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Tracking intracellular uptake and localisation of alkyne tagged fatty acids using Raman spectroscopy

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Intracellular uptake, distribution and metabolism of lipids are tightly regulated characteristics in healthy cells. An analytical technique capable of understanding these characteristics with a high level of species specificity in a minimally invasive manner is highly desirable in order to understand better how these become disrupted during disease. In this study, the uptake and distribution of three different alkyne tagged fatty acids in single cells were monitored and compared, highlighting the ability of Raman spectroscopy combined with alkyne tags for better understanding of the fine details with regard to uptake, distribution and metabolism of very chemically specific lipid species. This indicates the promise of using Raman spectroscopy directly with alkyne tagged lipids for cellular studies as opposed to subsequently clicking of a fluorophore onto the alkyne for fluorescence imaging.

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1. Introduction

Lipids are one of the four main classes of macromolecules. As such, they are important cellular components since tightly regulated uptake, distribution and metabolism of lipid species are vital for normal cell functioning. Dysregulation of these processes has been linked to disease states such as neurodegenerative disease [1] and cancer [2]. In order to develop new and effective treatment for such diseases it is important to gain a detailed understanding of underlying lipid biochemistry with respect to uptake, distribution and metabolism and changes during disease onset and progression. Better understanding will enable drug design for improved efficacy and therapies. In addition, one potential strategy for diagnostics and treatment monitoring is to detect divergence from the healthy lipid state and subsequent return through, for example, drug treatment by measuring a characteristic 'snapshot' of the state in healthy cells.

In order to gain insight into detailed aspects of lipid biochemistry and its regulation, it is vital to have analytical techniques capable of measuring these species. Lipids are a particularly understudied class of biomolecules due to the limitations in analytical techniques available to accurately image and characterize these species intracellularly. Many molecular biology based approaches for biochemical analysis require cells to be lysed and individual cell components isolated, such as the polymerase chain reaction, electrophoresis and Western blotting [3]. While these techniques can give a high level of chemically specific information, they are destructive and could introduce artefacts and modifications to the sample during the process of lysing and isolation of cell components. In addition, lipidomics is an emerging technique for lipid analysis but this is on a more global scale, requiring large sample quantities, which undergo extraction procedures, again making this a destructive technique [4]. Most conventional imaging strategies rely on fluorescently tagging biomolecules for detection of specific species [5]. This approach allows species to be imaged in their native environment non-destructively but there are disadvantages associated with the need for an external label to be added to the sample, which could itself interfere with the native state of the system. In particular, these techniques are less well established and limited in their ability to analyse lipids. Lipids lack inherent fluorescence, and the dyes available for staining lipid species tend to be very similar in size to the lipid species themselves, therefore having the potential to significantly alter innate distribution, dynamics and metabolism [6].

Raman spectroscopy is a promising technique for analysis of intracellular lipid species, due to the high Raman cross section associated with the largely non-polar lipid species giving rise to strong signals in the Raman spectra of these molecules [7]. While Raman scattering is a weak physical process, meaning measurements can be lengthy, it allows biological samples to be analysed in a label free manner with minimal

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sample preparation and non-destructively, allowing them to remain in their native state [8,9]. One of the major drawbacks when using Raman to analyse biological samples is that it is difficult to extract information about very specific species as opposed to a global response in, for example, lipid composition, due to the presence of broad peaks resulting from the overlapping of multiple peaks at slightly different frequencies [10]. Therefore, while this technique gives useful information regarding global distribution and dynamics of lipid species, it is difficult to assign signals to very specific lipids such as individual fatty acids.

In order to gain insight into lipid distribution and dynamics with regards to very specific biomolecules, a combination of a labelling approach with Raman imaging could be key. Alkyne tags have become important functional groups for labelling species for tracking using Raman spectroscopy. This utility stems from the unique alkyne signal observed in the biologically 'silent' region of the Raman spectrum between approximately 1800 and 2800 cm^{-1} [11]. Alkyne functionality is not an inherent characteristic of native biological systems and therefore in recent years a number of studies have created biorthogonal alkyne tagged reporters in order to image specific biological species or follow the dynamics of biological processes. Alkyne tags have also been incorporated into fluorescent imaging approaches, where they are added to biomolecules as precursors to later undergo click reactions with fluorescent tags typically introduced after cell fixation [12,13]. This approach has been used with alkynyl fatty acids previously to visualise lipid-modified proteins [13] using click chemistry-based detection and also more recently using a proximity-ligation strategy for detection of specific acylated protein [14]. The tags were introduced due to their small size, resulting in minimal interference with native biochemical processes, in comparison to traditional fluorescent imaging approaches. However, using Raman spectroscopy improves this technique further by removing the need for additional click reactions due to the ease at which the alkyne signal can be detected in the biological silent region of the Raman spectrum.

EdU (5-ethynyl-2′-deoxyuridine), a thymidine analogue with alkyne functionality, is one of the most studied alkyne tags for Raman tracking [12]. Tracking has moved from traditional Raman spectroscopy to the non-linear techniques of coherent anti-Stokes Raman and stimulated Raman spectroscopy to improve speed [15–18]. Studies have incorporated alkyne modified fatty acids previously, however, these were primarily used as broad markers for lipids in comparison to proteins and nucleic acids, monitored using different alkyne tagged biomolecules [19,20]. To our knowledge, no previous work has directly compared the uptake and distribution of multiple different alkyne tagged fatty acids in a single study. In addition to the use of alkyne tagged biomolecules, deuterium tagging has been employed in a number of studies in an analogous approach [21-23], including comparison of uptake, metabolism and distribution of deuterated palmitic acid, oleic acid and cholesterol by macrophages [24]. However, in a comprehensive study, Yamakoshi et al. [11] demonstrated the superior capability of alkyne tagged compared to deuterium tagged molecules for Raman imaging studies. This study highlighted that although fully deuterated molecules could display relatively high Raman intensities with, for example, fully deuterated acetonitrile showing similar intensity for the nitrile compared to C—D stretch, when C—D bonds were not all identical the resultant signals were much more complex and individually lower in relative intensity. The study compared alkyne, nitrile, azide and fully deuterium tagged hexanoic acid, showing the alkyne tag to have the highest relative intensity for the alkyne stretching frequency, approximately 2.5× more intense than the nitrile equivalent, which from reference to acetonitrile had comparable signal intensity to incorporation of three identical C—D bonds. Therefore, alkyne tags have vast potential for probing uptake, distribution and metabolism of biomolecules, which has not yet been fully explored, particularly for comparing these characteristics for multiple structurally similar biomolecules.

2. Materials and Methods

2.1. Fatty Acids

Myristic acid, palmitic acid and stearic acid were purchased from Sigma Aldrich.

The synthesis of fatty acid alkyne probes is described in Greaves et al. [25].

2.2. Cell Culture

HEK293T cells were plated on glass coverslips in DMEM supplemented with 10% fetal bovine serum. For labelling with alkyne fatty acids, DMEM containing 1 mg/mL defatted BSA was added to the cells and the cells were incubated at 37 °C for 30 min. The medium was then removed and replaced with DMEM/BSA containing 100 μM of the fatty acid alkyne probe (C14, C16 or C18) and incubated for 4 h at 37 °C. Cells were then washed three times in PBS, fixed in 4% formaldehyde for 20 min, washed in PBS then H_2O and air-dried overnight.

2.3. Raman Measurements

Raman spectra were acquired on a Renishaw inVia Raman microscope equipped with a 532 nm Nd:YAG laser giving a maximum power of 500 mW, 1800 lines/mm grating, and an Olympus 100×/NA 0.90 MPlanFL N objective.

For pure fatty acids and alkyne tagged fatty acids, a small sample of each was transferred onto a CaF_2 window. Spectra of the solid crystals were acquired using 10 s acquisition time and 50% (*ca.* 10 mW) laser power for extended scanning from 200 cm^{-1} to 3200 cm^{-1}. Three measurements were taken for each sample.

Fixed cells were mapped using a step size of 1 μm in x and y, 1 s acquisition time, 100% (*ca.* 20 mW) laser power and a spectral centre of 2000 cm^{-1}. Three maps were acquired per condition.

2.4. Data Processing

2.4.1. Pure Compounds

Spectra of pure compounds were processed using MATLAB® R2016a. Spectra were smoothed using Savitzky-Golay filtering with a polynomial order of 3 and a frame length of 9 followed by baseline subtraction using a custom script. Three spectra for each compound were min-max scaled and the average spectrum was calculated. For spectra of alkyne tagged fatty acids, the ratio of the intensity of the peak at 2110 cm^{-1} and the total spectral intensity between 1181 cm^{-1} and 2740 cm^{-1} was calculated for each spectrum.

2.4.2. Cells

Cell maps were pre-processing using Renishaw Wire 4.2 software. Cosmic rays were removed using the nearest neighbour algorithm following by noise filtering and baseline subtraction. Pre-processed maps were then imported into MATLAB® R2016a. Cell regions were selected using a mask based on total spectral intensity to remove non-cell background. A false colour map of the intensity of the peak at 2118 cm^{-1} was created to map the presence of each alkyne in the cells. False colour images of the following peak intensity ratio for each cell were created:

- peak intensity 1448 cm^{-1}/(peak intensity 1657 cm^{-1} + peak intensity 1448 cm^{-1}).

The total intensity at 2118 cm^{-1} over each cell area was divided by the total spectral intensity over the respective cell area to give an indication of level of alkyne uptake and this was scaled based on the inherent intensity differences of the alkyne tagged fatty acids calculated previously.

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