

Identifying and localizing intracellular nanoparticles using Raman Spectroscopy:

Jennifer Dorney^{1,2}, Franck Bonnier^{3*}, Amaya Garcia³, Alan Casey¹, Gordon Chambers^{1,2},
Hugh J. Byrne¹

*1. Nanolab Research Centre, Focas Research Institute, Dublin Institute of Technology (DIT),
Camden Row, Dublin 8, Ireland*

*2. School of Physics, Dublin Institute of Technology (DIT), Kevin Street,
Dublin 8, Ireland*

*3. Radiation and Environmental Science Centre, Dublin Institute of Technology (DIT),
Camden Row, Dublin 8, Ireland*

Supplementary Information

S.1 Physico Chemical Characterisation of the Nanoparticles

Physico-chemical characterization of the particles was performed by dynamic light scattering using a Malvern Zetasizer ZS. A HeNe laser with a wavelength of 633nm and an avalanche photodiode detector, Q.E. >50% at 633nm at 173° (backscatter detection) was used. Particle size measurements and zeta potential measurements were carried out in the respective biological media at a concentration of 1×10^{12} particles per ml at 37° C. The number of particles per ml of suspension may be determined from the following equation, as specified by the supplier:

$$\text{Number of particles/ ml} = \frac{6C \times 10^{12}}{\rho \times \pi \times \Phi}$$

where: C = Concentration of suspended beads in g/ml

ρ = Density of polymer in g/ml

Φ = Diameter of suspended particles in μm .

The size and zeta potential of both the nominally 50 nm and 100 nm sized particles differed slightly according to the medium in which they were suspended, when measured by dynamic light scattering. Nominally 50 nm particles were found to have an average particle size in H₂O at 25°C and 37°C of 52 nm and 53 nm, respectively, while 50 nm particles in 10% FBS RPMI media at 25°C and 37°C were determined to have particle sizes of 49 nm and 38 nm respectively. The average particle sizes for the 100 nm samples in H₂O at 25°C and 37°C were found to be 169 nm and 138 nm respectively, while average particle sizes for 100 nm samples measured in medium at 25°C and 37°C were found to be 149 nm and 116 nm respectively. Zeta potential measurements of the 50nm samples revealed that particles dispersed in H₂O at 25°C and 37°C were found to be -62 mV and -53 mV respectively, and in media at 25°C and 37°C to be -13 mV and -12 mV indicating that the solution was moderately stable at the temperatures measured. 100 nm particles were found to have zeta potential values, in H₂O at 25°C and 37°C, of -18.62 mV and -15.09 mV respectively. Similarly, zeta potential measurements of 100 nm particles in H₂O at 25°C and 37°C were found to have values of -42.32 mV and -38.27 mV respectively, also indicating the solution was moderately stable at both temperatures. Changes in zeta potential between H₂O and cell culture medium are well documented and are proposed to result from the interaction of the nanoparticle surface with the molecular components of the cell culture medium¹. Despite the uncertainty in the exact nanoparticle sizes, for the purpose of brevity, the nanoparticles will be referred to as 50nm and 100nm particles throughout the manuscript.

S.2 Cytotoxicity assays

The cytotoxicity assays employed during this experiment were Alamar Blue™ (AB), Neutral Red (NR), Coomassie Blue (CB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). For cytotoxicity evaluation, cells were seeded in 96-well micro plates

(Nunc, Denmark) in triplicate for each of the four time points studied 24, 48, 72, 96 hr. Plates were seeded at a density of 1.5×10^5 cells/ml for 24 hr, 5×10^4 cells/ml for 48 hr, 3×10^4 cells/ml for 72 hr and 2×10^4 cells/ml for 96 hr exposure. These densities were found to be optimal to achieve the desired confluence at the end of the exposure period. After an initial 24 hr of cell attachment, the media was removed and the plates were washed with 100 μ l/well phosphate buffered saline (PBS). Cells were then treated with increasing concentrations of each nanomaterial and with a positive control of a 10% DMSO 90% media solution. The cells were then incubated for the desired time period and the cytotoxic effects evaluated. For each independent experiment, six replicate wells were used for control, six replicate wells were employed for the positive control and six replicate wells were used for each test concentration per micro plate. For cytotoxicity evaluation, fluorescence and absorbance were all quantified using a microplate reader (TECAN GENios, Grodig, Austria). Further details for each of the assays performed can be found in ².

For all assays employed, at all timepoints, no significant cytotoxicity was observed over the concentration range employed for both 50 and 100 nm polystyrene nanoparticles. As an example, Figure S.1 shows the MTT response to exposure of 50 nm polystyrene nanoparticles. The observations are consistent with previous reports of exposure to neutral nanoparticles ³ and indicate that the labelling of the nanoparticles with the fluorescent moiety does not impact significantly on the toxic response.

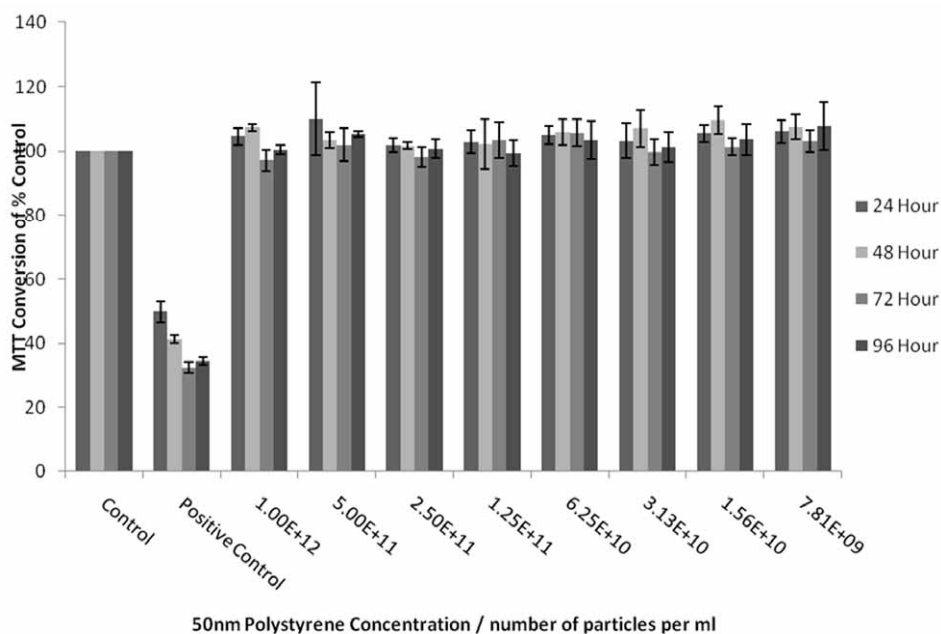


Figure S.1: Cytotoxicity of 50 nm Duke Scientific Nanopolystyrene after 24, 48, 72 and 96 hour exposures determined by the MTT assay. Data are expressed as percent of control mean \pm SD of three independent experiments.

S.3 Sample preparation and cell morphology

When working with A549 cells, the first observation made was the inconsistency of the cell morphology while preparing the samples. The cells were seeded on CaF₂ substrates, left overnight in the incubator and used for Raman analysis the next day for both live cell and fixed cell analysis. The protocols used were standard, but still the cells could exhibit particular characteristics and “stressed” samples seemed to be regularly observed. The main characteristic of these cells was the presence of microscopic “droplets” (~1-5 μ m) in the cytoplasm. Their number was seen to vary considerably from sample to sample and thus the ability to resolve different structures present in the cytoplasm varied significantly. This phenomenon is independent of the cell fixation using formalin as the features can be seen in both live cells (figure S2.A) and fixed cells (figure S2.B). The droplets are observed to exhibit consistent Raman spectra which vary little in fixed (figure S2.D) compared to live

cells (figure S2.C), and comparison with spectra of common lipids such as phosphatidylinositol (figure S2.E) and phosphatidyl-L-serine (figure S2.E) confirms that they are lipidic in structure, an obvious candidate being peroxisomes. Regardless of their origin, the main concern was the strong Raman scattering of these droplets and therefore the probable interference with the detection of nanoparticles in the cells. It was therefore deemed imperative to optimize the protocols for the cell sample preparation and to reproducibly obtain cells with similar morphologies.

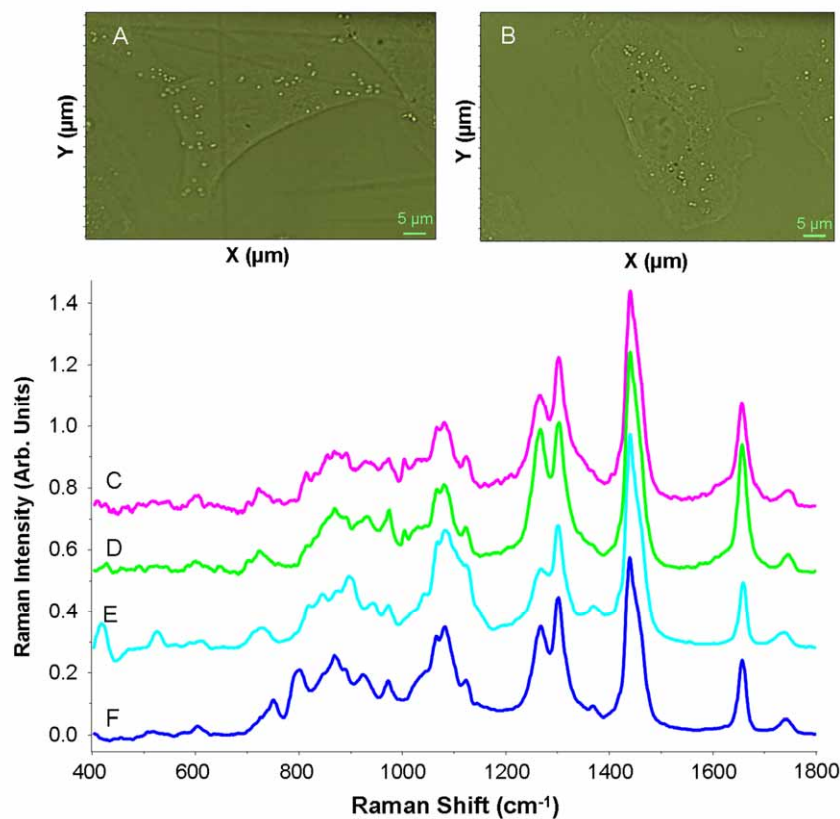


Figure S2: Microscopic images of live (A) and formaline fixed (B) A549 cells with “stressed” morphologies. Raman Spectra of droplets in (C) live cells (D) fixed cells compared to (E) phosphatidylinositol and (F) Phosphatidyl-L-serine. Spectra are offset for clarity

Different parameters have to be taken into consideration during the cell culture and the preparation of the samples. The cells are commonly split when they have reached between 80-85% of confluency in the cell culture flasks. However, performing a splitting when the

cells were only about 60% confluent greatly diminished the number of the droplets per cell. Under such conditions, after a few splits, almost no droplets can be seen in cells either in the cell growth flasks or on the substrates used for confocal imaging or Raman mapping. Subsequently, the temperature of the PBS solution used to wash the cells has to be 37 °C, and it was further noted that the quality of the fixation can be affected by the temperature of the formalin used. Although, the formalin is usually kept at room temperature, warming to 37 °C before fixation showed a better conservation of the cell morphology after fixation. Once the sample preparation procedures have been optimised, cells of consistently “unstressed” morphology can be routinely obtained, as shown in Figure S3. Fewer vacuoles or lipid droplets are evident, and there is a better definition of the endoplasmic reticulum (marked with arrows in figure S3).

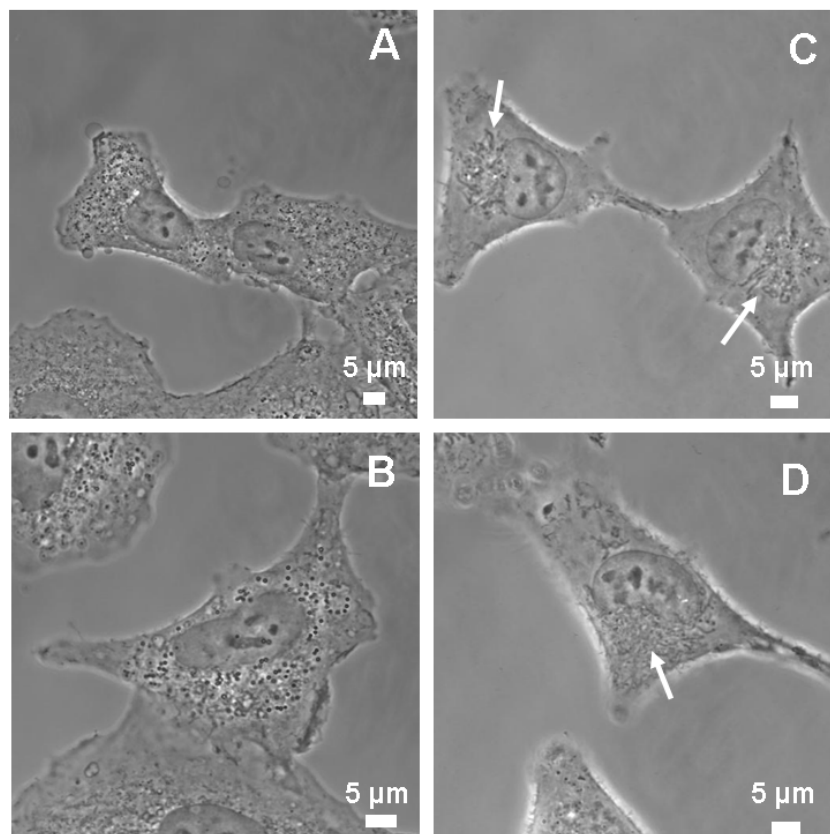


Figure S3: Microscopic images of cells with “stressed” (A, B) and “unstressed” morphology (C, D).

S.4 Effect of the cell fixation on the Raman spectra

Mapping of single cells using Raman spectroscopy can be time consuming and, depending on the quality of spectra required, many hours are usually needed to complete a single map. As the viability of cells removed from an incubation environment is compromised, fixation of cells remains a popular option for extended measurements. Different approaches to fixation can be employed, including dry fixation, alcohol fixation or formalin fixation. These different methods have been recently compared by Raman spectroscopy⁴⁻⁶. In order to preserve the cell morphology, dry fixation has been excluded for this study, as the modification in the cell shape and thickness due to the drying will make comparison with live cells difficult. Formalin fixation is the more promising approach, as the cells are maintained in a hydrated state and, therefore, as close as possible to the living state⁴. Based on the observations made above, the protocols for cell fixation were optimised and the sample preparation standardised. Under these conditions, the localisation of the different subcellular organelles can be precisely visualised and Raman spectra recorded from similar structures more accurately. Figure S4 presents spectra recorded from the nucleus and cytoplasm from live and formalin fixed cells. The spectra exhibit similar intensities and no contribution from the CaF₂ substrate can be seen. The spectral signatures obtained after fixation using formalin are identical to those recorded from the live cells. Using adapted protocols for the cell culture and fixation, the effect of the fixatives used can be greatly reduced.

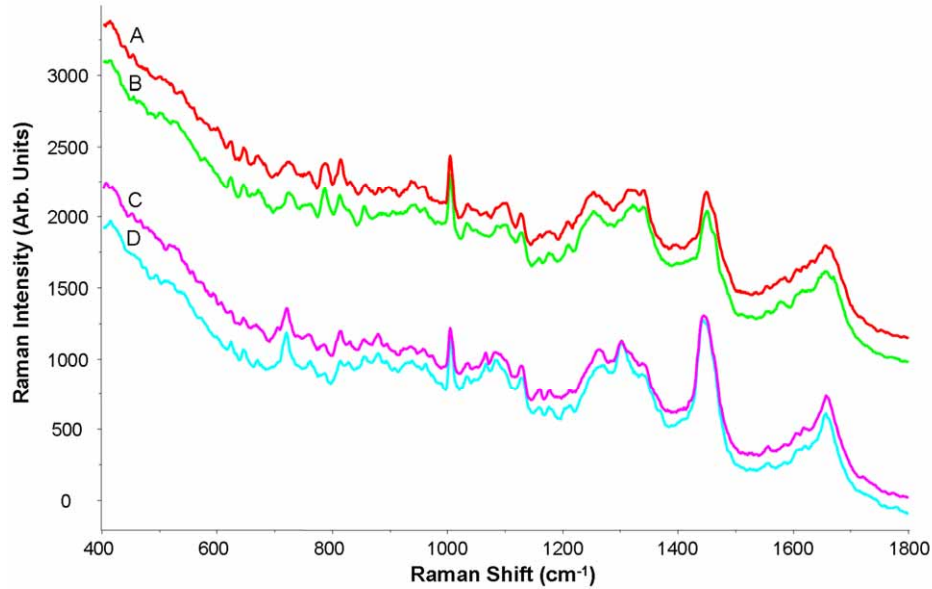


Figure S4: Raman spectra of the nuclear region of fixed (A) and live (B) cells and the cytoplasmic region of fixed (C) and live (D) cells. All Spectra have been recorded using the 785 nm laser source and are the result of two accumulations of 20 seconds per spot. Spectra are offset for clarity

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