFI measurements. One should keep in mind that very low concentrations would require analysis of large suspension volumes to detect a sufficiently large number of particles (in this study the choice was detection of at least 50,000 particles in total) for the statistical power of the analysis. Analysis of large volumes would require tremendously prolonged measurement times. Therefore, finding a compromise between particle concentration, statistical power and measurement time is necessary for individual batches to be measured. In our study, typical analysis times per sample were between 20 - 40 minutes.

Decreasing the concentration of a microparticle batch with a broad size distribution cannot fully assure that coincidental particle overlap in the field of view will not occur. This has to do with the sedimentation of particles during the measurements. Particulate sedimentation in a fluid depends mainly on the size of the particle and the density difference between the particle and the fluid<sup>16</sup>. In case of a sample containing a broad size distribution (*e.g.*, PLGA microparticles), a big density gap between the particles and the suspending fluid will lead to rather fast settling particles. Since larger particles will settle faster, this can create locally high concentrations of (in particular large) particles. Decreasing the density difference between the particle and the fluid is an effective way to avoid this phenomenon. Cesium

chloride is known to be an inert salt and has the great advantage of being able to change the density of aqueous solutions with very limited change in the viscosity<sup>17</sup>, and therefore a good choice to be used for adjusting the fluid density.

Our results presented in Figure 1 clearly indicate that the bigger the difference between the density of the particle and the suspending liquid, the higher the chance of sample heterogeneity. This heterogeneity caused by settling/floating of particles during measurement, would hypothetically result into locally high numbers of non-isolated particles. We have proven this hypothesis for the condition where we had the largest gap in density between particle and the suspending liquid, *i.e.*, for the PMMA microparticle beads suspended in a CsCl free solution with a density of 1 g/mL. The low counts at start are caused by dilution with particle free liquid that was used to perform the 'optimize illumination' step and the low counts in the last part of the measurement are caused by creation of locally decreased particle concentration due to settling of particles earlier during the measurement. We have calculated the volume percentage of non-isolated particles at each time point, excluding the first and last minutes of the run where the low number of particles compared to the rest of the run could result in unreliability in calculation of the percentage of aggregates. The data in the Supplementary Figure S1 indicates that the percentage of non-isolated particles resembled a parabolic shape over the course of MFI analysis. This confirms the abovementioned hypothesis that the settling of particle during the experiment can cause a locally high concentration of non-isolated particles (*i.e.* the peak of the parabola). Consequently this could lead to larger errors in estimation of the total volume of the particles.

Both the suspending medium and the PMMA particles may interact not only with the container, but also with the pipette tip on top of the MFI inlet port and the MFI tubing. This is also evident from the results in Figure 3A, where the first part of the measurement has a lower total particle volume than the rest of the measurement until post-rinsing steps. This could be overcome by a post-sample rinsing step with particle free suspending medium: the expected porosity (0% for control PMMA particles) was reached after 3 rinsing steps were included in the measurement. The tendency of suspending medium and suspended particles to interact with the tip and tubing materials will determine these rinsing conditions. Despite the attempts presented here, it appears to be difficult to have a homogenous suspension throughout the whole measurement. Also the loss of particles due to interaction with the tip and tubes would jeopardize the accuracy of the method even when fluid density is adjusted and a rather long-term homogeneity during the measurement is attained. Therefore, the presented method cannot be further simplified by calculating the particle volume (per volume of suspension) from a specific fraction of a MFI measurement; rather, it is advised to measure the entire sample and include post-sample rinsing steps to ensure measurement of all the particles within that sample.

After validation of the method with PMMA beads, we were able to find fairly similar porosities for 7 batches of PLGA microparticles compared to MIP derived ones. That is seen from the average slope of the linear regression that had a value of  $1.17 \pm 0.06$ . Our investigation shows also that the method can be applied for a considerably wide range of porosities. For the batch with the highest MIP derived porosity (batch G), we found a relatively large standard deviation of the measured particle porosity. The reason for this is likely the nonspherical shape of the particles, resulting in irreproducible sizing and hence total particle volume determination. The latter is also obvious from the aspect ratio value, which is lower for batch G compared to all the other PLGA microparticle batches (see Table 2).

Overall, the method we propose in this study is very applicable as a tool to assess the porosity of microparticles. In addition, the instrument delivers particle size distribution of the batch and images of the particles as well as several morphological parameters. For the porosity determinations certain preparatory studies, as we described here, need to be performed in order to achieve accurate measurements.

# Conclusion

In this study, we have investigated a novel method for the porosity determination of microparticles based on measurement using MFI. We have shown that optimization of the particle concentration, suspending fluid density and post-sample rinsing steps during measurement are advisable for obtaining the most reliable porosity outcome. The described method allowed for successful measurement of the porosity of (spherical) PMMA beads and 7 different PLGA microparticle batches. The method requires only a few milligrams of the particle powder, which is an asset for early stage formulation and process development operations.

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# **Supplementary Information**

The sizing accuracy of MFI was tested for bead larger than 10  $\mu$ m. Therefore, 3 NIST-traceable polystyrene beads were used of different sizes (mean ± standard deviation), namely 29.8 ± 0.4  $\mu$ m; 50.2 ± 0.5  $\mu$ m; 69.1 ± 0.8  $\mu$ m. The results are shown in **Supplementary Table 1**. The measured values for each bead size were compared with the size provided by the manufacturer (using a paired t-test). All MFI derived sizes were shown to be statistically similar (p > 0.05) to the manufacturer's specifications.

Declared size by the man-	MFI derived size	P value
ufacturer (µm)	(μm)	
29.8 ± 0.4	29.7 ± 0.1	< 0.0001
50.2 ± 0.5	50.1 ± 0.1	< 0.0001
69.1 ± 0.8	69.0 ± 0.2	0.0001

Supplementary	/ Table 1:	Sizina	accuracy	/ of r	olvstv	vrene	standards	with	MFI
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**Supplementary Figure 1:** Percent of non-isolated particles (based on volume) detected at each time point of the measurement presented in Figure 1A of the main document.