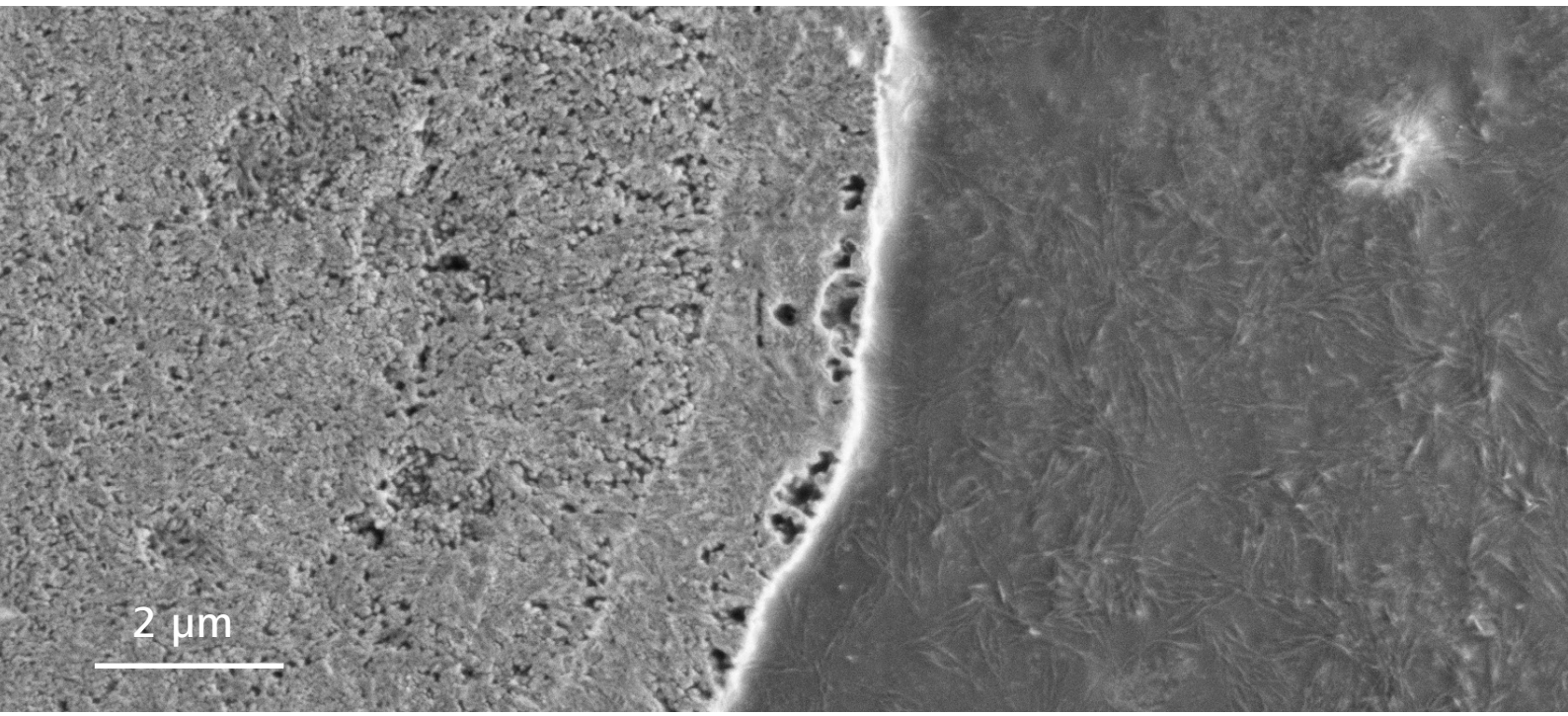
Application Note

Correlative SEM-Raman Microscopy to Reveal Nanoplastics in Complex Environments using ZEISS Sigma 300 with RISE



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Seeing beyond

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Due to the increasing number of reports on the occurrence of microplastics in our environment, plastic pollution of soils and water systems has become an important area for research in the scientific community. Microplastics is a common word nowadays, used to describe particles of plastic 5 mm or smaller. Nanoplastics, like those investigated in this study, are defined as plastic particles smaller than 1 µm.

In the last year global plastic production reached a roughly estimated 500 million tons, with between 4.8 and 12.7 million tons of waste plastic ending up in the world's oceans each year. The smaller size of nanoplastics relative to microplastics means they are increasingly found in consumer products from sea salt (Iniguez et al. 2017) to fish meat (Wang et al. 2020), with the effects on humans being largely unknown.

Scanning electron microscopy (SEM) is a critical tool employed by many research labs where particle analysis required. SEM is able to find particles down to a few nanometers, which is well within the range of both microplastics and nanoplastics. The problem lies in the fact that SEM alone cannot distinguish between types of particles (especially organic particles) in a sample. Hence, in this study SEM was coupled with Raman imaging technology to enable the detection and identification of nanoplastics, using ZEISS Sigma 300 with WITec Raman Imaging and Scanning Electron Microscopy (RISE).

Challenges of nanoplastic analysis

The two main challenges identified with the qualitative study of nanoplastics are spatially locating the particles and the subsequent identification of the chemical composition of the target nanoplastic polymer. Hence, Raman microscopy is a suitable analysis technique for this study, as it can identify the different polymers of plastics down to resolutions of 0.5 µm and trace detection below that range.

However, the finite resolution of light microscopy presents a limitation of Raman when it comes to the analysis of nano-plastics, as many of the particles intended for identification will be below the wavelength of the laser source used in the technique. This problem can be alleviated through application of the RISE system.

Introduction to RISE

Polymers are often highly functional, protective, and insulating materials used for a wide range of applications. Due to the non-conductive and beam-sensitive characteristics of polymers, they are a challenge to image using SEM techniques. To overcome this, field emission scanning electron microscopy (FE-SEM) is used at low accelerating voltages, removing the need to sputter coat samples with a conductive film. This means that it is possible to analyze polymers without any sample preparation and is also necessary if subsequent Raman measurements are to be performed. The ZEISS Gemini electron optics design has excellent low kV imaging performance with the highest sample flexibility, making it possible to analyze non-conductive, beam-sensitive materials more easily.

Raman is a well-established spectroscopic imaging technique used to characterize the chemical and molecular compositions of components of a sample. In the case of polymers and more narrowly nanoplastics Raman can provide valuable insight into their identity, allowing the researcher to determine the chemical fingerprint, visualize stress and strain in the sample as well as differentiate between different polymorphs of polymers in the sample.

RISE microscopy is the combination of Raman imaging and SEM all in one instrument. Combining the high-resolution imaging capabilities of SEM with the chemical sensitivity of Raman allows the researcher to analyze polymers on a variety of scales more efficiently, accurately, and reliably than before.

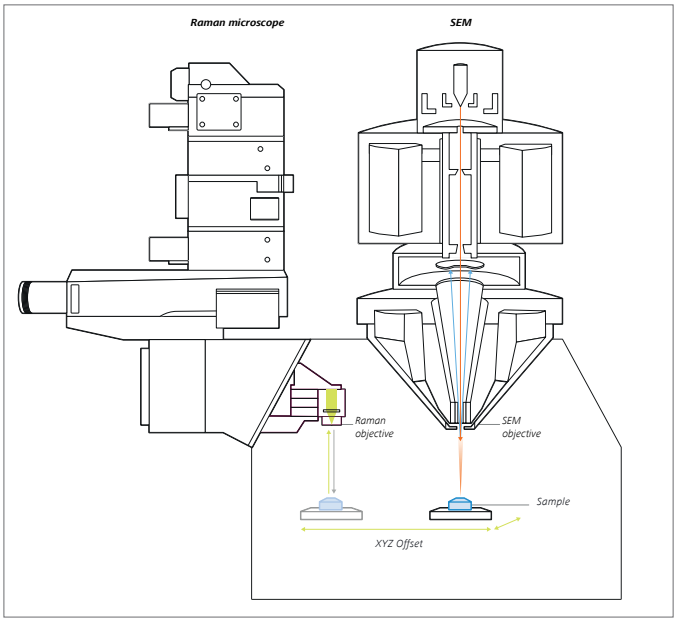
RISE allows for diffraction-limited confocal Raman imaging of the exact same sample position from which the SEM image was taken. It is also able to generate 3D images and depth profiles to visualize the distribution of compounds within a sample volume. Full integration of the Raman system into the SEM chamber enables automated sample transfer for seamless, fast, and precise correlation of SEM imaging and Raman analytics. The precise translation stage allows it to automatically transfer the sample inside the microscope's vacuum chamber and reposition it between measurements. The results acquired from the combined techniques can be overlaid to provide a more complete understanding of the sample in question.

The study "Correlative Microscopy and Spectroscopy Workflow for Microplastics" by Saura et al. (2020) provides a good account of why a combined SEM-Raman approach is useful when studying microplastics. For the study, optical microscopy as well as a SEM-Raman combination was used. Firstly, the whole sample surface was optically scanned at low spatial resolution to obtain an overview image of the substrate (in this case, the filter on which the microplastic particles were present). This first stage allowed visualization of microplastics down to sizes of approx. 10 µm. Secondly, SEM images of the microplastic particles are acquired at a high spatial resolution. Thirdly, the SEM stage was moved over to the Raman objective within the SEM chamber. The same ROIs were precisely retrieved and optically scanned at a spatial resolution of approx. 1 µm and overlaid with the corresponding SEM images. Finally, micro-Raman measurements were carried out in a single microplastic agglomerate. This method provided a comprehensive study of micro- and nanoplastics ranging from 13 µm down to 100 nm. This ability to analyze a range of particle sizes is especially important when studying samples gathered from environmental sources such as bodies of water and soil samples, as many of them will have adsorbed surface species and consequently resemble agglomerated microplastics; hence the ability to distinguish between different sizes of microplastics and identify the target of interest is of high importance.

Complementary to the work of Sarau et al. (2020), the study "Correlative SEM-Raman microscopy to reveal nanoplastics in complex environments" by Schmidt et al. (2021) focused on detecting polystyrene nanoplastics of a diameter of 200 nm or less in a variety of realistic environments ranging from ideal (distilled water) to challenging (sea salt and human amniotic fluid). The results of this study will be the main focus of this application note.

To test the approach in theory, standardized polystyrene (PS) beads of approx. 200 nm were mixed into the various environments (distilled water, sea water, and human amniotic fluid) in concentrations ranging from 20 ppm to 0.002 ppb. The range of diluted samples were then dropped onto cleaned glass slides coated with 200 nm of gold before being dehumidified in a desiccator ready for analysis. Methodology and details from Schmidt et al. (2021).

To test their approach in a more realistic and practical way, Schmidt et al. (2021) ensured both the sea salt and amniotic fluid samples were prepared without PS beads to check for native nanoplastics in the samples. Due to the particularly problematic organic background of the amniotic fluid sample, a second sample was analyzed after having been filtered through a 0.22 µm cellulose filter to remove some of the organic background, giving a more acceptable Raman spectrum.



Graphic shows the principle of RISE microscopy: The Raman microscope is attached to the chamber of the SEM. The sample is investigated with both microscopic techniques under vacuum in the SEM chamber. An integrated software module facilitates the workflow (Raman beam green, SEM beam orange).

RISE microscopy for nanoplastic identification

The identification of nanoplastics is challenging in a number of ways: Firstly, the size of nanoplastics is below the resolution of Raman instruments, making it difficult to accurately identify the polymer which the plastic of interest is made of. Secondly, in practice many samples will contain material other than the target nanoplastics; due to the nanoscale nature of the experiment, many of these non-plastics have indistinct characteristics and can be mistaken for the desired nanoplastics. Thirdly, fluorescence of certain functional groups within the polymers (in particular additives and plasticizers) or within background organic materials can make interpretation of the resulting spectra difficult and lead to inaccurate and unreliable results.

Finally, the environmental persistence and degradation of nano- and microplastics are poorly understood. Nanoplastics may undergo disintegration into smaller particles or organic compounds, or they may subject to chemical transition. All these changes can lead to a mismatch between practical samples from a variety of sources and standardized reference samples, again leading to ambiguous results.

Electron microscopy

Potential nanoplastics were located using ZEISS Sigma 300VP. The instrument parameters were adjusted on a sample-by-sample basis to give the best contrast between candidate particles and the background. The most sensitive setup was a comparatively low acceleration voltage in the 2–7 kV range, using the SE2 detector (secondary electrons) in high vacuum. In variable pressure mode (VP), where nitrogen is used as an

imaging gas at pressures between 10 and 133 Pa, the dedicated C2D detector images the SE contrast. VP mode enabled the investigation of non-conductive samples at conventional beam energies without the need of an additional conductive surface layer (Schmidt et al., 2021).

Raman microscopy

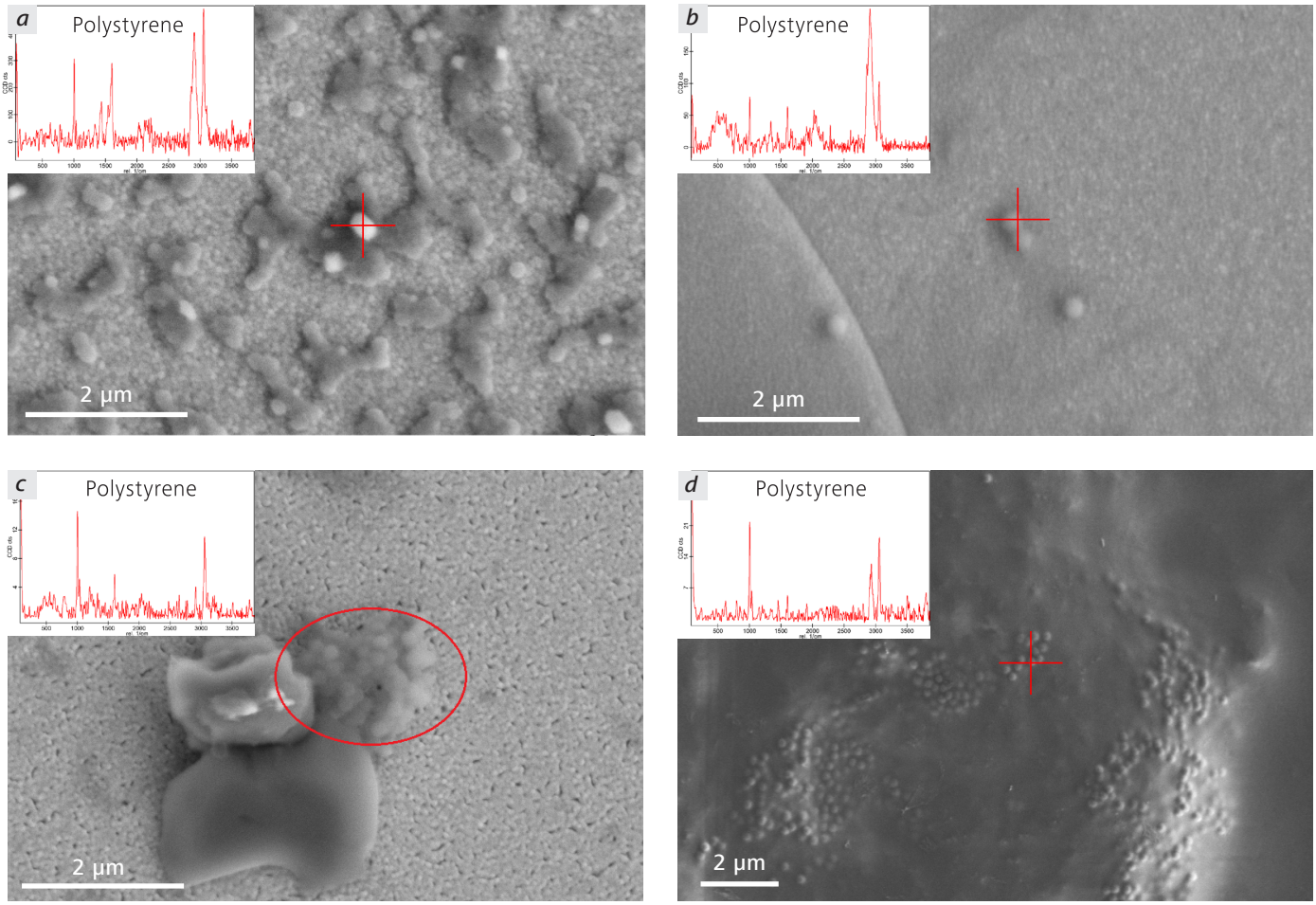
At the SEM integrated confocal Raman microscope (RISE, WITec), a laser with a wavelength of 532 nm and the objective ZEISS LD EC Epiplan-Neofluar 100x/0.75 HD DIC were used for the Raman measurements. The laser power and integration time were adjusted on a sample-by-sample basis to afford reasonable spectral quality. Typical laser powers ranged from 0.5 mW to 3.5 mW. Typical used integration times ranged from 5 s with 9–30 accumulations to 100 s with 2 accumulations (Schmidt et al., 2021).

Results and discussion

Firstly, a negative test was performed with distilled water to confirm that potentially detected particles were not introduced by preparation steps for the experiment. Results from the positive test series indicate a reasonable detection limit for particles in the range of 200 nm. At a concentration of 20 ppm the PS beads are easy to find and identify in distilled water. But even at a dilution of 10^{-10}, which is as low as 0.002 ppb, PS beads can still be found within a reasonable searching time (about 0.5 –1 h). At a concentration of 0.0002 ppb, candidate particles could not be detected by SEM within the search time of about 1 h.

This test of theory afforded promising detection limits of 0.002 ppb (2x10-3 µg/L) for the distilled water environment, 20 ppb (20 µg/L) for the sea salt environment, and 200 ppb (200 µg/L) for the human amniotic fluid environments.

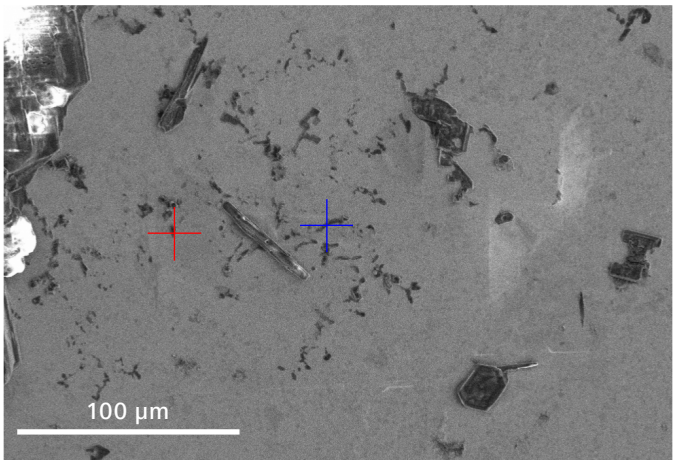
In both the sea salt and amniotic fluid tests, due to background signals from native contamination, it became necessary to search for small particle clusters to improve the signal-to-noise ratio with Raman spectroscopy. In the amniotic fluid samples, finding the particles was further complicated by biological components that resemble PS beads in the SEM micrographs. Furthermore, Raman signals from the organic biological background also overlap in part with the PS beads' spectrum, further complicating identification of the particles.



SE image of analyzed particles in a) distilled water with a PS concentration of 0.0002 ppb, b) distilled water with a PS concentration of 0.002 ppb, c) Fleur de Sel salt saturated solved in distilled water with a PS concentration of 20 ppb, d) human amniotic fluid with a PS concentration of 200 ppb. All samples were taken from the surface of the solutions and applied on a gold-sputtered slide (Schmidt et al., 2021).

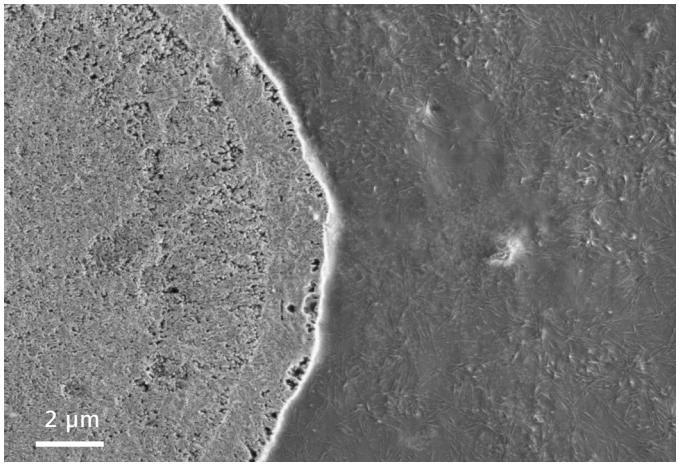
Application sea salt and amniotic fluid

The investigation of the untreated salt solution shows the difficulty of particle identification due to native contaminants in the salt. The background signal hinders an accurate analysis of the individual nanoplastics. The Raman bands found on these particles are around 3000 cm^{-1}, clearly showing that there are C-H bonds present in the selected particles and making them viable candidates for nanoplastics. However, the "fingerprint region" (below 1500 cm^{-1}) could not be measured with a sufficient signal-to-noise ratio to identify the specific type of polymer or even to exclude that it is not some kind of non-polymeric organic compound.



SE image of analyzed microplastics in the sea salt solution (Schmidt et al., 2021).

This problem is exaggerated in amniotic fluid samples due to the presence of biological materials such as cells, cell fragments, lipid droplets, etc. As a remedy, the amniotic fluid was filtered to reduce the overall concentration of biological materials in the sample. The Raman spectra from the filtered amniotic fluid sample were compared with the "KnowItAll" database, yielding nylon as a result. Since the medical sampling equipment for the amniotic fluid contains nylon, this particle might have originated from the equipment itself. However, due to the size of the particle (100–200 nm), an alternative explanation is that the nylon nanoplastic was already contained in the amniotic fluid itself before sampling had taken place.



Overview SE image of analyzed particle in amniotic fluid filtered through a 0.22 µm cellulose filter. (Schmidt et al. 2021)

Practical detection and identification problems

Due to the high resolution of SEM, detection of the experimental PS beads was unproblematic in all environments with acceptable detection limits.

To avoid excessive deterioration of the nanoplastics by the electron beam, only fast scan speeds were able to be used in the initial search. This means that the operator had to work at a reduced signal-to-noise ratio thus, impeding recognition of smaller particles. A higher signal-to-noise scan was able to be completed after Raman measurements, thus allowing a more accurate analysis of the particle shape and size.

For Raman studies, laser beam-induced damage may thermally compromise the nanoplastics even at low powers (0.5 mW). Low laser powers combined with particle sizes smaller than the resolution of the Raman microscope mean that the signal-to-noise ratio is below acceptable limits, even at high integration times. A choice between high spectral quality of the Raman measurement to enable complete identification of the nanoplastic (potentially destroying the particle) and the ability to to gather a high-resolution image of the particle must be made by the operator.

Summary

RISE has been proven an extremely valuable tool for imaging nanoparticles in challenging environments; good detection limits for PS beads were obtained across all the test conditions. Problems arise when background signals from organic matter, native to the sample, cause ambiguity in the particle identification process. To remedy this, filtration was used to some level of success. Further research into the imaging and identification of nanoparticles in challenging environments is needed as well as a more in-depth look into the sample preparation process. This work provides a useful pilot study on the benefits as well as the limitations of SEM-Raman-based methods and shows the suitability and versatility of this tool for the detection and analysis of nanoplastics.

References:

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