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Supplementary Information to:

Ecotoxicological assessment of nanoparticle-containing acrylic copolymer dispersions in fairy shrimp and zebrafish embryos

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1 Introduction to Raman scattering microscopy as relevant for the specific study

Adapted from: Moger *et al.*;[1] Galloway *et al.*;[2] Alfonso-Garcia *et al.*;[3] Zhang *et al.*[4]

Spontaneous Raman scattering (applied as pre-tests in the present study to select the most appropriate test materials) involves two beams (or photons, fields, or laser), an incident beam, called *pump* (or signal) beam, and a scattered beam, called *Stoke*s (or idler) beam. Assuming that the molecule under investigation is initially in its ground state, the Raman scattered beam is of lower frequency than the incident beam. The energy difference between the two beams corresponds to the vibrational energy of the molecule, which is left in a vibrationally excited state after the light-matter interaction. In spontaneous Raman microscopy, the intensity of laser light scattered from a sample is measured as a function of the energy shift (Raman shift) with respect to the incident beam, in units of wavenumbers (cm^{-1}). This energy shift is a result of interactions of the incident beam with Raman-active chemical bonds within the sample. The Raman spectrum consists of narrow peaks which correspond to particular chemical bonds, thereby forming a unique ‘fingerprint’ of the particular sample. The intensity of the Raman scattered light is directly proportional to the number of vibrationally active chemical bonds, which often allows straightforward quantitative analyses. However, spontaneous Raman scattering provides only weak signals (and it further requires long acquisition times >100 seconds). Typical Raman cross-sections per molecule range from 10^{-25} to 10^{-30} cm^2, whereas typical cross-sections obtained, e.g. in fluorescence

microscopy, range from 10^{-16} to 10^{-17} cm^2. In theory, the signal-to-noise ratio could be improved by increasing the intensity of the incident laser beam. However, in practise, this rapidly causes photo-thermal damage to biological samples.

Coherent Raman scattering (CRS), i.e. *Coherent anti-Stokes Raman Scattering (CARS) or Stimulated Raman Scattering (SRS)*, uses visible or near-infrared excitation light. By contrast to *spontaneous Raman scattering*, in CRS techniques, two (or more) incident beams, instead of only one, drive the molecule, i.e. the *pump beam* (or signal beam) and the *Stokes beam* (or idler beam). These two beams are made to overlap perfectly in time and space before being directed into a microscope, with lenses focussing the beams onto the sample under investigation. This results in a non-linear dependence on the incident light, and coherent Raman signals are produced. Thereby, the detected Raman signal is sufficiently strong to obtain contrast from even relatively weak Raman-active molecular vibrations, that would not be detectable by spontaneous Raman scattering.

Coherent anti-Stokes Raman Scattering (CARS; applied in the present study to confirm the presence of nanoparticle-containing polymer dispersions in biological samples) derives its contrast from intrinsic molecular vibrations in a sample. The use of infrared excitation gives CARS an increased depth penetration over conventional optical microscopy, which removes the need for sample sectioning, and it further minimises sample damage. By simultaneously illuminating the sample with two excitation wavelengths with the frequency difference chosen to match the vibrational frequency of chemical bonds within the desired sample component, CARS generates a strong *anti-Stokes signal* which is used as image contrast. This beam (signal) varies with the square of the number of Raman-active chemical bonds under investigation. Thereby, CARS is especially suited for the investigation of polymers with a high concentration of repeating chemical bonds. However, the CARS signal contains an inherent non-resonant background, which results in a distorted spectral profile as compared to the spontaneous Raman scattering spectrum.

Anti-Stokes signal, anti-Stokes Raman scattering: By contrast to 'Stokes Raman scattering', anti-Stokes Raman scattering implies that the molecule loses energy to the emitted beam so that it has a higher energy than the incoming light.

Stimulated Raman scattering (SRS; predominantly used method of the CRS part of the present study, applied to investigate the uptake and bioavailability of the nanoparticle-containing polymer dispersions): In SRS, the energy difference between the pump and Stokes fields is transferred to the molecule for vibrational excitation. This stimulated excitation leads to subtle changes in the excitation field. The SRS signal

appears at the same wavelengths as the excitation fields. It is manifested as a loss to the pump beam (stimulated Raman loss) or a gain to the Stokes beam (stimulated Raman gain). SRS requires the use of ultrafast lasers with high peak powers (thereby providing excellent signal-to-noise contrast) but low time-averaged powers at the sample (thereby preventing photo-thermal sample damage). SRS offers the benefit of the chemical selectivity of spontaneous Raman scattering without the problem of low sensitivity, combined with the benefits of confocal microscopy, such as 3D sectioning capabilities and sub-cellular resolution. While the enhanced sensitivity of SRS allows investigating materials that would not be detectable with spontaneous Raman scattering, the spectral profile obtained with SRS is identical to that of spontaneous Raman scattering. This enables straightforward comparisons between the two techniques. SRS is inherently a non-linear technique since the signal intensity of SRS does not scale linearly with the concentration of the test material under investigation. Nevertheless, SRS measurements allow semi-quantitative determination of test material concentrations, when signal intensities are compared between pixels and hyperspectral profiles are compared against positive controls.

Hyperspectral imaging: Each pixel of an image is associated with a spectral information.

2 Preliminary tests to optimise the preparation of fairy shrimp and zebrafish and microscopy settings

Transmitted light images were recorded from fairy shrimp, wild-type zebrafish embryos and *Casper* using an Olympus SZX16 microscope equipped with an Olympus XC10 camera (Olympus, UK). Glass coverslips were used to direct a portion of the transmitted light onto a FDS1010 Si photodiode (Thorlabs, UK) whose output was not demodulated. To optimise the technique of preparing fairy shrimp and zebrafish for CRS imaging, a fairy shrimp was placed in a droplet of water directly onto a glass coverslip. Clear transmitted light images (Figure SI-9; left) showed the shrimp moving within the droplet in an oscillating manner, leading to 'wobbly' images at scan times of approx. 20 seconds per frame. Application of faster scan times of approx. 2 seconds per frame led to clear transmitted light images without motion artefacts (Figure SI-9 right). Since scan times of approx. 20 seconds per frame are necessary to obtain a good signal-to-noise ratio in the SRS images (and approx. 14 seconds per frame in the CARS images), animals were fixed in paraformaldehyde (Sigma-Aldrich) and mounted on their left sides between glass coverslips using low melting point agarose gel (Thermo Fisher Scientific, UK) prior to CRS imaging.

Setup of the combined SRS – CARS microscopy

A neodymium vanadate laser source (High-Q Picotrain, Austria) was used to provide a 1064 nm wavelength beam with a 6 ps pulse-width and a repetition rate of 76 MHz. This 1064 nm beam was used as Stokes beam. To generate the pump beam, a 1064 beam was frequency-doubled to 532 nm to pump the near-infrared light source, a Levante Optical Parametric Oscillator (Levante Emerald, APE, Germany). The 1064 nm Stokes beam was amplitude-modulated by an acoustic optical modulator (AOMO 3080-197 Crystal Technology) driven by an 80 MHz driver (AODR 1080AF-A1F0-1.0, Crystal Technology). The collinear Stokes and pump beams were directed into a confocal scan unit (FV300, Olympus, UK) and from there into a modified commercial inverted microscope (IX71, Olympus, UK). Depending on the degree of magnification required, an air-immersion objective lens (UPLSAPO 20x 0.75 NA, Olympus, UK) or a water-immersion objective lens (UPLSAPO 60x 1.2 NA, Olympus, UK) was used to focus the Stokes and pump beams onto the same spot on the sample. When the pump and Stokes beams were focussed onto the same spot on the sample and tuned such that their frequency difference matched a Raman-active molecular vibration in the sample, the amplitude modulation was transferred to the pump beam.

For **SRS microscopy**, the pump beam was then directed onto a wide-area silicon photodiode detector (i.e. the stimulated Raman loss detector, applied reverse bias of 64 V; FDS1010; Thorlabs, UK), which was positioned at the back-aperture of the condensing lens. The output from the silicon photodiode was demodulated using a sophisticated lock-in amplifier (SRS 844; Stanford Research Systems, UK) that was set to pick out and amplify fluctuations in the pump beam occurring at the modulation frequency. The choice of modulation frequency is important, since the sought for stimulated Raman loss signal is so small that normal intensity fluctuations of the laser beam could drown it out. A high frequency of 1.7 MHz was chosen for modulation, since the laser fluctuations only occur at low frequencies. The lock-in amplifier's time constant (the time over which the input signal is integrated) was set to match the pixel dwell time (or voxel dwell time to reflect the three dimensions of the point of focus). Finally, the magnitude of the stimulated Raman loss signal output from the lock-in amplifier was fed into a computer for digital analysis.

For simultaneous **CARS microscopy**, an air condenser (0.55 NA) collected and directed the transmitted light towards a band-pass filter (CARS 890/220 nm, Chroma Technology) to block the 1064 nm Stokes beam thereby generating the anti-Stokes signal. CARS images were detected in the backwards epi-direction of the beam using a 750-nm long pass dichroic mirror (750dcxr 229; Chroma Technology) and two filters

centred at 660 nm (660.0 IF 40D 230; Ealing Inc.) to separate the signal from the fundamental laser.

To acquire **hyperspectral images**, the SRS lasers were tuned to match one specific Raman shift, an image was acquired, and then the lasers were tuned to match a different Raman shift, etc. Thereby, multiple images were acquired which probed across the respective relevant region of the Raman spectrum. The images were normalised to account for any variation in laser intensity, and each pixel's intensity was plotted as a function of Raman shift thereby producing a hyperspectral data set. The location of any pixels which exhibited signals of the test material was noted. After the desired series of frames had been taken, each frame was false-colour-coded following a “rainbow” lookup table. Once the coloured hyperspectral data stack was complete, the frames were merged into one single red-green-blue image, using the public domain image processing programme ImageJ developed by the USA National Institute of Health (http://imagej.nih.gov/ij/). Thereby, the composite hyperspectral images exhibit different colours depending on the spectral features of each individual pixel. Composite images for CARS were generated using the same software and techniques.

Pre-tests to determine the laser power tolerance of the biological samples determined the optimal total laser power incident on the biological samples (i.e. the laser power at the focus) to be <20 mW. These power tolerance experiments involved gradually increasing the laser power and observing the sample for signs indicative of photo-thermal damage (such as blebbing of the sample). Each organism investigated has a different tolerance to laser intensity, due to its unique scattering and absorption properties. Hence, for each test species the power tolerance experiments were repeated to ensure consistency in sample integrity.

3 Semi-quantitative SRS to determine limit of detection

The detected SRS signal is linearly proportional to the concentration of the molecular bonds being probed, and it further varies linearly with both the pump and Stokes laser beam powers. To determine the lowest detectable concentration of the test material with the setup of the present study (applying all parameters as selected for SRS imaging of the aquatic organisms), hyperspectral profiles across the CH stretch region from 2850 cm^{-1} to 3150 cm-1 were generated from 110 nm-ACP successively diluted in standardised dilution water. The detected signal was normalised relative to the water background, and the aromatic CH stretch peak height at 3055 cm^{-1} was measured in the CH stretch region.

- The mass of polymer detected in a certain number of voxels in tissue was calculated based on the limit of detection (LoD) as follows: $Mass_{detected}$ = LoD [mg/L] * voxel volume [L] * number of voxels.
- The mass of a particle was calculated as follows: $Mass_{particle}$ = Pi /6 * density [g/cm^3] * diameter^3 [nm^3]. (A 100nm particle has a volume of 5.24×10^{-16} cm^3. Assuming a polymer specific density of 1.2 g/cm^3, the mass of a single particle is 6.3×10^{-16} g.)
- The number of particles present in a given sample was calculated as follows: Number of particles in the sample = $mass_{detected}$ / $mass_{particle}$.

If the latter quotient undercut 1, this implied that no integer particles, but rather styrene-containing impurities of the ACP dispersions were detected. If, however, the quotient exceeded 1, one could not exclude that particles of the test material were detected.

4 Quality control values for acute aquatic toxicity tests

Dissolved oxygen and pH values of the test material preparations

Fully saturated standardised dilution water was assessed as having 100% dissolved oxygen (8.4 ppm), and its pH was 7.5. The readings of the exposed media were undertaken upon exposure of the *D. rerio* embryos to the test materials. Neither dissolved oxygen nor the pH value were significantly altered at either 1000 or 2500 mg/L (Table SI-2A and SI-2B).

5 Abbreviations to Supplementary Information

CARS: Coherent anti-Stokes Raman Scattering; CRS: Coherent Raman scattering; LoD: Limit of detection; SRS: Stimulated Raman Scattering.

6 References to Supplementary information

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