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Deuterated Carbohydrate Probes as 'Label-Free' Substrates for Probing Nutrient Uptake in Mycobacteria by Nuclear Reaction Analysis

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Understanding and probing small molecule uptake in cells is challenging, requiring sterically large chemical labels, or radioactive isotopes. Here, the uptake of deuterated sugars by *Mycobacterium smegmatis*, a non-pathogenic model of *Mycobacterium tuberculosis*, has been investigated using ion-beam (nuclear reaction) analysis demonstrating a new technique for label-free nutrient acquisition measurement.

Mycobacterium tuberculosis is the etiological agent of tuberculosis (TB). TB remains a leading cause of death worldwide and in 2013 there were 9.0 million new cases and 1.5 million people died from TB.[1] *M. tuberculosis* has a complex, unique cell wall that is rich is diverse carbohydrates and lipids that protects the bacterium from environmental stresses and chemotherapeutic agents. Despite the global threat of TB there are limited studies to investigate the nutrient requirement of this organism. Recent studies have implicated a role for putative sugar-transporters in *M. tuberculosis* to have an essential role during intracellular infection. Despite the obvious importance in gaining a detailed understanding of how *M. tuberculosis* processes carbohydrates, there exist very few detailed studies. This is due to the inherent lack of chromophore/fluorophore moieties on the sugars that significantly limits the analytic tools available to probe these essential biological processes *in vitro* and *in vivo*. Probes for such studies to date have been limited to radiolabelled carbohydrates, fluorescently modified sugars, or azido-modified sugars. Radiolabelled $^{14}C/^{3}H$ carbohydrates are expensive and non-standard carbohydrates are not readily available from commercial sources nor easy to synthesise. Fluorescently labelled carbohydrates have been used for such studies.[2] However, their synthesis is often non-trivial and, more importantly, the large size of the fluorophores gives significant changes to the molecule. Extrapolating the function of the native carbohydrate from such derivatives is challenging, and non-specific uptake due to the lipophilic character of most dyes cannot be ruled out. The use of azido-sugars to metabolically label cells followed by Cu-free click,[3] or Staudinger-ligation[4] chemistry has been successfully undertaken.[5] However, this method requires chemical synthesis of the desired azido-sugar, and pre-requisite knowledge about the intracellular processing of the sugar to ensure the structural modification will not influence its metabolism, relative to the native carbohydrate.[6]

Considering the above challenges, alternative biochemically passive labelling strategies are required. Deuterium is a stable, safe and abundant isotope of hydrogen that has near identical chemical reactivity, and has a low natural abundance in water of < 0.02%, essential for any analytical method. We therefore reasoned that Nuclear Reaction Analysis (NRA), which is uniquely sensitive towards the detection of deuterium could be employed to monitor the uptake of deuterated nutrients into bacteria.[7]. NRA is one of a family of MeV ion beam analysis techniques often used for quantitative materials (including polymer) analysis and depth profiling[8-9]. The $^{2}H(^{3}He,p)\alpha$ nuclear reaction is well established in materials analysis to quantify variation in ^{2}H content with depth.[10-11] This method is also appealing as data acquisition is rapid (less than 10 minutes) and does not rely on external calibration.

Here we have employed nuclear reaction analysis to study the uptake of deuterated carbohydrates by the non-pathogenic model organism *Mycobacterium smegmatis.*

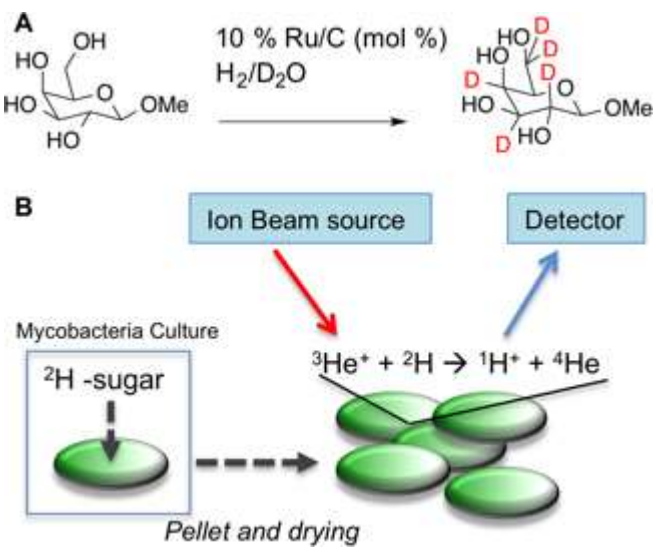

Figure 1. A) Synthetic scheme for carbohydrate deuteration, shown for 1-O-β-methyl galactose B) Schematic illustration for assessing ^{2}H-carbohydrate uptake by *M. smegmatis* and ion beam analysis.

To obtain deuterated carbohydrates, the regio-selective method of Sawama et al. was employed.[12] Briefly, the carbohydrates were dissolved in D_2O, which serves as both solvent and the source of deuterium ions. Ruthenium on carbon (10 mol %) was added as the catalyst, and the reaction heated under a H_2 atmosphere. In the case of the reducing sugars: glucose; galactose; mannose and arabinose it was necessary to use their anomeric methyl ethers, since previous studies have shown that non-methylated protected sugars undergo decomposition under these reaction conditions with Ru/C-catalysed hydrogenation possibly due to the hemi-acetal moiety of non-reduced sugars. For the non-reducing sugar trehalose this was not necessary. Following isolation, the modified carbohydrates were analysed by ESI-mass spectrometry and ^{1}H NMR to estimate the degree of deuteration, summarised in Table 1 and also in the Supporting Information.

Table 1. Deuterated Carbohydrates

Carbohydrate	M_R (^{1}H)[(a)]	M_R (^{2}H) [(b)]	% ^{2}H [(c)] (NMR)
Glu-OMe	180	217-219	83
Gal-OMe	180	217-219	73
Man-OMe	180	217-219	60
Arab-OMe	150	187-189	81
Trehalose	342	365-369	62

(a) Molar mass of starting carbohydrate; (b) Main peak range in ESI-MS following deuteration of $[M+Na]^+$; (c) Average deuteration as evaluated by ^{1}H NMR.

ESI-MS revealed that each of the carbohydrates had an increase in mass from the single molecular ion to a heavier, distribution of ESI-MS peaks. The ESI-MS results indicated that there were typically 3/4 deuterons per sugar. It should be noted that the method used here only deuterates protons adjacent to a hydroxyl group, hence 100 % deuteration of the carbohydrate is not possible (Figure 1).[12] For our intended application complete deuteration was, however, unnecessary with ease and scale of the synthesis being the key requirements. ^{1}H NMR confirmed deuteration by a clear decrease in the number of proton signals relative to non-exchanged peaks. The ^{1}H NMR spectra of methyl-α-D-glucopyranoside is shown in Figure 2 (Supp. Info. Figs XX for other sugars) showing the change in peak intensity follow deuteration to give ^{2}H-Methyl-α-D-glucopyranoside.

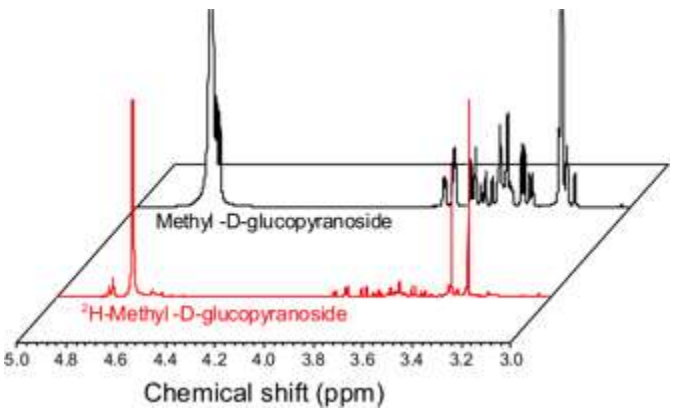

Figure 2. ^{1}H NMR spectra of methyl-α-D-glucopyranoside before and after deuteration, showing reduction in ring-protein intensity following deuteration, but retention of the methyl protons.

With this diverse range of ^{2}H carbohydrates to hand it was possible to assess the uptake of these carbohydrates by *M. smegmatis* (a non-pathogenic model for *M. tuberculosis*) using NRA ion beam analysis. In an initial screening experiment, ^{2}H trehalose was incubated with *M. smegmatis* at a final concentration of 50 mM for 60 minutes. After this time, the cells were pelleted, washed with PBS, heat-killed (95 °C, 15 mins) and freeze dried to provide a solid lyophilised sample suitable for subsequent ion beam analysis. The solid pellets were compressed to a homogenous disc and it was ensured that the thickness of this pellet was greater than the penetration depth of the $^3He^+$ ion beam (~ 4 microns). Full experimental details can be found in the supporting information. Briefly, the samples were irradiated with the $^3He^+$ ion beam and protons resulting from the nuclear reaction were detected at 170°. The low natural abundance of ^{2}H, along with the high energy of the detection of the emitted protons meant that this gave extremely good signal to noise ratio, and is ideal to detect low levels of deuterated carbohydrates. Encouragingly, the ion beam analysis detected the deuterium signal of the ^{2}H labelled *M. smegmatis* heat-killed whole cells, compared to zero signal for cell-only *M. smegmatis* control. This indicated that the amount of ^{2}H trehalose being taken up by *M. smegmatis* is in a range that is detectable by this method.

To probe the utility of this new analytical method, the panel of sugars in Table 1 were investigated for uptake into *M. smegmatis*. The ability to probe such a panel of sugars is crucial to gain information about the carbon sources employed by mycobacteria that are transported into the cell and metabolised both *in vitro* and *in vivo* The ^{2}H carbohydrate library were again added at a final concentration of 50 mM and incubated with *M. smegmatis* for 60 minutes. Following acquisition using NRA and analysis of the data we were able to calculate the relative molar uptake of each ^{2}H-carbohydrate as shown in Figure 3. This calculation included a correction for the total level of deuteration per-sugar (determined from Table 1) to ensure that a direct comparison of uptake of each ^{2}H carbohydrate can be made.

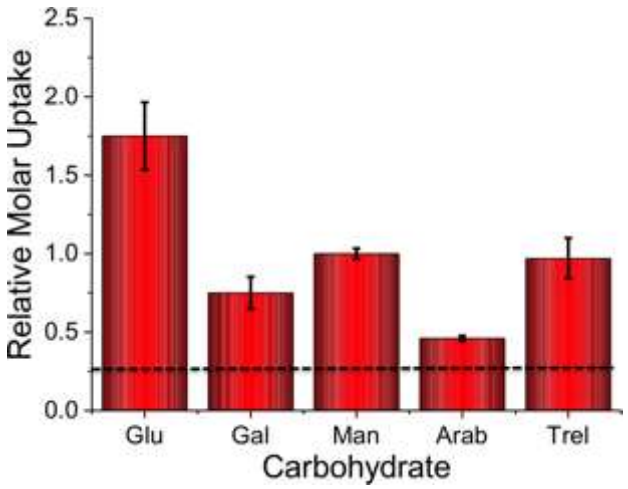

Figure 3. Relative molar uptake of carbohydrates into *M. smegmatis* determined by ion beam analysis. Uptake corrected for relative degrees of deuteration. Dashed line is the background signal from the cell-only fractions.

The results obtained from the NRA ^{2}H-carbohydrate uptake assay are comparable to that obtained from previous studies (REFS) that have utilised either ^{14}C carbohydrates, fluorescently labelled carbohydrates or azido-modified carbohydrates (REFS). In addition our results are comparable with studies of mycobacteria grown on carbohydrates as the sole carbon source (REF Titgeymer plus others). The metabolic utilitsation of carbohydrates by mycobacteria differs from study to study depending on the experimental conditions used. Our study therefore highlights the benefit of this straightforward, label-free and rapid method.

Recent studies have shown that trehalose is recycled from the cell wall of *M. tuberculosis* and is taken up by an ABC-transporter that is essential for the virulence of this pathogen (REF DEL). *In vitro* studies have demonstrated that trehalose can serve as a sole carbon source for mycobacteria and that it is an essential precursor for cell wall metabolites.[13] Intriguingly, trehalose uptake has been shown to be tolerant to a range of chemically altered trehalose analogues indicating its potential role as a new drug and biosensing target. Given the importance of trehalose in mycobacteria we sampled uptake of ^{2}H trehalose by *M. smegmatis* from 0-60 mins. A clear increase in the uptake of ^{2}H trehalose over the 60 minute time interval was found, with a significant uptake of ^{2}H trehalose at 30 mins, Figure 4.

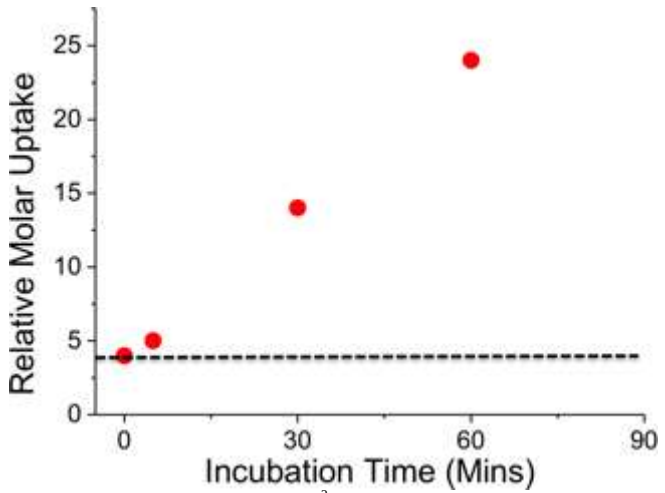

Figure 4. Time dependant uptake of ^{2}H-trehalose into *M. smegmatis*. Dashed line indicates cell-only background.

Crucially, these results demonstrate that this NRA method can be used for monitoring dynamic uptake processes, especially with relatively slow growing organisms such as mycobacteria. A key feature to emphasise is the novel use of ion-beam analysis to evaluate biological uptake processes utilising deuterated probes that are structurally more analogous to the 'native' sugars, than chemically modified probes, such as FITC-modified-, azido-modified-sugars and are easier and cheaper to handle than radio-labelled carbohydrates.

Conclusions

In summary, we have taken advantage of the ability to deuterate carbohydrates in a facile, regio-selective scalable manner enabling rapid access to a wide-range of carbohydrates that has allowed us to determine the relative uptake of a panel of ^{2}H-carbohydrates by *M. smegmatis.* This ^{2}H-carbohydrate uptake was probed experimentally and analysed by making use of the ^{3}He nuclear reaction analysis using an ion-beam. To our knowledge this is the first use of such ion-beam analysis in the discovery of small molecule uptake in bacteria and is comparable to data obtained by other methods. Using this method, the unusual uptake of trehalose into mycobacteria is observed, which is of particularly importance in the development of new treatments and diagnostics for pathogenic mycobacteria such as *M. tuberculosis*.

Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

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