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Tracking glycosylation in live cells using FTIR spectroscopy

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Abstract

This is the first demonstration of the study of glycan protein turnover in living cells by FTIR with commercially available tetraacetylated N-Azidoacetyl-D-Mannosamine (Ac_4ManNAz) label. The FTIR analysis has shown to be able to monitor the metabolism of glycans in living cells in real time. The method is simple, quantitative and requires equipment that are available in many laboratories. It can be used in a wide range of applications such as the study of glycosylation and cell-signalling.

Keywords: FT-IR; live cells; ATR; metabolic labelling; azide; alkyne; vibrational spectroscopy

Introduction

Metabolic labelling has established to be a powerful imaging method, which overcomes the major limitation of traditional fluorescent labelling methods caused by the large size of fluorophores without the disadvantages of radioactive labelling. Cell labelling kits based on this concept are now commercially available, creating significant impacts in many areas of biomedical research. The method is based on the use of an analogue biomolecule with an inert modification, e.g. a substitution of a methyl group by an azide or alkyne, or isotopically labelled molecules to metabolically label the target biomolecule. [1-12] Due to the small modification and the biorthogonal nature of the modified group, this method has found novel applications for labelling biomolecules such as glycans and lipids. However, the current labelling method requires the attachment of a fluorophore at the detection step, which is constrained by the diffusion and reaction rate, severely limiting the number of time points that can be collected per measurement and hence the potential for studying the dynamics inside a living cell.[13] Interestingly, these functional groups produce intrinsic distinct sharp peaks in the vibrational spectral region (~2120 cm^{-1}, so called the "bio-silent" region) where natural biological compounds do not produce bands, i.e. they are biorthogonal spectroscopically. This opportunity to directly study the metabolic of these compounds using vibrational spectroscopic techniques, has been thus far explored using spontaneous Raman,[14] surface enhanced Raman[15] as well as with stimulation Raman (SRS)[16-20] or synchrotron Fourier transform infrared (FTIR) using deuterated palmitic acid.[21]

FTIR application to the detection of these metabolic labels is a rather unexplored area despite FTIR being a fast method with a much simpler set up and has no photo effect on the living cells. The cost of the instrument is significantly lower than Raman systems with most analytical laboratories already possessing research grade FTIR spectrometers. It is important to note that many of the commercially available protein and glycan metabolic labels are azide based whilst Raman methods mainly target the alkyne ν(C≡C) band at 2120 cm^{-1} because azide groups produce weak Raman peaks.[13] In contrast, azide ν(N≡N) vibration produces a strong IR band[22] at ~2122 cm^{-1} opening opportunities to apply the existing library of azide labels that are already available, complementing the development in Raman. We have demonstrated that FTIR is a simple and direct method for studying living cells.[23-26] It is a simple quantitative method (absorbance is proportional to concentration

following the Beers' law) and can detect concentration at 15 μM level,[23, 26] which is significantly better than standard Raman approaches.[17] The precise FTIR method exploited the advantage of the total attenuation reflection (ATR) measurement mode where only the living cells attached to the measuring surface contribute to the signal without interference from the medium above the cell.[27] Incorporation of the azide sugar into the glycan structure can therefore be monitored directly by this FTIR method at μM level detection limit. The concept of this work has been illustrated in Fig. 1.

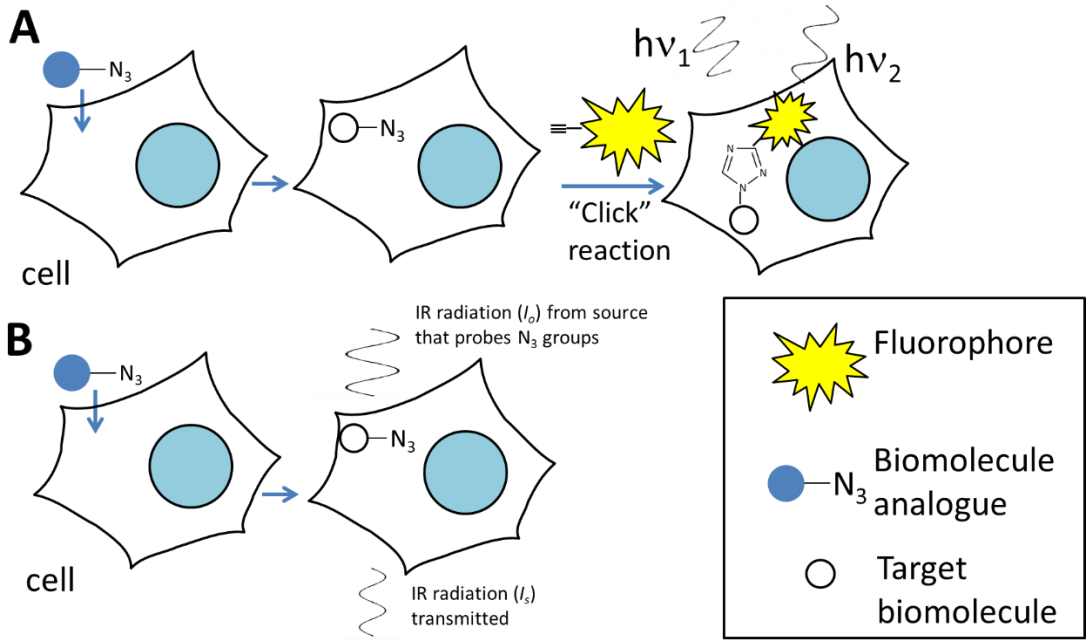


Fig. 1: (A) Azide labelling detected using fluorescence and (B) Azide labelling detected using IR.

To demonstrate that FTIR can be used to track metabolically labelled molecules in living cells, commercially available tetraacetylated N-Azidoacetyl-D-Mannosamine ($Ac_4ManNAz$), an azide labelled monosaccharide building block, which specifically labels sialic acid-containing glycoproteins on the cell membrane and can be used to study glycosylation in cells, was used. Our hypothesis is that once the azide functional group is metabolically incorporated into the cell membrane structure, it will accumulate to a level that can be detected and quantified directly using the IR absorbance band at ~2122 cm^{-1}.

Methods

Cell Culture/Stocks

MDA-MB-231 (MDA) breast cancer cells were used as a representative cell line for tracking of glycosylation processes. Cells with passage number of <20 were used for all experiments. Cells were grown up in DMEM (amended with 10% FBS, 2mM L-glutamine, 100 U mL^{-1} Penstrep, and 1% MEM non-essential amino acids) at 37 °C with a 5% CO2 atmosphere in vented culture flasks. Cells were passaged using standard cell line splitting procedures; in brief, upon reaching high confluence (70-80% surface coverage) cells were detached with 1X Trypsin (Sigma-Aldrich, Cat. No. 59427C) and transferred to a fresh culture flask. Trypan blue assay was conducted for all cell counts. L15 medium was amended with 10% FBS, 2mM L-Glutamine, 1% MEM non-essential amino acids, and 100 U mL^{-1} Penstrep. Cells in L15 medium were incubated at 37°C in moist air.

50 mM $Ac_4ManNAz$ (ThermoFisher, Cat. No. C33366) stock solutions were prepared by dissolving powder $Ac_4ManNAz$ in 100% DMSO and stored in the dark at –20°C. 10 mg/mL Alexa Fluor 488 stocks were prepared by dissolving powder Alexa Fluor 488 in 100% DMSO and stored in the dark at –20°C.

Attenuated total reflection (ATR)-FTIR

ATR-FTIR spectroscopy was used to track glycosylation processes in the MDA breast cancer cell line. Here, tracking was enabled by the cellular incorporation of the IR-active azide-functionalized sialic acid reporter, Ac_4ManNAz. First, MDA cells were directly seeded onto the measuring surface (zinc sulfide horizontal(H)-ATR crystal, Crystran Ltd. UK) of the ATR-FTIR measurement device as previously described[23, 24] at ~50% confluence so that the cells could be measured during exponential phase. For 24 hours, the cells were measured on the ATR-FTIR device while growing in L-15 (Leibovitz, Sigma-Aldrich, Cat. No. L4386) with 100 U mL^{-1} PenStrep amended with 50 μM Ac_4ManNAz. Ac_4ManNAz was then removed by replacement of culture medium with fresh Ac_4ManNAz-free L-15 medium; cells were then incubated and measured for an additional 48 hours. FTIR spectra were collected every 20 minutes, concurrent with incubation, using a FTIR spectrometer (Frontier, PerkinElmer) fitted with a DTGS detector. Each spectrum was measured across the range of 3000-900 cm^{-1} at a resolution of 8 cm^{-1}. Total scan time for each spectrum was 11 minutes with a scan speed of 0.2 cm s^{-1}. Spectra were produced and analyzed using the Spectrum 10 (PerkinElmer) software – applying a strong apodization function (effectively a smoothing factor of 7 compared to spectrum obtained by applying a weak apodization) to the interferogram. Three replicate experiments were performed based on 3 separate cultures.

Flow Cytometry

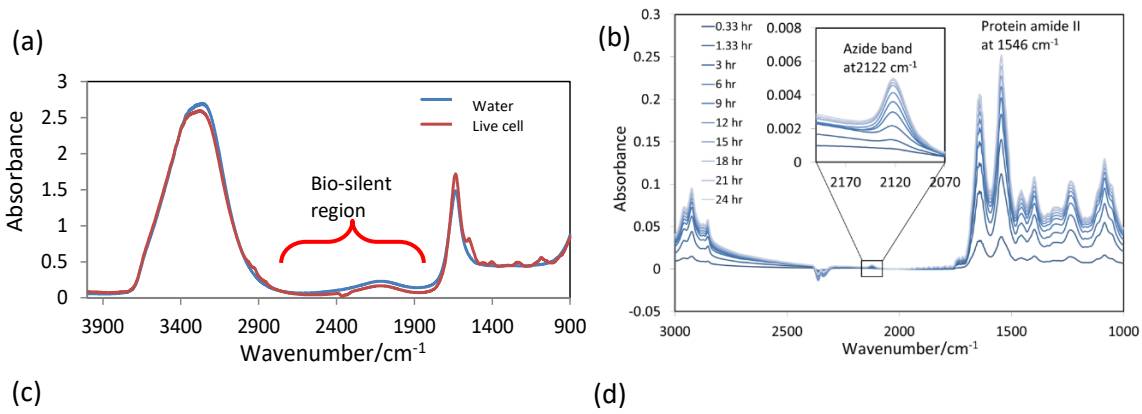
Successful incorporation of the azido-glycan, Ac_4ManNAz, was verified with flow cytometry (FC); making use of the azido-glycans click chemistry functionality to attach the alkyne fluorophore Alexa Fluor 488 (ThermoFisher, Cat. No. A10267). MDA cells in L15 medium amended 50 µM Ac_4ManNAz were seeded onto a 24-well plate at the density of 150,000 cells/well and incubated for 24 hours to allow for a high degree of glycan incorporation. Untreated cell populations, without any Ac4ManNAz, were used as a baseline and control for fluorescent measurements. Washing steps were conducted by suspension of cell pellet (in noted solutions), centrifugation at 800xg for 5 minutes, and careful removal of supernatant.

First, all cell treatments were detached using accutase (Sigma-Aldrich, Cat. No. A6964) and incubated at room temperature (~20 °C) for 10 minutes to best preserve cell surface features. L15 medium was added to inhibit accutase activity; cells were then pelleted and accutase/L15 mix was discarded. Resulting pellet was washed with 1X PBS. Cells were then fixed by suspension in 4% paraformaldehyde and incubated for 15 minutes at room temperature followed by a wash with 1X PBS. All cell treatments, with exception of the unpermeabilized cell populations, were then suspended in 0.25% Triton X100 in 1X PBS and incubated for 15 minutes followed by a wash in a blocking solution of 3% BSA in 1X PBS. The resulting pellet was then suspended in 500 µL of reaction cocktail prepared as directed in the Click-iT® Cell Reaction Buffer Kit (ThermoFisher, Cat. No. C10269) making use of addition of 1 mg/mL Alexa Fluor 488 (ThermoFisher, Cat. No. A10267) 1:1 Alexa Fluor 488/Ac4ManNAz mole ratio with an incorporation assumed on the order of 10^8 molecules Ac4ManNAz/cell, consistent with known incorporation rates of Ac4ManNAz. Cells were then incubated with reaction cocktail for 30 minutes at room termpature in the dark. Finally, cells were centrifuged and washed again with a blocking solution of 3% BSA in 1X PBS. The final cell pellet was suspended in 500 µL 1X PBS. FC measurements were taken using the Ex/Em of Alexa Fluor 488 (493 and 520 nm respectively).

Results

The cell morphology was monitored and there was no change observed after the exposure in 50 μM of $Ac_4ManNAz$ when compared to the control cells (i.e. cells without $Ac_4ManNAz$ treatment). The recorded spectra of the control cells and water are shown in Fig. 2a showing the typical spectra of live cells with most of the strong absorbance from water, proteins, lipids carbohydrates and nucleic acids in the 1700 cm^{-1}-900 cm^{-1} range but no prominent absorbance features in the bio-silent 2122 cm^{-1} region. Similarly, water bands dominate the 3700 to 3000 cm^{-1} region (OH stretching band) and the 1680-1600 cm^{-1} region (OH bending band) but not in the bio-silent 2122 cm^{-1} region. In order to show more clearly the spectra of the living cells, medium, which contains >99% water, was used as the background spectrum when the cells were treated in 50 μM of $Ac_4ManNAz$. The measured spectra have shown the cell absorbance have rapidly increased through the attachment and growth of the living cells in the first 24 hours. Importantly, a growing absorbance peak at 2122 cm^{-1} that is specific to the azide $\nu(N{\equiv}N)$ stretching mode vibrational band has been observed (Fig. 2b) as a result of the incorporation of the azide functional group into the cell structure.

To remove the effect of cell attachment and growth, a peak height ratio of the azide band at 2122 cm^{-1} against the protein amide II band at 1546 cm^{-1} has been plotted (Fig. 2c). The amide II band was used instead of amide I because it was noted that using medium as the background spectrum has led to an over subtraction of the water absorbance in the 1680-1600 cm^{-1} region, resulting in an apparently smaller amide I band. More importantly, Figure 2a shows that the amide I band absorbance was higher than 1, i.e. outside the linear response region of the detector, making it not suitable for quantitative analysis. However, the amide II band was not affected by these. The profile of the azide:protein band shows that the azide peak reached a plateau after 15 hours of incubation, suggesting that the azido-sugar incorporation into the glycan structure has reached saturation. At this point, the peak height of the azide band was ~3.5×10^{-3} (after baseline subtraction) and the spectral signal to noise ratio exceeded 700 (based on the peak to peak noise in the 1900-2000 cm^{-1} region, which is typically less than 5 $\times10^{-6}$), highlighting the high quality of the spectral data. Standard solutions made of medium containing 50 and 500 μM of $Ac_4ManNAz$ (without cell) were also measured to rule out the possible contribution of the azide absorbance from the medium or through passive diffusion of the azide molecules into the cells. At 50 μM and 500 μM, the 2122 cm^{-1} band was detectable with a respective absorbance peak height of ~ 3×10^{-5} and ~3×10^{-4}, both significantly less than what has been detected in the cell treated in 50 μM of $Ac_4ManNAz$. The result shows a high uptake of the azide sugar analogue $Ac_4ManNAz$ in the cell as a result of the glycosylation of the cell membrane proteins.



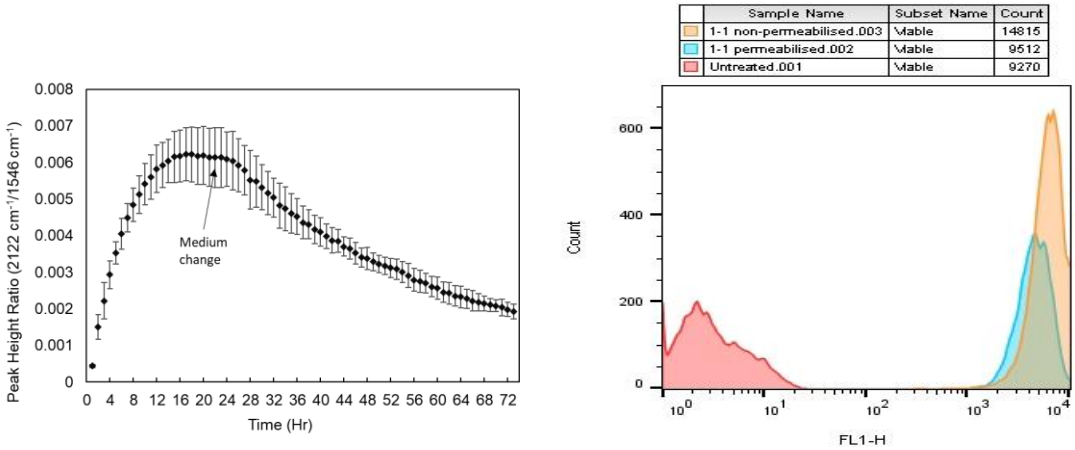


Fig. 2: (a) IR spectra of water and live cell (no water subtraction) showing the bio-silent region has no sharp peaks. (b) Spectrum of the live cell (water subtracted by using the medium, >99% water, as the background) treated in 50 μM of Ac_4ManNAz for 24 hours. (c) Azide: amide II peak ratio profile after the addition of Ac_4ManNAz from time 0 and replacement of the Ac_4ManNAz-free medium at 24 hr. Error bars shows the standard deviation from 3 independent experiments. (d) Flow cytometry data showing the cells treated with Ac_4ManNAz (blue and orange peaks) have high fluorescence signal relative to the low fluorescence signal of the untreated cells (red peaks).

After the 24 hours of exposure to Ac_4ManNAz, the azide sugar supplement was removed by replacing the medium without Ac_4ManNAz and the FTIR spectra of the cell were monitored for a further 48 hours. The amide II band absorbance, which represent protein, has continued to increase showing that the cells were growing continuously throughout the measurement. However, a decay in the azide peak of the live cells was observed (Fig. 2c) demonstrating the feasibility to monitor glycan turnover in live cells with this technique. The error bars on Fig. 2c represent the standard deviation from the three separate experiments, which has shown an averaged covariance of 10.9% representing the variation in response between the different batch of cultured cells. The origin of the observed biological variation was not investigated as it is not the key focus of the current work.

The incorporation of azide in the cells were validated using the Alexa Fluor 488 alkyne and click-iT cell reaction buffer kit to fluorescently label the azide tagged glycan on the cell at 24 hr treatment time (Fig. 2d). As expected, the results have shown that cells treated with Ac_4ManNAz has been labelled by the fluorescence dye while the non-treated cells were not labelled. Also noted is that permeabilised cells display lower fluorescent signal than non-permeabilised cells. A feature which may be explained by the localisation of the azido-glycan to the cell membrane; exposing it to higher disruption in the permeabilised cell treatments. In comparison to the fluorescent detection, which is destructive and only a single time point was obtained in an experiment, the single step FTIR has the advantage of the data being collected from the same batch of cells with significantly more time points and no need to add the fluorescent dye.

The results shown in this work have demonstrated that metabolic labelling combined with FTIR detection can monitor glycosylation, a post-transitional modification process, in living cell. Measurement of glycosylation in cell is important for both biomedical and biopharmaceutical research. A large proportion of biopharmaceuticals are glycoproteins, a method that allows the monitoring of glycosylation of a specific type of carbohydrate units in living cell would be a valuable tool in the optimisation and quality control of these products.

The metabolic labelling compound $Ac_4ManNAz$ used in this study selectively labels sialylated glycans, which is particularly important in the study of cancers. These glycans are noticed to be highly expressed in cancer cells and has been highlighted as a potential biomarker for diagnosis or therapeutic target. A potential application of the presented method is the studying of the effect of drugs on glycosylation in cell, which has extensive implication on cancer treatments. For example, inhibition of glycosylation has found to enhance the toxicity of cisplatin, an anticancer agent, to head-and-neck cancer cells including resistant strain.[28] The presented technique can be used as a screening tool to identify compounds that may suppress glycosylation in cells to enhance the sensitivity of cancer cells to anti-cancer agents. A recent study has demonstrated the use of metabolic labelling for ex vivo tissue imaging.[29] The method presented in this work can also be adapted in imaging mode and applied to simplify the detection of the azide labelled cells in a tissue while maintaining the specificity offered by the technique.

Conclusions

In summary, we have demonstrated live cell FTIR with azide labelling is a simple method in the study of glycan dynamics in living cells. The method has shown to be capable of performing real-time quantitative analysis of metabolic incorporation of azide sugar to cell membrane based on the absorbance of the azide peak at 2122 cm^{-1}. The presented method is not limited to the study of glycosylation. Other azide modified biorthogonal molecules such as azido- palmitic acid, myristic acid, geranylgeranyl alcohol, farnesyl alcohol and alanine are all already commercially available for real-time study of various biochemical targets using live-cell FTIR.

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