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Particle size analysis of nanocrystals: Improved analysis method

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ABSTRACT

The influence of optical parameters, additional techniques (e.g. PIDS technology) and the importance of light microscopy were investigated by comparing laser diffraction data obtained via the conventional method and an optimized analysis method. Also the influence of a possible dissolution of nanocrystals during a measurement on the size result obtained was assessed in this study. The results reveal that dissolution occurs if unsaturated medium or microparticle saturated medium is used for the measurements. The dissolution is erratic and the results are not reproducible. Dissolution can be overcome by saturating the measuring medium prior to the measurement. If nanocrystals are analysed the dispersion medium should be saturated with the nanocrystals, because the solubility is higher than for coarse micro-sized drug material. The importance of using the optimized analysis method was proven by analysing 40 different nanosuspensions via the conventional versus the optimized sizing method. There was no large difference in the results obtained for the 40 nanosuspensions using the conventional method. This would have led to the conclusion, that all the 40 formulations investigated are physically stable. However, the analysis via the optimized method revealed that from 40 formulations investigated only four were physically stable. In conclusion an optimized analysis saves time and money and avoids misleading developments, because discrimination between "stable" and "unstable" can be done reliably at a very early stage of the development.

1. Introduction

Nanotechnology became one of the hot topics in pharmaceutical research and with that also the need of appropriate techniques for analysing such systems (Carl Englert, 2007; Prosie et al., 2008; Warheit et al., 2008). If "nano" is used the definition will differ, depending on the discipline (colloidal physicists versus pharmacists) and on the application of the nanosized system. In pharmaceutics the word "nano" is defined as particles being below 1000 nm in size (Kreuter, 2001; Müller, 1998), other definitions are that real nanoparticles are below 100 nm (ISO/TS-27687, 2008; PAS71, 2005). Various techniques exist to analyse the size of pharmaceutical systems. Nowadays the most frequently used techniques for particle size measurements of nanosized systems are dynamic light scattering techniques, static light scattering techniques and microscopy. Each method has advantages but also disadvantages.

Dynamic light scattering, also known as photon correlation spectroscopy (PCS) is advantageous, because it yields accurate results and the measurements are fast and easy to perform. However, the disadvantage is that via this technique it is not possible to analyse particles being larger than 6 μm. Therefore additional techniques are required which ensure the detection of possible larger particles in nanosized populations. Techniques for the detection of larger particles are microscopy and low angle static light scattering, also known as laser light diffraction. The advantage of light microscopy is the visible and therefore doubt free result, however, disadvantages are the missing statistical significance, because it is not possible or very time consuming to analyse 10,000 particles or more, which would be required for a valid analysis (ISO-14488, 2000; Rawle, 2007). Laser diffractometry is a robust technique and has the advantage over all the other techniques to be able to analyse large particles, small nanoparticles and mixtures of small and large particles within only one single measurement. However, disadvantages do also exist for this technique, even though they are not as common knowledge as the disadvantages for dynamic light scattering and light microscopy (Keck and Müller, 2005a).

The disadvantages of laser diffraction techniques rose with the need of analysing nanoparticles with a technique being originally intended for the measurement of larger micrometer particles. Because laser diffraction is a simple and fast sizing method it was aimed to extend the measuring range (e.g. from 400 nm to 2000 μm) to a broader range, being able to analyse even very small particles (e.g. from 20 nm to 2000 μm). However, in principle it is only possible to analyse particles from about 400 nm and larger via this techniques. The prerequisite for this is the use of the Mie formula for the calculation of the particle size (ISO13320-1, 1999).

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For this the optical parameters of the particles which need to be analysed are required. For many solid compounds (e.g. new drug entities) these optical parameters are not known and moreover they are very difficult to access. Therefore in many cases the calculation of the result is performed by using guessed optical parameters, or simply the Fraunhofer approximation, which does not require the optical parameters of the compound (Keck and Müller, 2008). In theory Fraunhofer approximation can be used if the particles which need to analysed are at least five times larger than the wavelength of the light source used for analysis. Depending on the manufacturer of the LD instruments the wavelengths of the lasers range from 632.8 to 800 nm. Hence all particles being smaller than about 3.5 μm need to be analysed using the Mie formula and the corresponding optical parameters. In a previous study it could be shown that the influence of the optical parameters tremendously affects the final size results obtained. It could be shown, that the analysis of particles will lead to incorrect results if guessed optical parameters or Fraunhofer approximation are used (Keck and Müller, 2004, 2008).

Laser diffraction is not suitable for the analysis of particles being smaller than about 400 nm, because the intensity of diffracted light decreases with decreasing size. However, modern LD instruments can analyse particles from 20 nm up to 2 mm or even larger. The extension of the measuring range for very small particles was possible by introducing a second, additional technique, being different from laser diffraction, into the instruments. The additional technique gains more information about the particles by measuring other optical phenomena (e.g. scattering intensities in different directions) (Bott and Hart, 1990; Xu, 2003). Thus the additional techniques are different to pure laser light diffraction. The additional information from the additional technique is than incorporated into the size analysis of the LD measurement, which leads to a combined result of pure LD and additional technique. Therefore, strictly spoken, today's LD measurements are not only pure LD measurements but a combination of two different techniques. Also here it could be shown in a previous study that this combination of independent techniques can lead to false results. It could be proven that additional techniques can overestimate the presence of small nanoparticles by overlooking larger particles (e.g. large crystals and/or aggregates/agglomerates) (Keck and Müller, 2005b). This finding is of extraordinary importance, because most often LD is used to detect possible large particles besides a small sized main bulk population, or to prove the absence of such large crystals, which is not possible via PCS measurements. In conclusion; particle sizing of submicron particles will only lead to meaningful results if all the above-mentioned parameters are taken into account.

Of course problems in size analysis not only arrive from the instrumental setup alone, but also from the material to be analysed. As a rule of thumb it is said that the stability of the sample during analysis is the most important prerequisite for correct and reproducible results (Rawle, 2004). However this is not always easy to achieve and sometimes changes are not even recognized. Possible changes or instabilities of a sample are for instance agglomeration or dissolution. Therefore the size analysis of samples with high solubility and/or increased dissolution velocity might be especially sensitive to such changes.

A typical example for systems possessing an increased solubility and dissolution velocity are drug nanocrystals (Jinno et al., 2006; Merisko-Liversidge et al., 2003; Müller et al., 1999). Nanocrystals have been developed for the formulation of poorly soluble compounds, which number is increasing every year (Keck and Müller, 2006; Stegemann et al., 2007). Thus, also the research using nanocrystals as universal formulation approach is increasing in parallel. The principle of nanocrystals is the decrease in size of drug crystals, which leads to an increased total surface area of the compound and thus, according to the Noyes–Whitney equation and the Ostwald–Freundlich equation, to an increase in dissolution rate and solubility (Müller and Junghanns, 2006; Patravale et al., 2004; Rao et al., 2004).

The increase in dissolution velocity and solubility is advantageous for the formulation of poorly soluble drugs, but might be disadvantageous for the size characterization of such systems. Therefore the aim of this work was to investigate the influence of possible changes due to dissolution of nanocrystals during the measurement on the size result obtained and to further proof the importance of correct optical parameters, the influence of additional technique and the use of light microscopy and their impact on research and formulation development. The results obtained in this study can in principle be transferred to any particulate or nanoparticulate material, which shows partial or potential dissolution.

2. Materials and methods

2.1. Materials

Cyclosporine was purchased from Chemos GmbH (Regensstauff, Germany) and Rutin was obtained from Sigma–Aldrich (Germany). As stabilizers for the suspensions were used: TPGS (Eastman, Kingsport, USA); sodium dodecyl sulphate (SDS); polysorbate 80 (Tween 80) (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); polyoxyethylene block copolymers: Poloxamer 188 (Lutrol F 68) and Poloxamer 407 (Lutrol F 127) (BASF AG, Ludiwigshafen, Germany). Purified water was obtained from a MilliQ plus system (Millpore, Schwalbach, Germany).

2.2. Methods

2.2.1. Production of nanocrystals

The nanocrystals were produced as aqueous nanosuspensions by high-pressure homogenization (HPH, Micron LAB 40, APV Deutschland GmbH, Unna, Germany). The rutin nanosuspension, containing 5% (w/w) rutin and 1% (w/w) Poloxamer 188, was produced applying 20 cycles at 1500 bar. The cyclosporine nanosuspensions were produced applying 40 cycles of high-pressure homogenisation. The cyclosporine nanosuspensions contained 1% (w/w) cyclosporine and 1% (w/w) stabilizer (Poloxamer 188 (PLX 188), Poloxamer 407 (PLX 407), Tween 80 or TPGS). The suspensions stabilized with SDS contained 10^{-3} M of the stabilizer. For each of the five formulations four different production parameters were applied (V1–V4, V1: HPH as described above, V2: as V1 followed by dilution with stabilizer solution (1:1) after the HPH process, V3: as V1 followed by dilution with glycerol 85% (1:1) after the HPH process, V4: as V3 and subsequent application of further 10 cycles of HPH at 1500 bar), giving a total of 20 different nanosuspensions. The rationale behind the versions V2–V4 was: V2 – addition of surfactant solution was a challenge test for Ostwald ripening, because additional surfactant increases solubility, V3 – addition of glycerol increases the viscosity and thus slows down the crystallization processes by diffusion (e.g. potential Ostwald ripening), V4 – application of additional homogenization cycles to V3 should increase homogeneity and thus lead to physically more stable nanosuspensions. The effects of the process variations V1–V4 were investigated applying the different sizing methods. Each nanosuspension was stored at room temperature and in the refrigerator (4 °C) to identify the most appropriate storage condition.

2.2.2. Dynamic light scattering

Dynamic light scattering (photon correlation spectroscopy (PCS)) was performed using a Zetasizer Nano ZS (Malvern Instruments, UK). All measurements were performed in quartz cuvettes at 20 °C.

2.2.3. Low angle light scattering

Low angle light scattering, also known as laser diffraction (LD) was performed using an LS 230 (Beckman-Coulter, Germany). Measurements were performed with and without additional polarization intensity differential scattering (PIDS) technology. Measuring time was 60 and 30 s for the measurements with and without PIDS, respectively. The analysis of the raw diffraction data was performed using the Beckman-Coulter Software Version 3.19.

2.2.4. Light microscopy

An Orthoplan (Leitz, Germany) was used with magnifications of 160× and 1000×.

2.2.5. Influence of dissolution on size analysis

The rutin nanosuspension was analysed using dynamic light scattering and low angle static light scattering. In order to investigate the influence due to dissolution each measurement was performed using unsaturated dispersion media (us), partlially saturated (ps) and fully saturated (ss) measuring (dispersion) media.

2.2.5.1. Measuring media. Medium 1 was purified water (us, unsaturated). As medium 2 purified water was saturated with coarse drug powder with a particle size of approximately 50 μm (ps, powder saturated). One hundred milligrams coarse powder was added to 1000 ml water. The obtained dispersion was stirred at room temperature for 24 h. After 24 h the dispersion was filtrated (Sartorius® 0.2, Sartorius, Germany). To avoid a loss of dissolved molecules from the dispersion medium during filtration due to a possible adsorption of the dissolved drug molecules onto the filter material, the first 100 ml of the dispersion medium were filtrated to ensure the saturation of the filter used, and discarded. As nanosuspensions possess an increased solubility, a third medium (ss, super saturated) was saturated by adding the respective nanosuspension to 500 ml of medium 2, which leads to an entirely saturated solution. The dispersion was stirred at room temperature for 4 h and filtrated as described above.

2.2.5.2. Measurements.

2.2.5.2.1. Dynamic light scattering (PCS). All measurements were performed in a standardized way using the following experimental setup: the temperature of the original sample and the respective dispersion medium were adjusted to 20 °C prior to the measurement and prior to adding the sample to the dispersion medium. For the measurement, the desired volume of dispersion medium (1 or 2 ml) was filled into the cuvette and than 10 μl of sample was added. The cuvette was shaken for 10 s s by hand, placed into the instrument and the measurements was started immediately. To minimize the time delay between placing the cuvette into the instrument and the actual start of the measurement, the equilibrium time was set to 0 s. This was possible because the temperature was adjusted outside. All measurements were performed in triplicate and in 1 and 2 ml dispersion media, respectively.

2.2.5.2.2. Laser diffraction (LD). A constant volume (50 μl of the rutin nanosuspension) was added to the dispersion medium, previously filled into the instrument. The measurements were performed with PIDS technology included and a measurement length of 60 s. Changes in size were obtained by repeating each measurement 15 times without changing the sample inside the instrument. Despite the dispersion media used every measuring parameter (e.g. delay between adding the sample, delay between the single measurements) was kept constant between the different experiments. The same procedure was performed for a cyclosporine nanosuspension.

2.2.6. Influence of dissolution and optical parameters on size analysis

The LD results obtained from Section 2.2.5.2.2 with the rutin nanosuspension were analysed using Fraunhofer approximation and using the Mie formula, either with guessed optical parameters (real refractive index: 1.456, imaginary refractive index 0.001) or with the correct refractive index (real refractive index: 1.593, imaginary refractive index 0.02). In addition the LD results of two different cyclosporine nanosuspensions (measurements were performed with included PIDS and in nanosuspension saturated media) were analysed using the Mie formula with 30 different guessed optical parameters.

2.2.7. Influence of additional PIDS technology, comparison of conventional particle size analysis and optimized size analysis

Coarse cyclosporine was subjected to high-pressure homogenisation to develop an ultrafine, physically stable cyclosporine nanosuspension for oral administration. Different production methods, different formulations and different storage conditions were employed to identify the most appropriate production conditions, the most efficient stabilizer and the most suitable storage conditions. LD measurements were performed using correct optical parameters (real refractive index (RRI) 1.493; imaginary refractive index 0.03) and dispersion medium which was saturated with the nanosuspension itself. Measurements were performed with and without additional technique (PIDS). The results were compared to investigate the usefulness and the meaningfulness of the conventional and the optimized method regarding product development. Light microscopy was performed in parallel for all the formulations investigated.

3. Results and discussion

3.1. Influence of dissolution on size analysis

3.1.1. Dynamic light scattering

The extent of dissolution depends on the amount of dispersion medium added if the amount of the soluble compound (μl nanosuspension) is kept constant. Hence dissolution should be more pronounced if the volume of dispersion medium is higher. To investigate this difference the sample amount analysed was kept constant to 10 μl and the sample was analysed in 1 and 2 ml of dispersion medium, respectively. The results for the three different dispersion media (PCS diameter in nm and polydispersity index, as a measure for the width of the size distribution) are shown in Fig. 1.

The results clearly show the strong influence of dissolution on the size results. The sample analysed in water shows the smallest particle size, because the dissolution effects are most pronounced (Fig. 1, left). Furthermore the difference in the size obtained between the 1 ml dispersion medium and the 2 ml dispersion medium is large (1020 nm versus 946 nm). The PDI for the measurement in 2 ml dispersion medium is smaller, because the dissolution is more pronounced, which leads to a narrower size distribution (cf. Section 3.1.2 and Fig. 3). The effect on dissolution can be decreased if saturated medium is used. However, as nanocrystals possess an increased saturation solubility, saturation of the dispersion medium with coarse drug powder (Fig. 1, middle) is not sufficient, nanocrystals still dissolve during the measurement leading to differences in size and polydispersity index (PDI), depending on the volume of dispersion medium used. Dissolution can only be avoided if the dispersion medium is saturated with the nanocrystals themselves (Fig. 1, right). The size and the PDI obtained are the same and independent on the volume of dispersion medium used. Only at these measuring conditions correct size results are obtained.

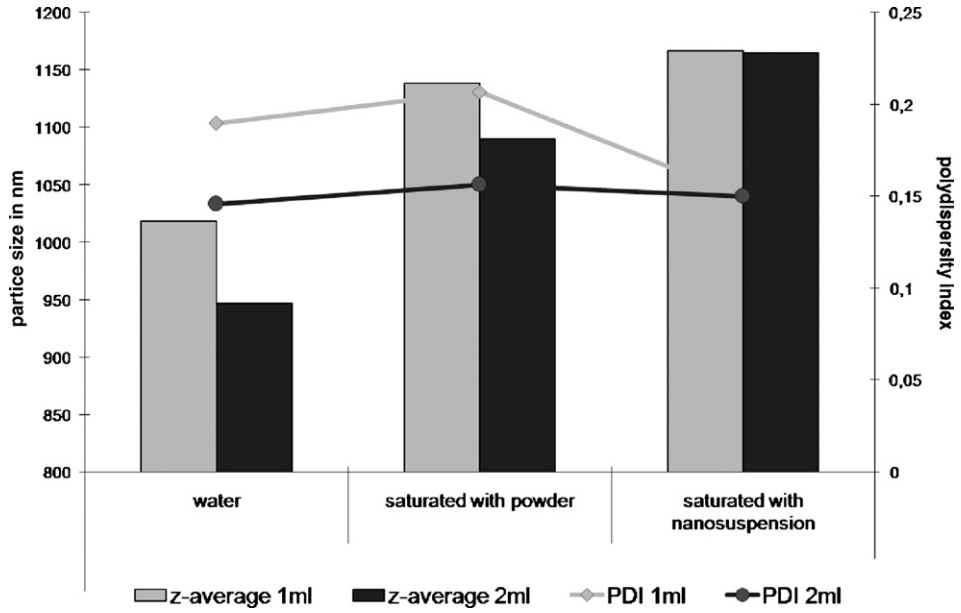


Fig. 1. PCS diameter (*z*-average) in nm and polydispersity index (PDI) of the rutin nanosuspension, analysed in either 1 or 2 ml of unsaturated (left), partly saturated (middle) and fully saturated (right) dispersion media.

3.1.2. Laser diffraction

The volume of the dispersion medium required for one measurement depends on the instrument and on the sample cell type of the respective instrument and cannot be changed. The typical time required for a measurement also depends on the respective instrument and the number of repetitions. A standard measuring time for the LS 230 is about 3 min (three repetitions) if PIDS is included into the measurements. However, the total time of the measurement can vary, because there is always a delay between adding the sample to the sample cell and the actual start of the measurement. In this experiment changes in size were followed over a time of 15 min (15 repetitions). The results obtained for the different types of dispersion media are shown in Fig. 2.

Pronounced dissolution takes place if pure water is used as dispersion medium (Fig. 2, left). The size strongly changed over the time of the measurements. Finally no result could be obtained anymore after 8 min, because the sample was dissolved completely. Less dissolution takes place if the dispersion medium is saturated with coarse drug powder (Fig. 2, middle). However, as the saturation equilibrium is only reached for large crystals, small nanocrystals dissolve, which leads to a fast drop in size at the beginning of the measurements. As a consequence, the size result obtained is too small. Saturation with the nanosuspension (Fig. 2, right) could avoid dissolution over the complete period of the measurement, as the size remained unchanged.

The results prove that dissolution strongly influences the size results obtained. To avoid any incorrect size results, it is necessary to use correctly, fully saturated dispersion media. In case nanosuspensions are analysed, medium saturated with the nanosuspension itself or even a smaller sized nanosuspension is required.

If measurements are performed without saturation the size results are too small in most of the cases. It is also important to note, that the size will not only decrease over the time! Depending on the progress of dissolution, the size can also increase during

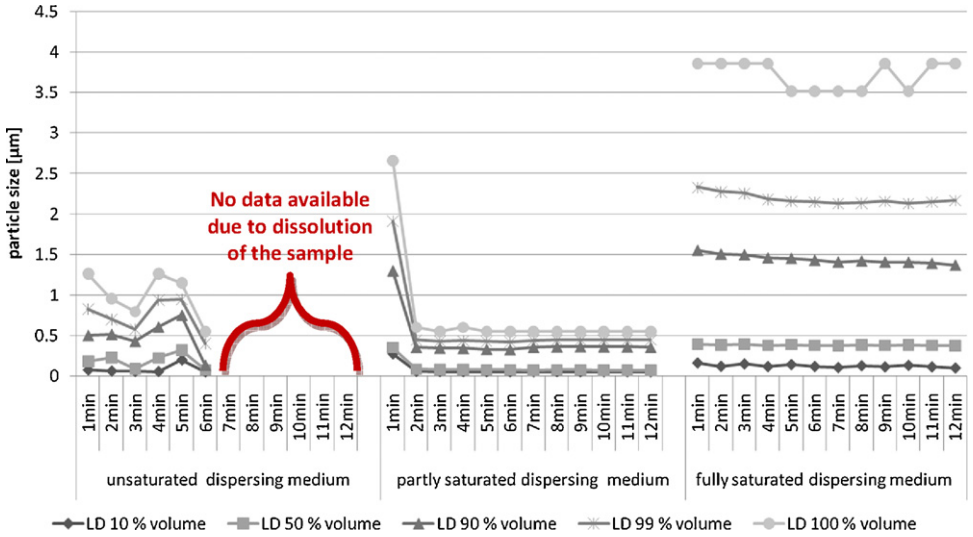


Fig. 2. LD data (diameters *d*(v) 10, 50, 90, 99 and 100%) for the rutin nanosuspension analysed in unsaturated (left), partly saturated (middle) and fully saturated (right) dispersion media.

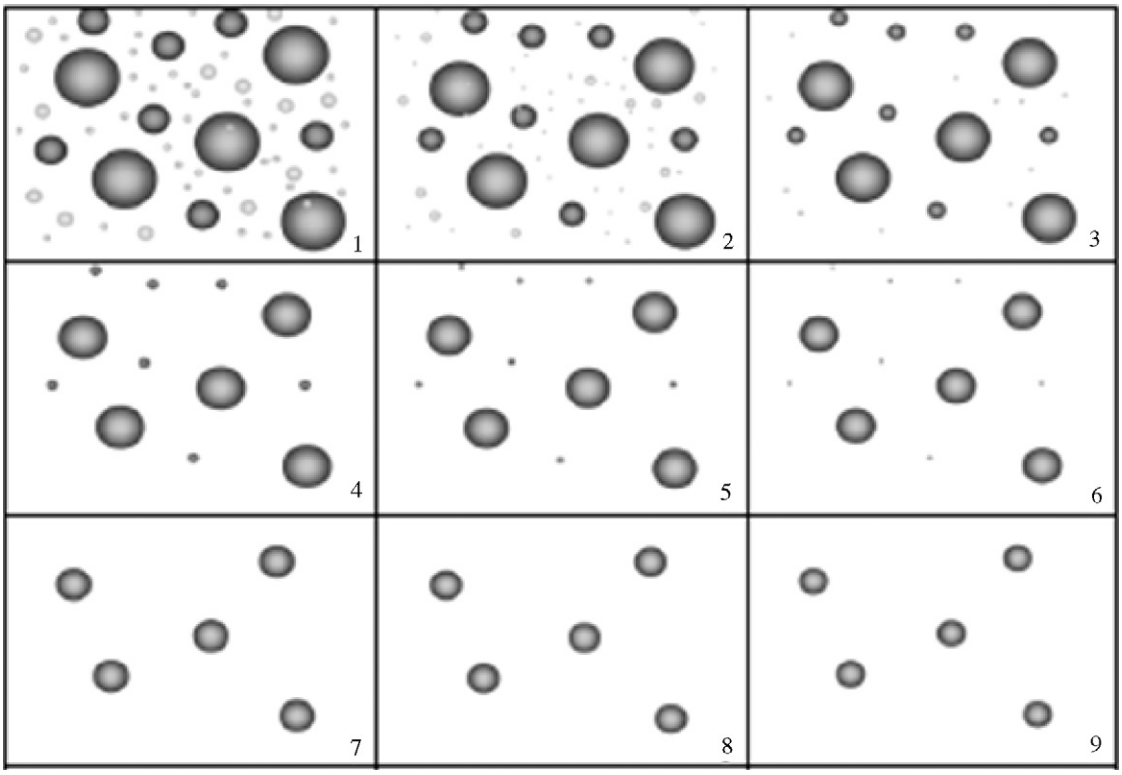


Fig. 3. (1–9): Principle of dissolution; 3.1: original sample, containing small particles, medium sized particles and larger particles. Dissolution takes place from 3.2 to 3.9; 3.2–3.3: decrease in size, because all particles dissolve, small particles dissolve faster than medium sized and larger particles; 3.4: complete dissolution of the small particles, leading to an increase in size; 3.5–3.6: decrease in size due to further dissolution of remaining particles; 3.7: increase in size due to complete dissolution of medium sized particles; 3.8–3.9: further decrease in particle size due to dissolution of the remaining large particles.

the measurement (cf. Fig. 2, left, after 3 min). This phenomenon is due to the complete dissolution of the smallest particles. The particle size will decrease until all the fine particles are dissolved, than after they have been disappeared, the size will increase suddenly. Further dissolution leads to a decrease in size again and so forth. Thus also the reproducibility of such measurements is not given. Fig. 3 depicts this phenomenon in more detail, showing that the size initially decreases, increases, decreases and increases again as a function of time dependent dissolution of differently size particle populations.

Fig. 4 shows the results of the analysis of a cyclosporine nanosuspension. Also here the influence of dissolution on the results obtained is clearly visible. However, the changes due to dissolution effects are different compared to the results from the rutin nanosuspension. Almost similar results were obtained for water as dispersion medium (Fig. 4, left). However, the changes in the

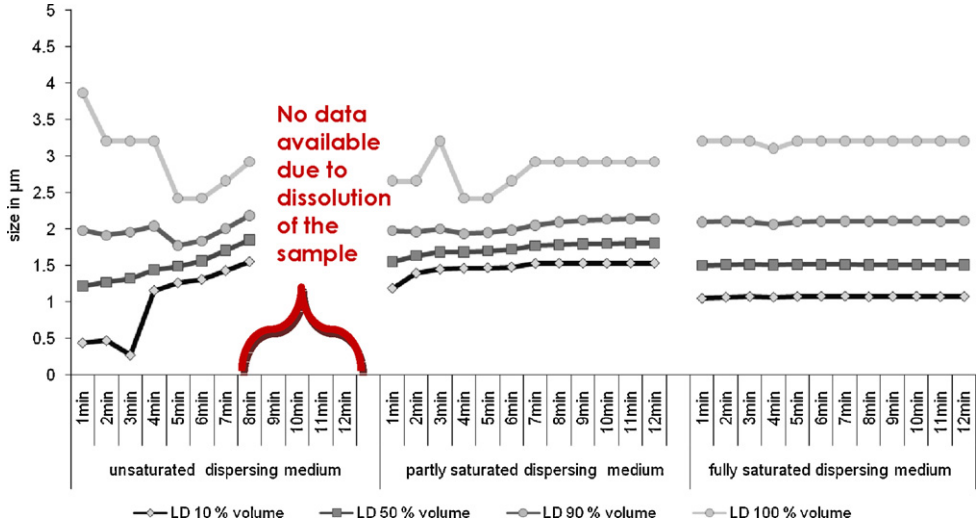


Fig. 4. LD data (diameters $d(v)$ 10, 50, 90, 99 and 100%) of a cyclosporine nanosuspension analysed in unsaturated (left), partly saturated (middle) and fully saturated (right) dispersion media. The dissolution effects are different than for the rutin nanosuspension (Fig. 2), indicating that dissolution is erratic and not reproducible. Dissolution can be avoided by saturating the dispersion medium with the nanosuspension itself. Therefore reproducible results can only be achieved using "correctly" saturated media for the measurements.

powder saturated medium (Fig. 4, middle) are different than for the rutin nanosuspension (Fig. 2, middle). In contrast to the rutin nanocrystals the size of cyclosporine nanosuspension increased after 3 min, than decreased (4 min) and finally increased and remained constant after 7 min of the measurement. The changes are related to the same phenomenon already explained in Fig. 3. However, the measurement in fully saturated medium (Fig. 4, right) clearly shows, that full saturation avoids dissolution effects during the measurement. The particle size stays constant over the whole time of the measurement.

By comparing the data obtained from the rutin nanosuspension and the cyclosporine nanosuspension it turns out, that reliable data can only be achieved by a full saturation of the measuring medium (i.e. in this case "supersaturation" with nanocrystals). The dissolution process depends on the solubility and the dissolution velocity of the compound, as well as on the particle size of the sample and the time of the measurement and therefore will be different for each sample. In conclusion, reproducible results are only achieved if fully saturated dispersion medium is used.

3.2. Influence of dissolution and optical parameters on size analysis

The very pronounced influence on the size result by the optical parameters was shown in a previous study for multimodal latex dispersions (Keck and Müller, 2008). In this study the LD data from the rutin nanosuspension were used to demonstrate the impact for nanosuspensions on the analysis result. For the model calculations the average of the first three measurements (repetitions 1–3), which corresponds to a standard measurement, was used for the analysis. The light scattering data which were obtained from the measurement in unsaturated medium and in fully saturated medium were analysed using either Fraunhofer approximation or the Mie formula. Analysis with the Mie formula was performed with incorrect and correct optical parameters, respectively. The results are shown in Figs. 5 and 6.

Fig. 5 shows the size distribution curves of the size analysis. All the results from the measurements in water lead to much smaller sizes ($D10$–$D99\%$), than the results obtained for the measurements in fully saturated medium. Also the size distribution curves vary for each analysis. The differences in results become even more visible when comparing the LD diameters, e.g. LD (v) 50% and LD (v) 90% (Fig. 6). Each analysis leads to a different result. Whereas the sizes for the measurements in unsaturated medium are too small (Fig. 6, left), the measurements in fully saturated medium are larger, but differ tremendously depending on the analytical model (Fraunhofer versus Mie) and optical parameters (correct versus incorrect). Importantly it needs to be noted, that the LD (v) 50%, often referred as a measure for the mean bulk diameter, is only around 400 nm for the analysis using Fraunhofer approximation and Mie formula with incorrect optical parameters. In contrast it is above 800 nm for the correct analysis result (Fig. 6, very right). Clearly, an incorrect analysis (model or parameters) will lead to a misinterpretation of the system analysed and to misleading conclusions for further investigations therefore.

As mentioned above, the LD (v) 50% is often referred to be a measure for the mean particle size, and very often the LD (v) 90% and LD (v) 99% are used as a measure for the presence of larger particles in the sample. On the base of these considerations the LD (v) 50% and the LD (v) 99% of different cyclosporine nanosuspensions are

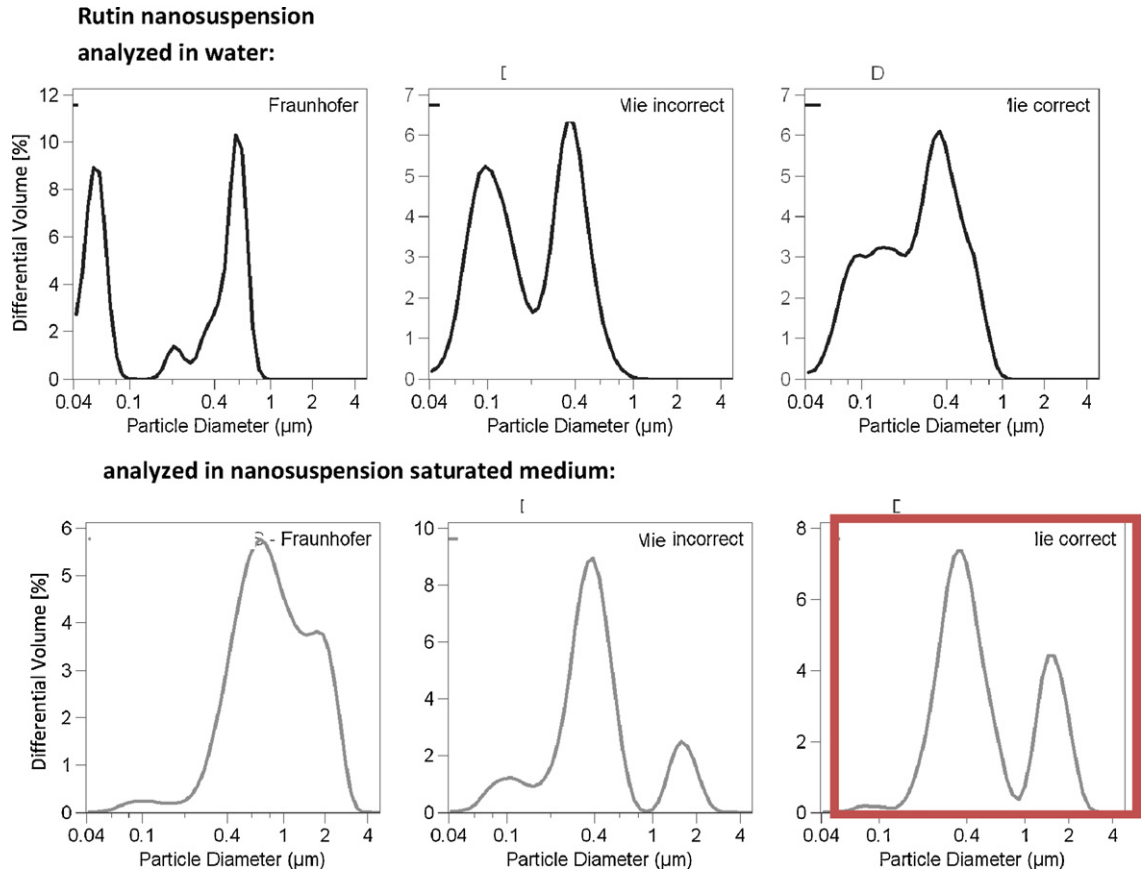


Fig. 5. Influence of optical parameters and dissolution on the size distribution curve (LD data, differential volume). The rutin nanosuspension was analysed using unsaturated medium, i.e. water (upper) and nanosuspension saturated medium (lower). The results obtained were analysed using Fraunhofer approximation (left column), Mie formula with incorrect optical parameters (RRI 1.456, IRI 0.01, middle) and with correct optical parameters (RRI 1.593, IRI 0.02, right). For only one sample analysed six completely different size results were obtained, being different in the mean size (D 50%) and the width of the size distribution and the all over distribution curves (modality).

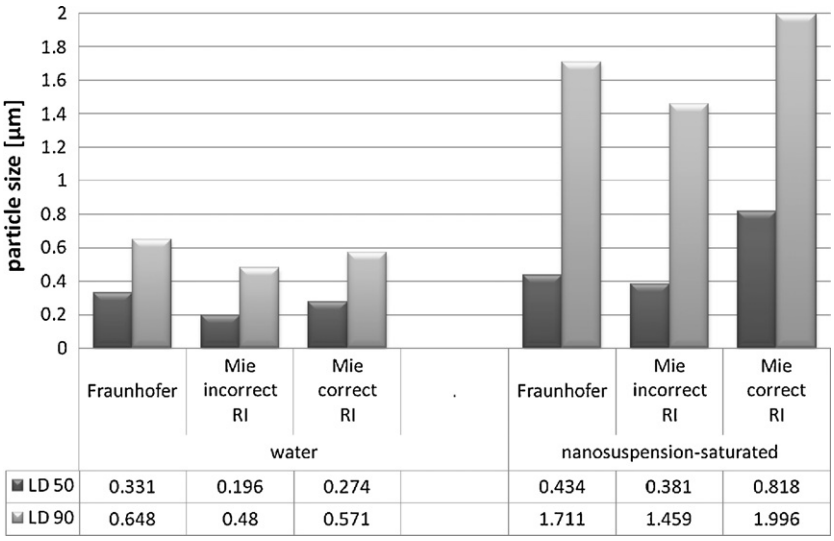
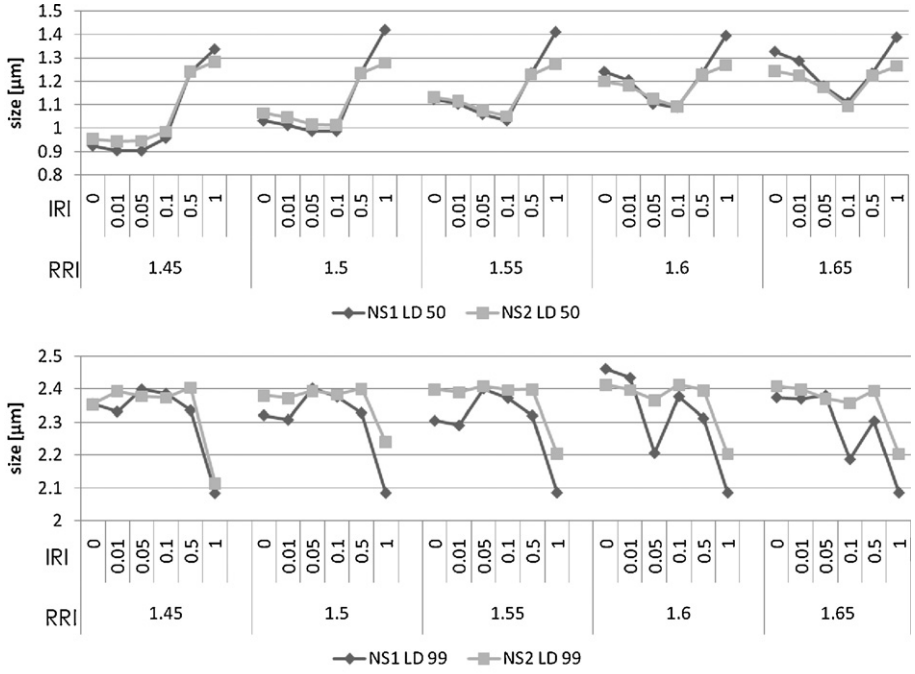


Fig. 6. Differences in LD size data (diameters $d(v)$ 50 and 90% of the rutin nanosuspension analysed in unsaturated (left) and fully saturated (right) dispersion media). Measurements in unsaturated media do not detect particles larger than 1 μm, leading to too small size results. The analyses in saturated medium detect large particles. Analysis with either Fraunhofer approximation or Mie formula using incorrect optical parameters leads to incorrect mean particle sizes being only about 400 nm. In contrast the use of the Mie formula with correct optical parameters leads to a mean particle size (D 50%) of 834 nm (0.834 μm).

compared between each other. In Fig. 7 two different cyclosporine nanosuspensions were analysed in fully saturated medium. The results obtained were analysed using different optical parameters (real refractive indices (RRI) used: 1.45, 1.5, 1.55, 1.60 and 1.65; imaginary refractive indices (IRI) used: 0, 0.01, 0.05, 0.1, 0.5 and 1). For the analysis each real refractive index selected was combined with each imaginary refractive index as one respective optical parameter, which gave a total of 30 results per nanosuspension. The results are presented in Fig. 7.

Depending on the optical parameter used, the size results vary for the two different nanosuspensions. Depending on the optical parameters used either nanosuspension 1 is smaller (e.g. RRI 1.45, IRI 0.01), larger than (e.g. RRI 1.6, IRI 0) or about the same size (e.g. RRI 1.55, IRI 0.05) as nanosuspension 2. Hence, depending



* Results were analysed using the real refractive indices (RRI): 1.45-1.65 and the imaginary refractive indices (IRI): 0-1, each RRI was combined with each IRI giving a total of 30 optical modules which were used for analysis. The corresponding results are shown above. Each diamond corresponds to one optical model.

Fig. 7. Influence of optical parameters (formulations: five different cyclosporine nanosuspensions stabilized with different stabilizers, each formulation produced with four different production parameters (V1–V4, cf. Section 2.2.1), stored either at room temperature (dark bars) or at 4 °C (grey bars)) on the LD parameters LD 50% (upper) and LD 99% (lower) for two different cyclosporine nanosuspensions (NS 1 and NS 2). Depending on which optical module is used for analysis NS 1 is smaller than NS 2 (e.g. RRI 1.45, IRI 0.01), larger than NS 2 (e.g. RRI 1.6, IRI 0) or identical to NS 2 (e.g. RRI 1.55, IRI 0.05).

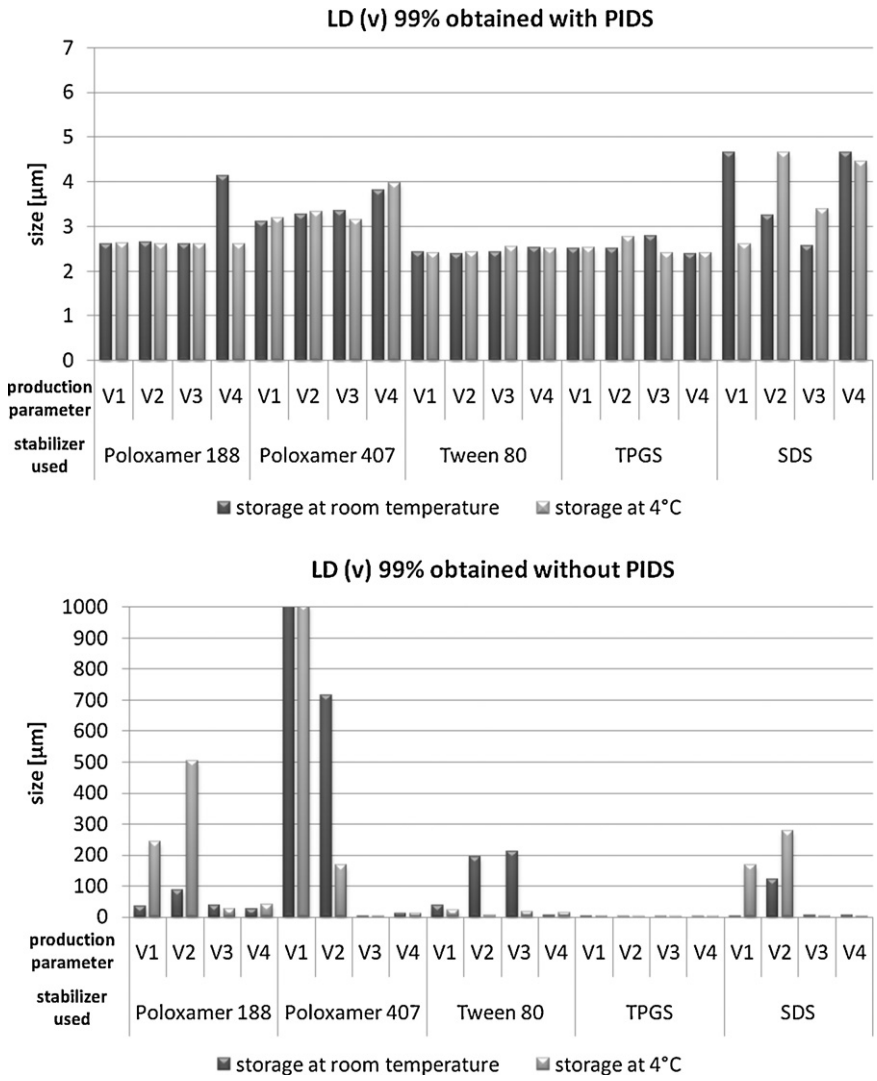


Fig. 8. Influence of additional technique (PIDS) on size results (LD *d*(v) 99%) for 40 different cyclosporine nanosuspensions (formulations: five different cyclosporine nanosuspensions stabilized with different stabilizers, each formulation produced with four different production parameters (V1–V4, cf. Section 2.2.1), stored either at room temperature (dark bars) or at 4 °C (grey bars)). Upper: analysis with included PIDS technology—none of the suspension shows particles above 5 µm, indicating good physical stability for all the suspensions investigated. In contrast analyses without PIDS (lower) clearly show the existence of large particles for the majority of the suspensions, indicating that most of the suspensions are not physically stable.

on which optical model is used nanosuspension 1 will be judged to be smaller, larger or identical to nanosuspension 2. This again proves the importance of correct optical parameters for research and development.

3.3. *Influence of additional PIDS technology, comparison of conventional particle size analysis and optimized size analysis*

To finally prove the importance of optimized size measurements, a case study was performed by developing a cyclosporine nanosuspension for oral formulation. The aim of the development was to identify the most appropriate production parameters, most efficient stabilizers and optimal storage conditions to obtain a small sized and physically stable nanosuspension. As shown before, the employment of the additional techniques (e.g. PIDS for the LS 230) can lead to the overestimation of small particles (e.g. nanoparticles) and a lack in detection of possible large particles (Keck and Müller, 2005b, 2008). Because very often LD is especially used for the detection of larger particles besides a small sized main population, this finding is of great importance. Therefore in this study all LD measurements were performed with and without included PIDS technology. Fig. 8 summarizes the results for some formulations from this study. It shows the particle sizes obtained for 40 different formulations after 4 weeks of storage. The upper part shows the results which were obtained from the measurement with included PIDS, the lower part shows the data from the measurements without included PIDS. All samples were measured in fully saturated medium and analysed with correct optical parameters (RRI 1.493, IRI 0.03).

The upper part of Fig. 8 shows the results obtained with included PIDS technology, no particle sizes above 5 µm (LD (v) 99%) could be detected via this analysis method. The interpretation of the data would lead to the conclusion, that all the formulations investigated are suitable for further investigation. No large influence was detected between the different stabilizers used (Poloxamer 188 (PLX188), Poloxamer 407 (PLX 407), Tween 80 (Tween), D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) and sodium lauryl sulphate (SDS)). Also the different production methods (V1–V4) and the storage temperature (room temperature or 4 °C) had no visible influence on the stability.In contrast large differences were found for the measurements without included PIDS technology (Fig. 8, lower). From the 40 formulations shown only

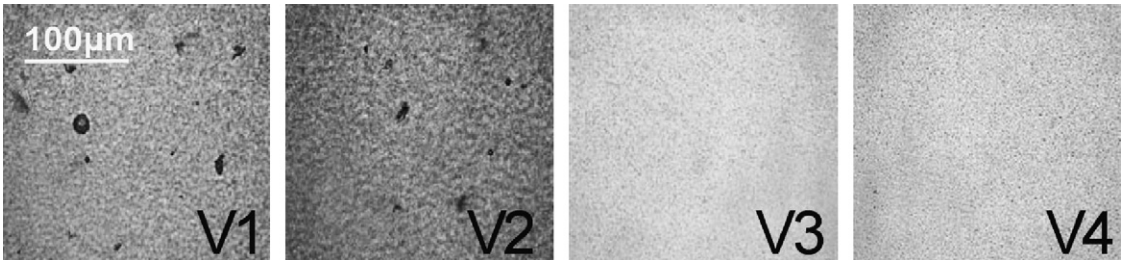


Fig. 9. Micrographs (magnification 160×) of the cyclosporine nanosuspensions stabilized with TPGS, stored at room temperature. Agglomerates can be detected for the suspensions with the production parameters V1 and V2, no agglomerates are found in the nanosuspensions with the production parameters V3 and V4. LD analysis (both conventional and optimized method) could not detect these large agglomerates, probably because they are loose and are destroyed by the stirrer of the LD instrument during LD analysis.

the formulations with the bioavailability enhancing TPGS appear to be physically stable, and formulations V3 and V4 stabilized with SDS, the PLX 407 stabilized formulations V3 and formulation V2, stored at 4 °C stabilized with Tween seem to be stable. The LD parameters (v) 50 and 99% obtained for these formulations are shown in Table 1, clearly proving that the formulation V4, stabilized with TPGS, stored at room temperature is the most suitable formulation for further investigations. Furthermore via this analysis method it was possible to differentiate the more appropriate production parameters (V4) and to detect differences in stability which depend on the storage conditions on the different stabilizers used.In addition light microscopy was employed for all the formulations investigated. A small magnification (160×) was used to detect large particles and a larger magnification (1000×) was used to characterize the morphology of the small particles. The images taken for the TPGS nanosuspensions, stored at room temperature, are shown in Fig. 9. The two micrographs left show the formulations V1 and V2. Both formulations contain agglomerates, which were not detected by laser diffraction. Probably they are loose enough to be destroyed by the stirrer of the instrument, which is needed to pump the sample through the measuring cell. Agglomerates are disadvantageous for the physical stability of nanosuspensions, as they can promote Ostwald ripening and crystal growth over time. Thus the detection of agglomerates is crucial for the evaluation of the physical stability. The two micrographs on the right side (V3 and V4) do not show any agglomerates, indicating good physical stability.Taken all results together; from 40 formulations, 36 formulations could be excluded from further investigations, due to optimized size measurements. This would not have been possible using the conventional sizing method.

Table 1
Particle sizes of the 15 best formulations of the screening (LD data, LD (v) 50 and 99%, measured using the optimized method). The formulations stabilized with TPGS, with the production parameter V4, stored at room temperature (bold) is the most suitable formulation. It possesses the smallest mean particle size (LD 50%) and no larger crystals (smallest LD 99%). The formulations stabilized with TPGS with the production parameters V3 and the formulation TPGS V4, stored at 4 °C (black ink) are also suitable. All other (grey ink) formulations show large LD 50% and a broader size distribution, which indicates less physical stability.

Formulation	Storage condition			
	Room temperature		4 °C	
	LD (v) 50%	LD (v) 99%	LD (v) 50%	LD (v) 99%
PLX 407				
V3	1.644	3.35	1.662	3.301
Tween 80				
V2			1.575	6.186
TPGS				
V1	1.298	2.508	1.298	3.295
V2	1.346	2.514	1.357	2.859
V3	1.135	2.791	1.121	2.843
V4	**1.098**	**2.387**	1.117	2.801
SDS				
V3	1.208	2.568	1.211	3.397
V4	1.162	5.794	0.993	4.445

4. Conclusions

Particle size analysis is one of the most important characterization parameters for product development. The characterization of nanoparticles using dynamic and static light scattering techniques can produce meaningful results, if essential prerequisites are fulfilled. The prerequisites are to ensure no dissolution of the sample during the measurement and to employ the Mie formula with correct optical parameters for laser diffraction analysis. As laser diffraction is used to detect possible larger particles, measurements should also be performed without the additional techniques built into the LD instruments. In addition light microscopy should be used to detect possible agglomerates, which can be destroyed during the LD measurements. If size characterization is performed in this optimized way, a clear discrimination between samples can be done, which is not possible in most of the cases when the “conventional” sizing method is used. Optimized particle size analysis enables the identification of possible candidates for further investigation steps at an early stage of the research.

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