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High content nanotoxicological screening using Raman spectroscopy

In recent years, the potential use of nanomaterials as novel agents in a wide range of areas, from industrial applications to Nanomedicine, has drawn attention to investigation of their toxicological properties. From their production, to use and disposal by consumers, nanomaterials interact with living systems via different exposure routes and can impact on human health and ultimately the environment. Although conventional *in-vitro* cytotoxicological techniques provide valuable information about the particle toxicity, the importance of gaining high content information in a single assay with the analysis of multiple parameters in a non-invasive and label-free way is still one of the biggest challenges in nanotoxicology due to possible physicochemical variations of the nanomaterials such as size, shape, surface charge.

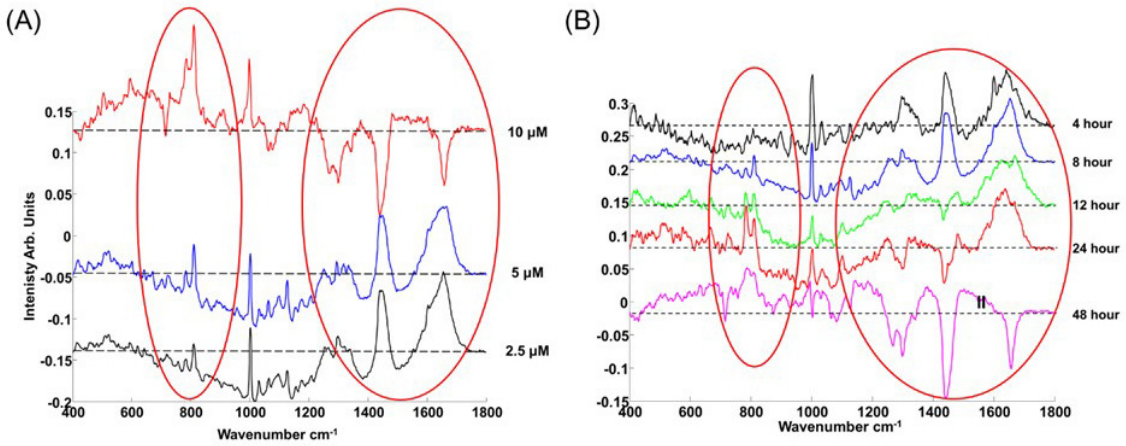

Fig. 1. (A) Loadings of PC1 for pairwise analysis of cytoplasm of exposed cells and controls for 10 µM (red), 5 µM (blue) and 2.5 µM (black) for 8 h. (B) Comparison of the Loading of PC1s for different PS-NH2 concentrations (cytoplasm). 4, 8, 12, 24 and 48 h are indicated with black, blue, green, red and magenta, respectively. Positive features of the PCs are related to exposed cells while negative features of the PCs are related to their controls. Loadings are offset for clarity. The dotted line represents the zero '0' point for each loading. Efeoglu et al. Analyst, 2016,141, 5417-5431.

As a vibrational spectroscopic technique, Raman spectroscopy has origin in inelastic collision of photons with molecules and provides fingerprint information about the specimen under investigation. Due to the nature of the technique, fingerprint information at a molecular level, easy sample preparation steps, narrow spectral bandwidth and minimal influence from water, a natural component of biological samples, the technique has attracted interest for the analysis of biological structures. The applicability of Raman spectroscopy as a tool for analysis of cells, tissues and biofluids has been demonstrated in recent years. The technique has also been widely used for the analysis of cell-drug and cell-nanoparticle interactions at a sub-cellular level.

In our study, the dose and time dependent cellular responses and effect of cytotoxic events on biochemical constituents of the cells are monitored using Raman spectroscopy. Aminated polystyrene nanoparticles ($PS\text{-}NH_2$) are chosen as model nanoparticles due to their well-documented cytotoxic mechanisms. Human

lung adenocarcinoma (A549) cells were chosen for consistency with other studies, which show common modes of action in a number of cell lines, and as they act as models for human exposure by inhalation. The conventional and commonly used cytotoxicity test, Alamar Blue (AB) is carried out for 4, 8, 12 and 24 h particle exposures to determine toxic effects. The 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay was also used for 8 h particle exposure to compare the responses from different cytotoxicity assays. A549 cells were exposed to 2.5, 5 and 10 μM concentrations of the PS-NH_2 for the evaluation of spectroscopic signatures of the dose-dependent toxic responses. Also, cells were exposed to 2.5 μM, a sub-lethal dose, for 4, 8, 12 and 24 h for the assessment of toxicity related changes in biomolecules such as the proteins, lipids and nucleic acids. Raman datasets were obtained from the cytoplasm, nucleus and nucleolus and unsupervised Principal Component analysis (PCA) was used for the elucidation and comparison of dose and time dependent biomolecular changes in the cells upon nanoparticle exposure.

For all exposure times and doses, the most prominent Raman spectral marker, reflecting the cytotoxic response to exposure to model PS-NH_2 nanoparticles, is found to be the bands at 785 and 810 cm^{-1} in the cytoplasm, reflecting changes in cytoplasmic nucleic acid content (Fig. 1). Notably, this response is not normally identified by conventional cytotoxicity assays. The concomitant and subsequent changes in the intensity of the bands corresponding to proteins (Amide I region (1550-1700 cm^{-1}) and lipids (1229 and 1438 cm^{-1}) can also be used to determine toxic effect of nanoparticles. The use of Raman spectroscopy helps to corroborate and further elucidate the mechanism of action of the nanoparticles within cells and Raman cytotoxicity spectral-markers identified for model nanoparticles can potentially be used to screen for the mode of action and degree of toxicity of novel nanoparticles, in a single label-free assay.

Esen Efeoglu [1,2], ***Alan Casey*** [2], ***Hugh J. Byrne*** [2]
[1]*School of Physics, Dublin Institute of Technology, Kevin Street, Dublin 2, Ireland*
[2]*FOCAS Research Institute, Dublin Institute of Technology, Kevin Street, Dublin 2, Ireland*

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