QUANTITATIVE CLASSIFICATION OF PARTICLES IN BIOLOGICAL LIQUIDS VIA SPES TECHNOLOGY

INTRODUCTION

A quantitative multiparametric analysis of particles incubated in target biological liquid matrix via Classizer™ ONE and SPES patented method is reported. Two application cases of capital importance are considered:

a) drug carriers based on particles. These systems are receiving every day increasing attention as **effective tools for the modulation of the pharmacokinetic and the pharmacodynamic profiles of delivered drugs**. One of the major points of weakness and limitation for therapeutic use of delivery systems resides on the lack of knowledge about their behaviour after reaching the heterogenous target media.

b) **eco-/cito-toxicology studies** facing nowadays global problems of **particle short and long-term impact** on ecosystems and human health. As a reference, nanoplastics (≤1000 nm) could pose real and uncontrolled challenge due to their small size and sharp ability to penetrate living organisms at any level. High quality and quantitative information, e.g. analysis and count of particle uptake in cell lysate, are needed to better evaluate particulate effects still far from being adequately understood.

Traditional analytical approaches rely on light scattering methods as DLS, SLS, NTA and Obscuration, which cannot provide reliable results due to the presence of the biological components in target real solution. EOS Classizer™ ONE quantifies and characterises particles in unfiltered liquids paving the way for ex vivo **eco-/cito-toxicology studies** of particles and improve product formulation evaluating the modifications that particles undergo in real target media.

PARTICLE ANALYSIS METHOD

Among the several methods currently adopted, optical ones have unique advantages, and therefore, have brought light scattering into the forefront of analytical methods in many scientific and industrial applications. Unfortunately, the number of parameters typically affecting the scattering properties of a given particle is such that the basic measure of the scattering power (or even the power removal from a light beam -extinction- from one particle) is far from being enough to recover something more than a rough estimate of its size. Things change appreciably when considering a collection of many scatterers, with the immediate drawback of introducing the need for mathematical inversion and ill-posed problems to interpret experimental real data.

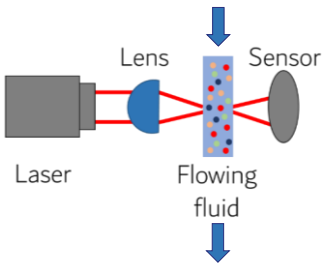
Figure 1 EOS Classizer™ ONE – front view

EOS Classizer™ ONE particle analyser is based on patented Single Particle Extinction and Scattering (SPES) method. It introduces a step forward in the way light scattering is exploited for single particle characterization.

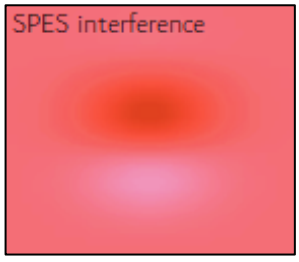
EOS Classizer™ ONE provides data that go beyond the traditionally optical approaches. EOS Classizer™ ONE discriminates, counts, and analyses single particles through their optical properties. It retrieves to the user several pieces of information such as: particle size distribution of the single observed populations, absolute and relative numerical concentrations, particle stability, information on optical particle structure and oversize. Classizer™ ONE works offline and online/real-time, enabling to verify consistency of intermediate and final formulations with target QbD, SbD, and Quality Control target expectations.

SPES TECHNOLOGY IN A NUTSHELL

The patented Single Particle Extinction and Scattering (SPES) method is based on a self-reference interferometric measurement of the scattered wavefront in the forward direction by a single illuminated particle.

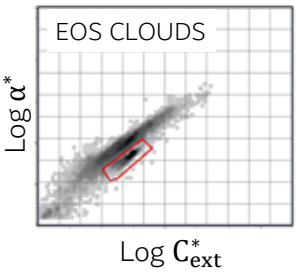


Particles are driven by a laminar fluid flow (liquid or gas depending on the application/CLASSIZER™ version) through the waist region of a tightly focused laser beam.



The intense transmitted beam interferes with the faint scattered wavefront in the far field, thus superimposing the two waves with the same curvature. This causes the interference pattern to exhibit intensity modulations on the spatial scale of the beam itself.

Two scattering features are sampled to follow the evolution of the intensity modulations during the passage of each single particle through the beam: i) the global attenuation given by the particle which removes a small fraction of the incoming power; ii) the fringes given by the partial constructive and destructive interference, proportional to the amplitude of the complex forward adimensional scattered field S(0). These two features are directly related to the real $\Re e\ S(0)$ and the imaginary $\Im m\ S(0)$ components of S(0), as stems from the Optical Theorem [H. C. van de Hulst, Light Scattering by Small Particles, 1981].



The Extinction Cross Section $\Re e\ S(0) = C^*_{ext} = \frac{k^2}{4\pi} C_{ext}$ and the Polarizability $\Im m\ S(0) = \alpha^* = k^3\ \alpha$, where $k = 2\pi n/\lambda$ is the wave number in the medium n at wavelength λ, **are thus retrieved for each single detected, validated, and counted particle** thanks to a robust Pulse Shape Analysis scheme and proprietary algorithms, without adopting ill-posed problems, like the inversion or deconvolution (other optical parameters could be alternatively retrieved, eg. particle optical thickness ϱ).

In a few minutes SPES/ CLASSIZER™ creates the unique **EOS CLOUDS**: a 2D histogram which is the optical fingerprint of the sample. Heterogeneous samples produce simultaneously different clouds for each particle population, which can be individually selected, analyzed, and compared. Particle size distribution, numerical concentration, oversize, and other statistical insights are retrieved accordingly to the selection, to the whole sample, or for each time frame acquired in CFA mode. Statistical approaches as PCA are furthermore viable to extract unique information typically inaccessible nowadays.

Added-value information is provided thanks to **SPES** and **EOS Classizer™ ONE** unique data and analysis libraries:

- **Optical Classification,** Absolute **Particle Size Distribution, Numerical Concentration** of each single population irrespectively of polydispersity/composition.

- Quality Control of particle **porosity, wetting**, **aspect ratio**, **payload**, **impurities**, **scraps**, and **shelf-life without intermediate steps** (purification/filtration).

- Measurement of **particle behavior and formulation stability** directly in real **heterogeneous non-filtered target biological, industrial, or environmental fluids**.

- Hi-Resolution **Continuous Flow Analysis**, also coupling SPES information with other analytical devices as CF3 separators, small chemical reactors, and pilot line.

- Statistical approaches as **Oversize Measure** and **PCA** for Hi-Quality Batch-2-Batch analysis and out-of-specifics identifications in product formulation and production.

Depending on the system configuration and sample, EOS Classizer™ ONE covers a dynamic range of 0.1 – 20 µm, concentration range of 1E5-1E7 ptc/mL @ 0.5-5ccm. External auto-dilution sampler and autosampler available.

EOS Classizer™ ONE, based on patented SPES method, is the ideal solution for improving formulations, for verifying product consistency with the target Quality-by-Design final expectations, and eco-/cito-toxicity studies.

This document presents representative examples of applications of EOS Classizer™ ONE and does not cover all the cases where the EOS Classizer™ ONE / SPES method solves the particle identification, classification, and characterisation of challenges in biological target heterogeneous fluids. EOS software release SW1.4.32 is used for the data analysis and for the export of the figures.

APPLICATION EXAMPLES

CASE A) STUDY OF **DRUG CARRIERS** BASED ON PARTICLES IN HUMAN PLASMA.

EOS Classizer™ ONE is exploited to characterize polydisperse poly(lactic-coglycolic acid) (PLGA) particles synthesized by Oil-in-Water (OW) solvent evaporation emulsion technique with and without PEG as surfactant. Commercial PLGA (Mw 25 kDa, 75:25 mol ratio D,L-lactide) has been selected for the emulsion and particle synthesis. ζ-potential of samples ranges from -30mV for PLGA to -40mV for PLGA+PEG particles.

The emulsion of PLGA is a typical drug delivery model with clinical relevance due to the PLGA biocompatibility and the possibility to finely tune particle size and drug payloads. For a general introduction of SPES data with standard samples as polystyrene spheres, refer to the Application Note AN001/2021, available for free online at EOS website: www.eosinstruments.com/publications/

SPES data of the sole PLGA will be presented at first. Then the time behaviour of the PLGA particles with and without the PEG stabilisation incubated in human plasma will be compared to enlighten the differences due to the synthesis.

Figure 2 shows SPES data for the emulsion of PLGA diluted in a filtered phosphate buffer solution. For this measurement, the numerical concentration of the particles is not subject to study and is adjusted for the purpose of analysis at about 3E6 ptc/mL. The sample is loaded in a syringe and flows at 4ccm using a lab pump. In few minutes data from some thousands of particles have been acquired and populate the SPES CLOUDS histogram in *Figure 2*.

The grey tones of the cloud are proportional to relative numerical particle concentration. Location of data in the 2D SPES CLOUDS is an optical fingerprint of the sample.

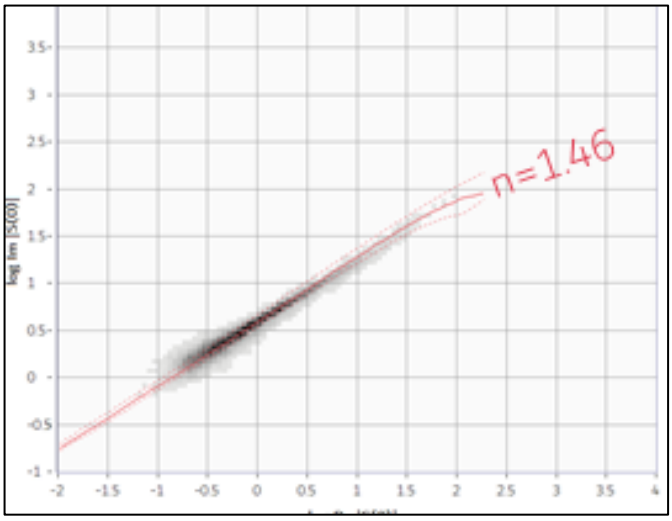


Figure 2 EOS CLOUDS histogram for the emulsion of PLGA filtered phosphate buffer solution. Position of experimental data (grey tones proportional to relative numerical concentration). Red line represents expected SPES position for PS spheres with different sizes.

Experimental data are compared to theoretical expected positions in the histogram for dielectric spheres of different sizes and refractive indexes. Different approaches can be considered, as tailored Mie or DDA. The best effective refractive index is thus automatically determined by EOS Classizer™ ONE, in this case n=1.46, in agreement with theoretical value at λ=640nm within experimental errors. Once retrieved the effective refractive index, particles are individually sized comparing their S(0) values with expected ones for spheres of different diameters. EOS Classizer™ ONE provides to user the Numerical Particle Size Distribution and other statistical values as AVG, CV, and quantiles (see *Figure 3*).

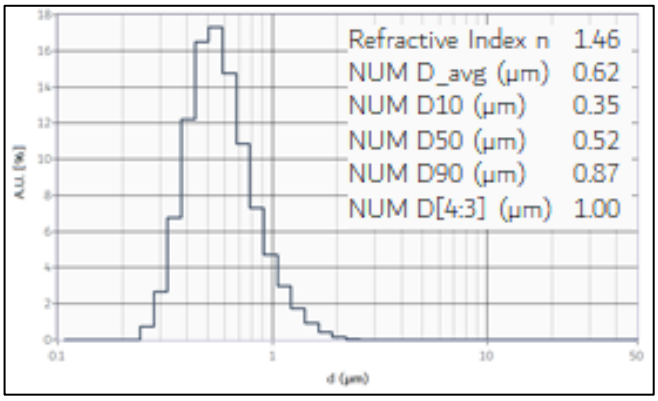


Figure 3 Experimental Numerical Particle Size Distribution of the emulsion of PLGA diluted in filtered phosphate buffer solution. Average particle diameter retrieved by EOS Classizer™ is 0.62 µm @ measured n=1.46. Mie scattering model is considered for particle sizing.

PLGA and PLGA+PEG samples in filtered phosphate buffer solution are incubated at 37°C for 48h. No relevant changes in optical properties of the particles as size and effective refractive are observed at scheduled times of 0h, 24h, and 48h. The conclusion of the preliminary test is that the particles are stable for the time of the incubation in phosphate buffer solution. Note. As a further preliminary observation, no optical difference between PLGA and PLGA+PEG particles are observed due to surfactant.

The protocol for the incubation in human plasma of the PLGA samples is a) aliquot 1mL of PLGA particle suspensions at concentration of about 2E10 ptc/mL; b) add 3mL of non-filtered human serum and incubated at 37°C; c) at scheduled times of 0h, 24h, and 48h, an aliquot of 40µL for each sample is withdrawn and subsequently diluted with 60 ml of milliQ water; d) SPES analysis. Three batches are prepared for each PLGA synthesis to guarantee the repetition of the analysis under controlled conditions.

In *Figure 4* SPES results of PLGA (top) and PLGA+PEG (down) in human plasma at 0h are presented. In both data, two particle populations can be observed. The first, narrow and elongated, corresponds to the well dispersed and stable particles. The other, broader, and corresponding to low effective refractive index particles (see arrows in *Figure 4*), is due to biological corpuscles of the human plasma.

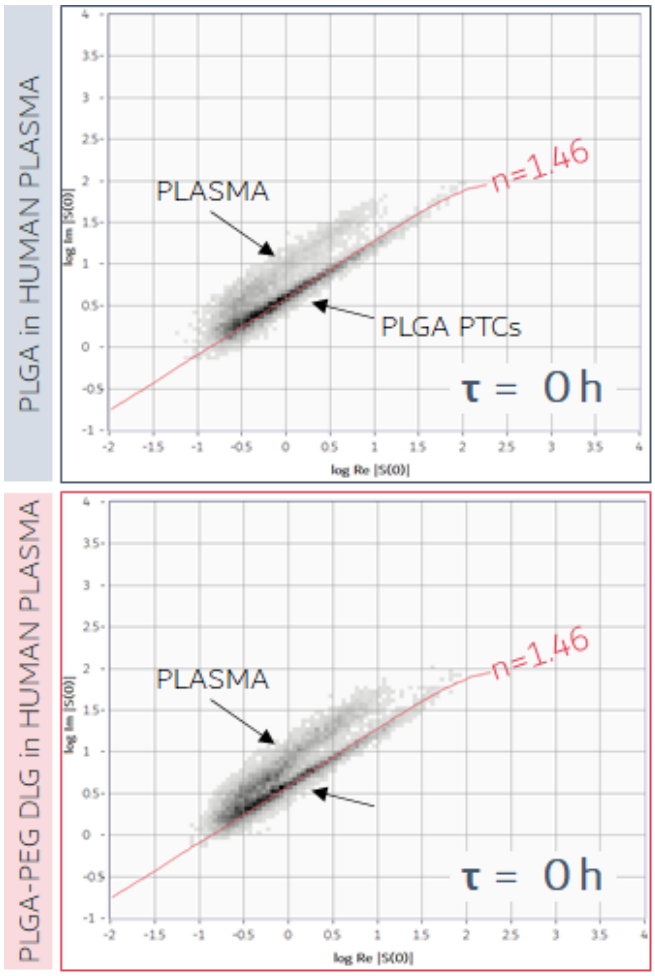


Figure 4 (top) EOS CLOUDS for a heterogeneous sample of human plasma mixed with an emulsion of PLGA particles. Two principal and separated populations are detected. Red line represents expected size trend for PLGA particles and agrees with data in *Figure 2* for PLGA. (down) EOS CLOUDS for a heterogeneous sample of human plasma mixed with an emulsion of PLGA stabilized with PEG particles. Two principal and separated populations are detected. Red line represents

expected size trend for PLGA particles and agrees with data in *Figure 2* for PLGA. No differences between the samples are observed at 0h time.

*Figure 5*Figure *4* reports SPES results obtained with PLGA (top) and PLGA+PEG (down) in human plasma after 24h of incubation at 37°C. Two EOS CLOUDS quantitatively show the aging of the PLGA in human plasma without the PEG stabilization. Data show appreciable shift indicating a lower refractive index. This effect is ascribed to the water absorption of the polymer with consequent swelling of the particle. The effective refractive index of such particles then decreases because of the average effect of the particle bulk material and the soaking liquid. On the other side the PLGA+PEG ptcs are stable and behave as measured at 0h.

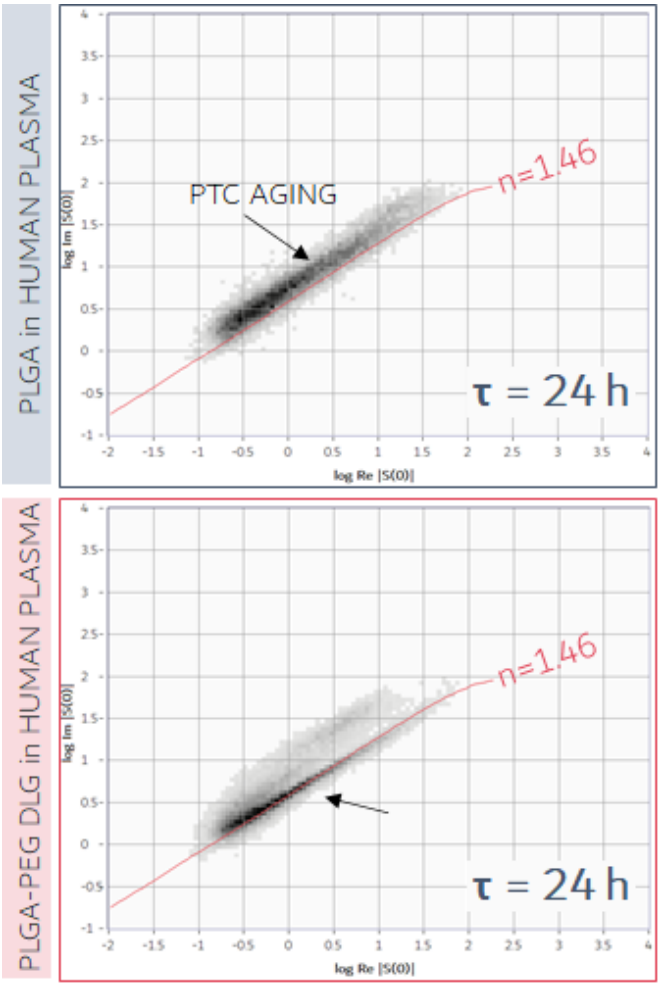


Figure 5 (top) EOS CLOUDS for a heterogeneous sample of human plasma mixed with an emulsion of PLGA particles after 24h incubation at 37°C. PLGA population changed its optical properties and shifted to a location corresponding to lower refractive index. Red line represents expected size trend for PLGA particles at 0h as a reference. (down) EOS CLOUDS for a heterogeneous sample of human plasma mixed with an emulsion of PLGA stabilized with PEG particles after 24h incubation at 37°C. Data are compatible with results at 0h, resulting in no significant change in optical properties of particles thanks to PEG stabilization. Red line represents expected size trend for PLGA particles.

Focusing on data of aged PLGA without PEG at 24h, some considerations can be done. First, data from the plasma contribution are enveloped by the PLGA data, the optical properties of the two populations being similar. Nevertheless, performing an accurate study of the plasma itself, the corresponding contribution can be statistically subtracted from the PLGA data. Note. This approach considers the two species as non-interacting. Otherwise, care must be taken to separate the effects due to swelling from those caused by the contribution of the interaction with the biological corpuscles.

Data in *Figure 5* (top) are compatible with an effective refractive index 1.30. EOS software provides the best Particle Size Distribution and other statistics as represented in *Figure 6*

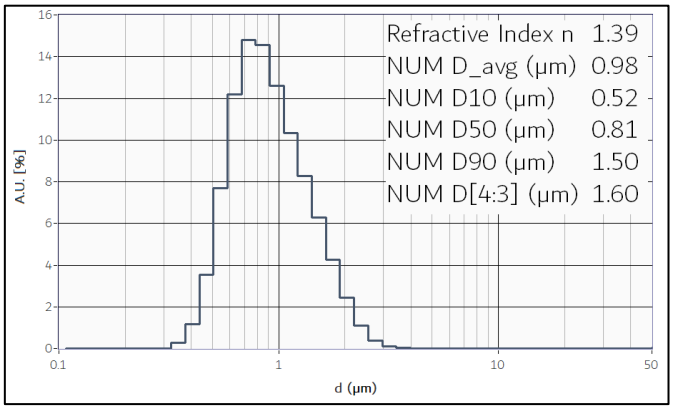


Figure 6 Experimental Numerical Particle Size Distribution of the aged PLGA population in human plasma after 24h incubation at 37°C. Average particle diameter retrieved by EOS Classizer™ is 0.98 µm @ measured n=1.39. Mie scattering model is considered for particle sizing.

Further information can be retrieved about the PLGA particle compactness via the **Average Filling Factor (AFF)** calculation provided by the EOS software. This tool can be used to give an estimate of the solid volume of a non-compact particle as an aggregate or a mesoporous particle. Average Filling Factor is based on the Mean Field Method: particles are modeled as spheres following the Lorenz-Mie approach, and the particle polarizability is approximated by assuming the mean-field approximation (MFA) (Chylek et al. 1988; Bohren and Huffman 2008). For the particles under study (bulk material refractive index n=1.46, effective aged refractive index n=1.39, and solvent n=1.33), the AFF model retrieve a **filling factor of 46.6 %.** In simple terms, slightly more than half of the aged particles is filled with water.

In *Figure 7*Figure *5*Figure *4* SPES results of PLGA (top) and PLGA+PEG (down) in human plasma after 48h of incubation at 37°C are presented. After this incubation period also the PLGA particles stabilized with PEG exhibit aging. Their optical properties are appreciably changed, thus indicating a change in structure and stability properties of the particulate. Similarly, to the previous case, EOS CLOUDS are shifted towards a region of the plot corresponding to lower refractive index.

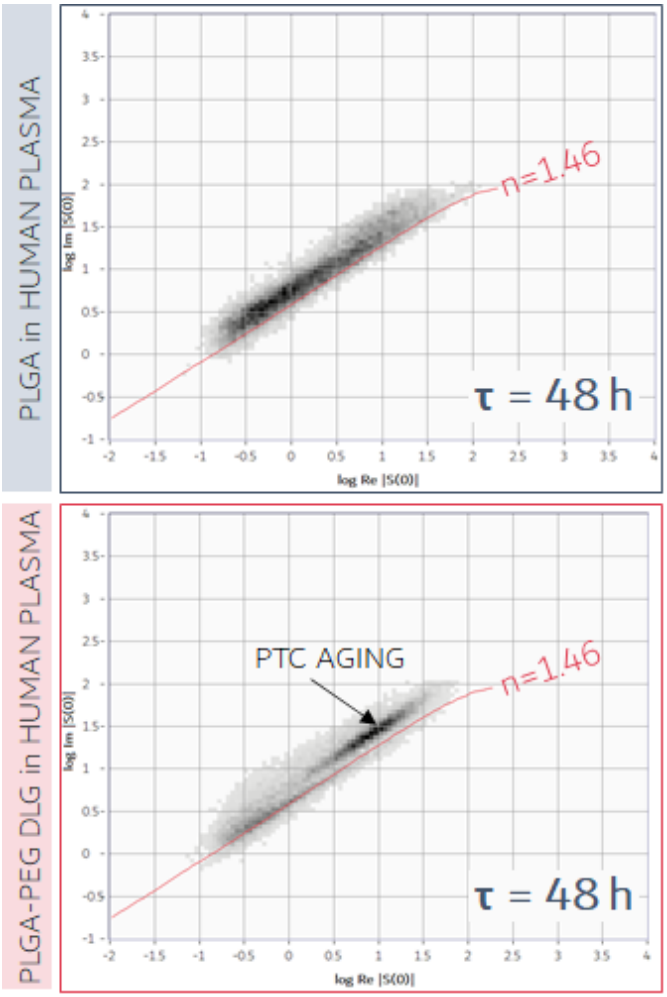


Figure 7 (top) EOS CLOUDS for a heterogeneous sample of human plasma mixed with an emulsion of PLGA particles after 48h incubation at 37°C. PLGA populations changed its optical properties and shifted to a location corresponding to lower refractive index. Red line represents expected size trend for PLGA particles at 0h as a reference. (down) EOS CLOUDS for a heterogeneous sample of human plasma mixed with an emulsion of PLGA stabilized with PEG particles after 48h incubation at 37°C. Secondary low refractive index particle population appear meanings that the PLGA+PEG particles aged changing their optical properties. Red line is a guide for the eye and represents the expected trend for PLGA particles at 0h as a reference.

The application example confirms the capability of EOS Classizer™ ONE and SPES patented technology to identify and classify particles in heterogeneous biological fluids as human plasma. This opens novel paths for Quality by Design and Safety by Design formulations analysing the particle behaviour directly in the target media where the chemical-physical characteristics of the liquids may affect the stability of the formulation. Problems as particle aggregation, degradation, and controlled release can be faced in real media optimising the choice of carrier material (e.g. type of polymer or molecular weight) and surfactant based on quantitative multiparametric information.

Case B) Identification of NanoPlastic particles in unfiltered cell lysate.

As a newly emerging pollutant, nanoplastics are easily to be ingested by organisms, and cause severe damage to biological functions because of their small size, high specific surface area and strong biological penetration. There are increasing reports of numerous airborne microplastics, including polystyrene, being detected in atmospheric samples, which implies a potential risk to the human respiratory system. Here a simple but effective feasibility study of the capability of SPES to detect the uptake of polystyrene particles in V79 cell studying the particles in the culture supernatant and in the cell lysate is reported.

Three cases are considered: a) polystyrene standard spheres of 0.5µm in diameter; b) polystyrene standard spheres of 1.0µm in diameter; c) a mix of 0.5µm and 1.0µm in diameter of polystyrene standard spheres. In all cases the treatment consists in feeding a culture of V79 cells with the three polystyrene samples at a numerical concentration of 1E6 ptc/mL.

Three pellets are prepared from the cell culture. The supernatants waters of the culture are measured to check the presence of polystyrene particles non internalized in the cells. The cell pellets are carefully cleaned to remove particles sticked outside the cell but non internalized. A lysate is prepared suspending the pellet in a solution of SDS 1% + Triton X-100 0.1% for 5 minutes.

In *Figure 8* SPES data of the cell lysates and related supernatants are represented. As enlightened with the coloured pano, the clouds related to the polystyrene particles can be discriminated in the large and broader mix of populations related to the corpuscles and impurities in the supernatants. As performed and presented for the supernatant case, focusing the selection to the polystyrene clouds, the estimates of the numerical concentrations of the particles in the supernatants are retrieved. In the case of cell lysate, considering the smaller numerical concentration and representativity respect the whole heterogeneous sample, this information can be retrieved carefully subtracting the contribution due to the cell fragment.

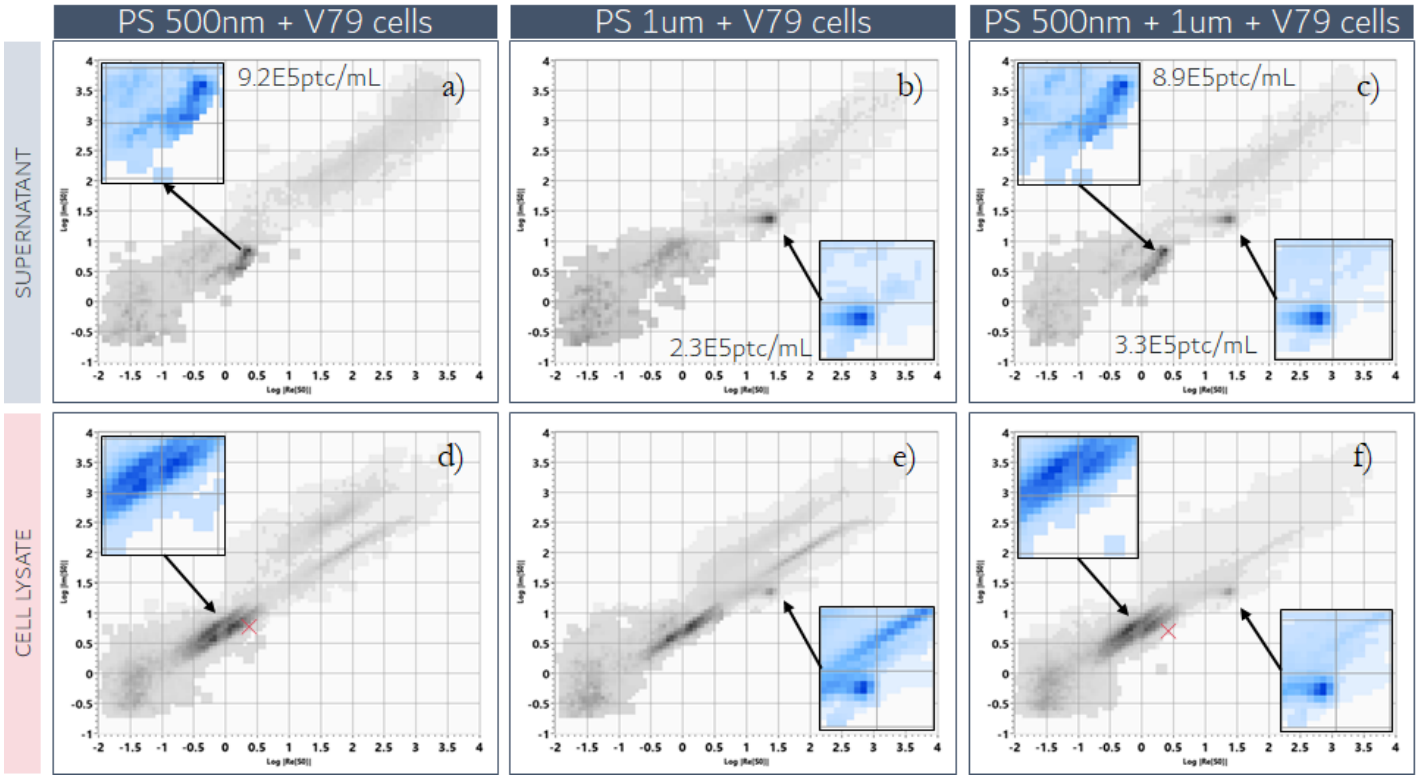


Figure 8 EOS CLOUDS for the heterogeneous samples of supernatants and cell lysates of polystyrene particles and cell V79. a) supernatant of cell culture V79 with PS 0.5µm spheres; b) supernatant of cell culture V79 with PS 1.0µm spheres; c) supernatant of cell culture V79 with a mix of PS 0.5µm and PS 1.0µm spheres; d) cell lysate of culture V79 with PS 0.5µm spheres; e) cell lysate of culture V79 with PS 0.5µm spheres; f) cell lysate of culture V79 with a mix of PS 0.5µm and PS 1.0µm spheres. Note. PS 0.5µm particles in cell lysate show different optical properties respect to PS 0.5µm particles in supernatant or in pure liquids as water (red cross indicates expected position). This effect suggests an interaction with the biological matter which modify the behavior and stability of the particles. On the contrary, the PS 1.0µm particles show no changes and are located where expected.

Experimental results suggest that EOS Classizer™ ONE and SPES technology could provide unique insights on exposure duration, diameter, and concentration of particles. These information are the key aspects for the evaluation of the toxicological effects of nanoplastics and other kind of particles.

Note. Should unknown particles be analysed in highly heterogeneous samples it is suggested to perform the SPES analyses of single particle populations in filtered liquid, e.g. filtered water, if possible. The independent knowledge of the expected position of the corresponding EOS CLOUDS for component of a formulation or a complex mix of particles can be of utmost importance and utility to identify the optical properties of the single populations. Results then effectively prove changes in particle behaviour, stability, and properties due to the surrounding media.

CONCLUSIONS

The capability of EOS Classizer™ ONE and SPES patented method of discriminating single particle basing on their optical properties is of capital importance with heterogeneous biological systems. In particular, the advantage is rampant and unique when particles have to be counted and analysed directly in the biological target media to tailor the effectiveness of the product formulation or to study **eco-/cito-toxicity effects of particle** on ecosystems and human health.

SPES data provide physical and statistical information, as particle size distribution and numerical concentration, as well as insight ~~on~~ into the particle structure and stability. Applications range from the aggregate concentration depending on the surfactant, e.g. for the improvement of the wetting of a powder or of the shelf life of a product, to the study of the behaviour of particles in target heterogeneous media to tailor the formulation. Oversize analysis can be performed also in presence of impurities. Scraps and out-of-specifics can be monitored in intermediate and final formulation.

RELEVANT PUBLICATIONS AND REFERENCES

Presentation of Single Particle Extinction and Scattering (SPES) method for particle analysis
AN001-2021 Analysis of Polymeric Particles via SPES Technology – a general introduction to SPES method

AN006-2021 Multiparametric Classification of Particles as a Pathway to Oversize Analysis in Complex Fluids via SPES Technology

Potenza MAC *et al.*, «Measuring the complex field scattered by single submicron particles », AIP Advances 5 (2015)

Example of CFA application of SPES technology
AN002-2021 Continuous SPES Flow Analysis CFA-SPES

Example of PCA application of SPES technology
AN005-2022 Multiparametric Principal Component Analysis of Heterogeneous Samples via SPES Technology

Classizer™ ONE with Sample Manager Autosampler
AN008-2022 Automatic Liquid Sample Management, Dilution, and System Cleaning with EOS Sample Manager

AN009-2022 Standardize SPES Operative Procedure of Liquid Samples Analysis via EOS Autosampler

Example of SPES application to aggregates
AN003-2021 Addressing the Issue of Particle Wetting and Clustering by means of SPES Technology

Potenza MAC *et al.*, «Single-Particle Extinction and Scattering Method …», ACS Earth Space Chem 15 (2017)

SPES application to non-spherical particles
AN004-2021 Addressing the Classification of Non-Spherical Particle by means of SPES Technology

Simonsen MF *et al.*, «Particle shape accounts for instrumental discrepancy in ice …», Clim. Past 14 (2018)

Example of SPES application to emulsions w/o payload in environmental waters
AN012-2021 Monitoring the Fate of a Lipid/ZnO Emulsion in Environmental Waters

Examples of SPES application to particle analysis and behavior characterization in biotech applications
AN011-2022 Quantitative Classification of Particles in Biological Liquids via SPES Technology

Sanvito T *et al.*, «Single particle extinction and scattering optical method unveils in real…", Nanomedicine 13 (2017)

Potenza MAC *et al.*, «Single particle optical extinction and scattering allows real time quantitative…», Sci Rep (2015)

Example of SPES application to oxide particles, abrasives, and industrial slurries w/o impurities
Potenza MAC *et al.*, «Optical characterization of particles for industries», KONA Powder and Particle 33 (2016)

AN002-2021 Analysis of Abrasives via SPES Technology

Example of SPES application to ecotoxicity analysis
Maiorana S *et al.*, «Phytotoxicity of wear debris from traditional and innovative brake pads», Env Int., 123 (2019)

Example of SPES application to aerosol analysis
Mariani F *et al.*, «Single Particle Extinction and Scattering allows novel optical …», J Nanopart Res 19 (2017)

Cremonesi L *et al.*, «Multiparametric optical characterization of airborne dust …. », Env Int 123 (2019)

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