^{13}C metabolic flux analysis of microbial and mammalian systems is enhanced with GC-MS measurements of glycogen and RNA labeling

Christopher P. Long[#], Jennifer Au[#], Jacqueline E. Gonzalez, Maciek R. Antoniewicz*

Department of Chemical and Biomolecular Engineering, Metabolic Engineering and Systems

Biology Laboratory, University of Delaware, Newark DE 19716, USA

*corresponding author:

Maciek R. Antoniewicz

Department of Chemical and Biomolecular Engineering

University of Delaware

150 Academy St

Newark, DE 19716

Tel.: 302-831-8960

Fax.: 302-831-1048

Email: mranton@udel.edu

[#] Equal contribution

ABSTRACT

^{13}C metabolic flux analysis (^{13}C -MFA) is a widely used tool for quantitative analysis of microbial and mammalian metabolism. Until now, ^{13}C -MFA was based mainly on measurements of isotopic labeling of amino acids derived from hydrolyzed biomass proteins and isotopic labeling of extracted intracellular metabolites. Here, we demonstrate that isotopic labeling of glycogen and RNA, measured with gas chromatography-mass spectrometry (GC-MS), provides valuable additional information for ^{13}C -MFA. Specifically, we demonstrate that isotopic labeling of glucose moiety of glycogen and ribose moiety of RNA greatly enhances resolution of metabolic fluxes in the upper part of metabolism; importantly, these measurements allow precise quantification of net and exchange fluxes in the pentose phosphate pathway. To demonstrate the practical importance of these measurements for ^{13}C -MFA, we have used *E. coli* as a model microbial system and CHO cells as a model mammalian system. Additionally, we have applied this approach to determine metabolic fluxes of glucose and xylose co-utilization in the *E. coli* $\Delta ptsG$ mutant. The convenience of measuring glycogen and RNA, which are stable and abundant in microbial and mammalian cells, offers the following key advantages: reduced sample size, no quenching required, no extractions required, and GC-MS can be used instead of more costly LC-MS/MS techniques. Overall, the presented approach for ^{13}C -MFA will have widespread applicability in metabolic engineering and biomedical research.

KEYWORDS

Pentose phosphate pathway; stable-isotope labeling; flux estimation; metabolism; glucose and xylose co-utilization.

1. INTRODUCTION

^{13}C metabolic flux analysis (^{13}C -MFA) is a powerful tool for quantifying cellular metabolism in a wide range of metabolic engineering and biomedical applications (Antoniewicz, 2015a; Young, 2014). A major factor governing the accuracy and precision of flux estimates from ^{13}C -MFA is the selection of informative isotopic labeling measurements. In most ^{13}C -MFA studies to date, flux analysis has been conducted using measurements of isotopic labeling of proteinogenic amino acids and/or isotopic labeling of extracted intracellular metabolites measured with techniques such as mass spectrometry (MS), tandem mass spectrometry (MS/MS), and nuclear magnetic resonance (NMR) (Antoniewicz, 2013a; Antoniewicz et al., 2007a; Masakapalli et al., 2014; Truong et al., 2014). Recently, improvements in flux precision were also achieved by simultaneously fitting multiple sets of labeling measurements from parallel tracer experiments (Crown et al., 2015).

In these data sets, however, metabolites derived from the upper half of central carbon metabolism (consisting of the upper portion of the glycolysis (EMP) pathway, pentose phosphate pathway (PPP), and Entner-Doudoroff (ED) pathway) are greatly underrepresented. For example, observability of PPP fluxes in *E. coli* largely depends on accurate measurements of phenylalanine labeling, which is synthesized from erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). Although there are other metabolites that are derived from upper metabolism, e.g. histidine and serine, these measurements are less informative. In our experience, histidine is difficult to detect reliably due to low abundance (Antoniewicz et al., 2007a), and both amino acids are related to one-carbon metabolism for which information may be incomplete. This leaves phenylalanine as the only relevant metabolite for flux analyses of

PPP. In mammalian systems, flux analysis is even more challenging given that histidine and phenylalanine are not synthesized by mammalian cells, i.e. these are essential amino acids. Thus, ^{13}C -MFA depends on more distant labeling measurements, for example, of intermediates in the lower glycolytic pathway such as 3PG and PEP (Ahn and Antoniewicz, 2013). LC-MS and LC-MS/MS based approaches have provided direct labeling information on pentose phosphate pathway intermediates and fragments (Hanke et al., 2013; Rühl et al., 2012); however, these methods are often costly and laborious, as analysis of these intracellular metabolites requires rapid quenching and efficient extraction techniques, and in many cases large sample sizes due to low concentrations of these metabolites inside cells.

A recent effort to provide additional labeling data that is more directly related to upper metabolism was focused on fragments of nucleosides derived from DNA and RNA (Miranda-Santos et al., 2015). Specifically, 31 nucleoside fragments were identified and the applicability of these fragments was illustrated in yeast cultures. While this method employed GC-MS instead of LC-MS, the presented protocols required large sample sizes (1.5 to 100 mL of biomass at $\text{OD}=3.5$) and the labeling data was not solely related to metabolism of PPP, but included other parts of metabolism, e.g. TCA cycle and one-carbon metabolism, which further complicated data analysis. Other research groups have measured ^{13}C -labeling of RNA, glycogen, and glycans, and have demonstrated the value of these measurements for flux analysis (Badur et al., 2015; Guzmán et al., 2014; Murphy et al., 2013).

Our work builds on these previous studies. Specifically, we present here a convenient GC-MS based method for measuring isotopic labeling of multiple fragments of glucose and ribose

derived from glycogen and RNA for ^{13}C -MFA studies. Our approach is less sample-intensive and more informative. We demonstrate the reliability of our approach in two biological systems: *E. coli* as a model microbial system and CHO cells as a model mammalian system. Specifically, we illustrate that isotopic labeling of glucose moiety from glycogen and ribose moiety from RNA permit precise quantification of net and exchange fluxes in PPP. We also use this approach to determine PPP fluxes during co-utilization of glucose and xylose. Overall, we demonstrate that incorporating labeling measurements of glycogen and RNA, which are stable and abundant in microbial and mammalian cells, greatly improves flux observability, thus paving the way for future applications of this approach in metabolic engineering and biomedical research.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals and M9 minimal medium were purchased from Sigma-Aldrich (St. Louis, MO).

Isotopic tracers were purchased from Cambridge Isotope Laboratories (Tewksbury, MA): [1,6- ^{13}C]glucose (99.2 % ^{13}C), [1,2- ^{13}C]glucose (99.7 %), [1- ^{13}C]glucose (99.5 %), [2- ^{13}C]glucose (99.5 %), [3- ^{13}C]glucose (99.5 %), [4,5,6- ^{13}C]glucose (99.5 %), and [1,2- ^{13}C]xylose (99.2 %).

The isotopic enrichment of all tracers and the composition of tracer mixtures used in parallel labeling experiments were validated by GC-MS analysis as described in (Sandberg et al., 2016) and (Cordova and Antoniewicz, 2016). SFM4CHO medium (GE Healthcare Life Sciences SH3054901) and DMEM medium (Corning 17-207-CV, without glucose, glutamine, and sodium pyruvate) were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Strains and culture conditions

For *E. coli* tracer experiments, wild-type *E. coli* BW21135 and a $\Delta ptsG$ (Keio collection) strain were used. All *E. coli* strains were purchased from GE Healthcare Dharmacon. *E. coli* was cultured aerobically in M9 minimal medium at 37°C in mini-bioreactors with 10 mL working volume as described previously (Crown et al., 2015). For the wild-type experiments, *E. coli* cultures were inoculated at OD₆₀₀ of 0.01. Tracers were added at the beginning of the culture. Cells were harvested (1 mL samples) for GC-MS analysis at mid-exponential growth when OD₆₀₀ was about 0.6. For the *E. coli* $\Delta ptsG$ tracer experiment, M9 medium was supplemented with 50 ug/mL of kanamycin (selection marker for the knockout). The culture was inoculated at OD₆₀₀ of 0.01, and [1,2-¹³C]glucose and [1,2-¹³C]xylose were added at the beginning of the culture, each at a concentration of 10 mM. Cells were harvested (1 mL samples) for GC-MS analysis at mid-exponential growth when OD₆₀₀ was about 0.6. For CHO cell tracer experiments, CHO-K1 cells previously adapted to serum-free suspension culture were used (Valente et al., 2015). CHO cells were cultured in 15 mL of SFM4CHO/DMEM (1:1, v/v) medium supplemented with 4 mM glutamine and 1 mM sodium pyruvate, in vented 125-mL flasks (Corning 431143) in a humidified 5% CO₂ incubator at 37°C with slow shaking (~100 rpm). CHO cell cultures were inoculated at approximately 1.0×10⁵ cells/mL. Tracers were added as a bolus when cell density reached 5.0×10⁵ cells/mL. Cells were harvested (4 mL sample for hydrolysis of glycogen and RNA, and 6 mL sample for intracellular metabolite extraction) for GC-MS analysis after 23.25 h, when cell density was approximately 1.0×10⁶ cells/mL.

2.3. Analytical methods

Cell growth of *E. coli* cultures was monitored by measuring the optical density at 600nm (OD₆₀₀) using a spectrophotometer (Eppendorf BioPhotometer). The OD₆₀₀ values were converted to cell

dry weight concentrations using a previously determined OD₆₀₀-dry cell weight relationship for *E. coli* (1.0 OD₆₀₀ = 0.31 g_{DW}/L) (Long et al., 2016) . After centrifugation (5 min at 14,000 rpm), the supernatant was separated from the cell pellet, and the cell pellets were washed twice with glucose-free M9 medium. Acetate and xylose concentrations were determined using an Agilent 1200 Series HPLC (Au et al., 2014).

Cell growth of CHO cell cultures was monitored by measuring the cell concentration and cell viability using a Moxi Z Cell Counter and Moxi Z Cassettes Type S (ORFLO Technologies, Ketchum, ID). After centrifugation (2 min at 1,000 rpm), the supernatant was separated from the cell pellet, and the cell pellets were washed twice with D-PBS (Mediatech, Inc., Manassas, VA). For intracellular metabolite extraction of CHO cells, the methanol/chloroform/water extraction method described in (Ahn and Antoniewicz, 2013) was followed. Glucose and lactate concentrations were determined using YSI 2700 biochemistry analyzer (YSI, Yellow Springs, OH).

2.4. Hydrolysis of glycogen and RNA

For hydrolysis of glycogen and RNA, the following two-step hydrochloric acid hydrolysis procedure was used. First, 50 µL of 6N HCl was added to dry cell pellets and the samples were incubated for 30 min at 30 °C. Next, 250 µL of water was added (thus diluting the acid to 1 N) and samples were incubated for 60 min at 110 °C. The samples were then cooled to room temperature, neutralized with 50 µL of 5 N NaOH, and dried under air flow at 65°C. The development of this method was recently described in (McConnell and Antoniewicz, 2016).

2.5. Derivatization of glucose and ribose

Glucose and ribose released from hydrolysis of biomass (i.e. from glycogen and RNA, respectively) were derivatized using the aldonitrile propionate derivatization method described in (Antoniewicz et al., 2011). Briefly, 50 μ L of 2 wt% hydroxylamine hydrochloride in pyridine was added to dried samples, which were then incubated for 60 min at 90°C. Next, 100 μ L of propionic anhydride was added followed by incubation at 60°C for 30 min. The samples were then immediately transferred to injection vials for GC-MS analysis.

2.6. Gas chromatography-mass spectrometry

GC-MS analysis was performed on an Agilent 7890B GC system equipped with a DB-5MS capillary column (30 m, 0.25 mm i.d., 0.25 μ m-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977A Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230°C, the MS quad temperature at 150°C, the interface temperature at 280°C, and the inlet temperature at 250°C. GC-MS analysis of *tert*-butyldimethylsilyl (TBDMS) derivatized proteinogenic amino acids was performed as described in (Leighty and Antoniewicz, 2013). GC-MS analysis of TBDMS derivatized intracellular metabolites was performed as described in (Ahn and Antoniewicz, 2011). For GC-MS analysis of glucose and ribose, 1 μ L of a derivatized sample was injected at 1:2 or 1:10 split ratio. The column was started at 80°C and held for 2 min, increased to 280°C at 10°C/min, and held for 12 min. The m/z 173 and m/z 370 fragments of the glucose derivative (containing the last two and first five C-atoms of glucose, respectively (Antoniewicz et al., 2011)), and the m/z 173 and m/z 284 fragments of the ribose derivative (containing the last two and first four C-atoms of ribose, respectively (Long and Antoniewicz,

2014)), were measured in single ion monitoring. Mass isotopomer distributions were obtained by integration (Antoniewicz et al., 2007a) and corrected for natural isotope abundances (Fernandez et al., 1996).

2.7. Metabolic network models and ^{13}C -metabolic flux analysis

The metabolic network models used for ^{13}C -MFA in this study are provided in Supplemental Materials. For *E. coli*, the full model described in (Crown et al., 2015) was used, as well as a simplified model containing only the upper portion of central carbon metabolism. For CHO cells, the model described in (Ahn and Antoniewicz, 2013) was used. All ^{13}C -MFA calculations were performed using the Metran software (Yoo et al., 2008), which is based on the elementary metabolite units (EMU) framework (Antoniewicz et al., 2007b). Fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the experimentally measured and model predicted mass isotopomer distributions using non-linear least-squares regression (Antoniewicz et al., 2006). For integrated analysis of parallel labeling experiments, the data sets were fitted simultaneously to a single flux model as described in (Antoniewicz, 2015a; Leighty and Antoniewicz, 2012). Flux estimation was repeated 10 times starting with random initial values for all fluxes to find a global solution. At convergence, accurate 68% and 95% confidence intervals were computed for all estimated fluxes by evaluating the sensitivity of the minimized SSR to flux variations (Antoniewicz et al., 2006).

To model fractional labeling of metabolites, G-value parameters were also included in ^{13}C -MFA (Antoniewicz, 2015b). The G-value represents the fraction of a metabolite pool that is produced during the labeling experiment, while 1-G represents the fraction that is naturally labeled, i.e.

from the inoculum. By default, one G-value parameter was included for each measured metabolite in each data set. Reversible reactions were modeled as separate forward and backward fluxes. Net and exchange fluxes were determined as follows: $v_{\text{net}} = v_f - v_b$; $v_{\text{exch}} = \min(v_f, v_b)$. For visual representation of exchange fluxes, the exchange fluxes were rescaled as follows: $\text{exchange flux (\%)} = 100\% \times v_{\text{exch}} / (100 + v_{\text{exch}})$.

2.8. Goodness-of-fit analysis

To determine the goodness-of-fit, ^{13}C -MFA fitting results were subjected to a χ^2 -statistical test. In short, assuming that the model is correct and data are without gross measurement errors, the minimized SSR is a stochastic variable with a χ^2 -distribution (Antoniewicz et al., 2006). The number of degrees of freedom is equal to the number of fitted measurements n minus the number of estimated independent parameters p . The acceptable range of SSR values is between $\chi^2_{\alpha/2}(n-p)$ and $\chi^2_{1-\alpha/2}(n-p)$, where α is a certain chosen threshold value, for example 0.05 for 95% confidence interval.

3. RESULTS AND DISCUSSION

3.1. Measuring glycogen and RNA labeling with GC-MS

Glycogen and RNA are abundant components of microbial and mammalian biomass. The glucose moiety of glycogen is derived from the glycolytic intermediate glucose-6-phosphate (G6P), and the ribose moiety of RNA is derived from the PPP intermediate ribose-5-phosphate (R5P). Here, we present a convenient procedure for measuring the labeling of glucose and ribose from glycogen and RNA using GC-MS for applications in ^{13}C -MFA. To observe the sugar monomers, the polymers are first hydrolyzed with hydrochloric acid and then derivatized as

shown in Figure 1. Previously, we validated that this approach is selective for detecting the stable biomass components glycogen and RNA, rather than intracellular metabolites (Long and Antoniewicz, 2014). For GC-MS analysis, we use the aldonitrile pentapropionate derivatization method that generates two reliable GC-MS fragments for each sugar. The first fragment, m/z 173, contains the last two carbons of each sugar, i.e. C5+C6 of glucose and C4+C5 of ribose. The second larger fragment contains the first five carbons of glucose (C1-C5, m/z 370), and the first four carbons of ribose (C1-C4, m/z 284). The resulting positional labeling information provided by these fragments is critical for precise flux analysis, as is demonstrated in the next sections. The ribose and glucose peaks are clearly identifiable in the chromatograms as shown in Figures 1B and 1C for *E. coli* and CHO cells, respectively. For CHO cells, we also detected mannose and galactose peaks (Figure 1C), which could become useful for other applications such as glycan analysis (Badur et al., 2015).

3.2. Glycogen and RNA labeling data improve resolution of PPP fluxes in *E. coli*

To demonstrate that glycogen and RNA labeling data can improve flux resolution, we first applied this approach to *E. coli* as a model microbial system. ^{13}C -MFA of *E. coli* currently relies on measurements of protein-bound amino acids. To assess the complementarity of glycogen and RNA measurements with amino acid measurements, a test case was performed using the tracer [1,6- ^{13}C]glucose and exponentially growing wild-type *E. coli*. In preliminary studies, we identified [1,6- ^{13}C]glucose as a promising tracer for ^{13}C -MFA with good performance throughout central carbon metabolism (Crown et al., 2016). After performing the tracer experiment, isotopic labeling of amino acids from hydrolyzed biomass, as well as glucose and ribose moieties of glycogen and RNA, were measured by GC-MS (all measurements are

provided in Supplemental Materials). Fluxes were then estimated, first using only the amino acid labeling data, and then using the amino acid data along with the glycogen and RNA data. The flux results are summarized in Figure 2 (complete flux results are provided in Supplemental Materials). Acceptable fits were obtained in all cases, assuming a constant measurement error of 0.3 mol% for all GC-MS measurements, demonstrating that all data were in good agreement. Figure 2A shows the estimated net fluxes and Figure 2B shows the 68% and 95% confidence intervals of several key fluxes in central carbon metabolism. Importantly, the addition of glycogen and RNA labeling data significantly improved the precision of fluxes in upper glycolysis (e.g. PGI flux) and PPP (e.g. oxPPP, TKT, TAL fluxes). The confidence intervals of these fluxes were improved by 4-fold when glycogen and RNA measurements were included (Figure 2B).

3.3. Estimation of net and exchange fluxes in *E. coli* upper metabolism with glycogen and RNA data

Next, we evaluated if glycogen and RNA data alone (i.e. without amino acid data) could be used for reliable flux estimation in the upper half of metabolism, defined here to include the upper portion of the glycolysis (EMP) pathway, pentose phosphate pathway (PPP), and Entner-Doudoroff (ED) pathway. For this analysis we used a simplified network model shown in Figure 3A. Of the commonly measured amino acids, only phenylalanine could be used in this case (see Introduction section). To demonstrate the usefulness of glycogen and RNA measurements, a novel parallel tracer experiment scheme was employed here. Specifically, three mixtures of tracers were used (Ahn et al., 2016): [1-¹³C]glucose + [4,5,6-¹³C]glucose (1:1), [2-¹³C]glucose + [4,5,6-¹³C]glucose (1:1), and [3-¹³C]glucose + [4,5,6-¹³C]glucose (1:1). These tracers were

selected based on the approaches for optimal tracer experiment design described in (Antoniewicz, 2015a, 2013b; Crown and Antoniewicz, 2012).

After performing the parallel labeling experiments, isotopic labeling of phenylalanine from hydrolyzed proteins, as well as the glucose and ribose moieties of glycogen and RNA, were measured by GC-MS (all data are provided in Supplemental Materials). Flux analysis was then performed three times: first, using only phenylalanine labeling data (using fragments m/z 302, 308 and 336); second, using only glycogen and RNA labeling data; and third, using phenylalanine, glycogen and RNA labeling data together. All fits were statistically acceptable, assuming a constant measurement error of 0.3 mol% for all GC-MS measurements. The flux results are shown in Figure 3 (complete flux results are provided in Supplemental Materials). The differences in flux precision for the different data sets are shown in Figure 3B, where for most reactions the glycogen and RNA measurements performed better than phenylalanine measurements alone, and the combined fits provided the most precise flux estimates. This was the case for all of the reactions except for the ED flux, where glycogen and RNA produced larger confidence intervals than phenylalanine. This is because while phenylalanine has some observability of the products of the ED reactions (i.e. PEP from GAP), glycogen and RNA are upstream and thus have little information about that flux. As shown in Figure 3C, the addition of glycogen and RNA measurements allowed good estimation of exchange fluxes. These exchange fluxes are notoriously difficult to estimate with amino acid labeling data alone across a wide spectrum of glucose tracers (Crown et al., 2015). The PGI exchange flux was unobservable using only phenylalanine, but with the addition of glycogen and RNA measurements it was estimated with a narrow confidence interval. Dramatic improvements in precision were also observed for

the exchange fluxes of transketolase (TKT) and transaldolase (TAL) half reactions leading to fructose-6-phosphate (F6P). Estimates were not improved in other half reactions (Supplemental Materials).

3.4. Elucidation of glucose and xylose co-utilization using glycogen and RNA data

To demonstrate a novel and practical application of our methodology, we applied it to elucidate the metabolism of *E. coli* $\Delta ptsG$ mutant, which can co-utilize glucose and xylose (Chiang et al., 2013; Li et al., 2007). A tracer experiment was performed using a 1:1 molar ratio of [1,2- ^{13}C]glucose and [1,2- ^{13}C]xylose. After performing the labeling experiment, isotopic labeling of phenylalanine, glycogen, and RNA were measured by GC-MS.

^{13}C -MFA was then performed three times as described above using different data sets. All fits were statistically acceptable, assuming a constant measurement error of 0.3 mol% for all GC-MS measurements. The flux results are shown in Figure 4 (complete flux results are provided in Supplemental Materials), and the differences in flux precision for the different data sets are shown in Figure 4B. For ^{13}C -MFA, we did not include the measured glucose and xylose uptake rates as constraints; instead, xylose uptake rate was fixed at 100 and the relative glucose uptake rate was estimated by ^{13}C -MFA so that it could be compared to the measured rate. Overall, including glycogen and RNA labeling data resulted in significantly more precise flux estimates, i.e. narrower confidence intervals, compared to using phenylalanine data alone (Figure 4B). Importantly, the addition of glycogen and RNA allowed the glucose uptake rate to be determined with high precision, which was not possible with phenylalanine data (Figure 4B). Based on ^{13}C -MFA results the glucose-to-xylose uptake ratio was 0.23 ± 0.02 , which matches perfectly with

the measured ratio of 0.22 (i.e. the measured glucose uptake rate was 1.93 mmol/g_{DW}/hr and the measured xylose uptake rate was 8.69 mmol/g_{DW}/hr). Previous studies on glucose and xylose co-utilization have relied on intracellular metabolite and amino acid measurements for flux elucidation (Aristilde et al., 2015; Cordova and Antoniewicz, 2016). Here, we demonstrate the value of adding glycogen and RNA labeling data for precise analysis of glucose and xylose co-utilization.

3.5. Estimation of net and exchange fluxes in CHO cell upper metabolism with glycogen and RNA data

Lastly, we applied the methodology to analyze the metabolism of CHO cells as a model mammalian system. To demonstrate the efficacy of glycogen and RNA labeling data for ¹³C-MFA of mammalian cells, the same parallel tracer scheme was used as described in section 3.3. CHO cells were grown in serum-free suspension culture to the mid-exponential phase ($\sim 0.5 \times 10^6$ cells/mL), at which point tracers were introduced as a single bolus. After ~ 24 hours, cells were harvested for analysis of 3PG and PEP labeling (intracellular metabolites), and glycogen and RNA labeling by GC-MS (all data are provided in Supplemental Materials).

Flux analysis was then performed three times: first, using only 3PG and PEP labeling data, representing the current standard of using intracellular metabolites for ¹³C-MFA; second, using only glycogen and RNA labeling data; and third, using 3PG, PEP, glycogen and RNA labeling data together. All fits were statistically acceptable, assuming a measurement error of 0.3 mol% for glycogen and RNA labeling measurements, and an error of 0.4 mol% for 3PG and PEP measurements which were more noisy. The flux results are shown in Figure 5 (complete flux

results are provided in Supplemental Materials). As shown in Figure 5B, the confidence intervals of fluxes in upper metabolism were largest when fluxes were estimated with 3PG and PEP data alone, were reduced when glycogen and RNA data was used, and were greatly reduced when all measurements were used for ^{13}C -MFA. A similar trend was observed for confidence intervals of exchange fluxes (Figure 5C), where the precision of TKT and TAL exchange fluxes was greatly improved when all four metabolites were fitted at the same time. Together, these results demonstrate that glycogen and RNA labeling data are complementary to 3PG and PEP labeling measurements for estimating net and exchange fluxes in upper metabolism of CHO cells.

3.6. Determining turnover rates of glycogen and RNA

In addition to determining intracellular fluxes, glycogen and RNA labeling data can also be used to determine turnover rates of glycogen and RNA (Murphy et al., 2013). For this purpose, the estimated G-values for glycogen and RNA are first used to calculate apparent labeling rates. These apparent labeling rates reflect the generation of new biomass (i.e. growth) as well as turnover (i.e. breakdown and regeneration) of the macromolecules:

$$\text{Apparent labeling rate} = -\ln(1-G)/t = \text{growth rate} + \text{turnover rate} \quad (1)$$

Here, G is the estimated G-value of glycogen (or RNA) from ^{13}C -MFA, and t is the length of the labeling experiment (in this study, $t = 23.25$ h). To illustrate this approach for determining turnover rates of glycogen and RNA, the growth rate of CHO cells was determined directly by cell counting (Figure 6A), and the apparent labeling rates were determined from the MFA-estimated G-values (Figure 6B). Based on cell counting, a specific cell growth rate of $0.035 \pm$

0.001 h⁻¹ was determined, and from on the estimated G-values for glycogen ($G = 0.65 \pm 0.01$) and RNA ($G = 0.61 \pm 0.01$), apparent labeling rates of 0.045 ± 0.001 and 0.040 ± 0.001 h⁻¹ were determined for glycogen and RNA, respectively. Thus, the turnover rate of glycogen was estimated to be 1.0% per hour (i.e. $0.045 - 0.035 = 0.010$ h⁻¹), and the turnover rate of RNA was 0.5% per hour (i.e. $0.040 - 0.035 = 0.005$ h⁻¹).

4. CONCLUSION

In this work, we have presented a practical approach for GC-MS based analysis of isotopic labeling of glycogen and RNA, and demonstrated the usefulness of these measurements for ¹³C-MFA. Compared to alternative approaches, our method requires relatively little biomass (<0.2 mg of dry biomass for *E. coli*, and $<4 \times 10^6$ CHO cells), and provides multiple fragments of glucose and ribose moieties with valuable information regarding metabolic fluxes in upper metabolism, including glycolysis and pentose phosphate pathway. Additionally, we demonstrate that these measurements are complementary to other commonly used measurements for ¹³C-MFA, including amino acids in microbial systems and intracellular metabolites in mammalian systems. We also demonstrate that glycogen and RNA labeling data are valuable for estimating precise glucose and xylose uptake rates when both substrates are co-utilized.

Beyond facilitating more precise flux estimates in combination with other common measurements, glycogen and RNA measurements provide unique observability of net and exchange fluxes in upper metabolism. In *E. coli*, these measurements are sufficient for precise quantification of fluxes in a simplified model of upper central carbon metabolism, performing better and with excellent complementarity to current measurements. In CHO cells, these

measurements perform similarly to the commonly measured intracellular metabolites, which require much more laborious sample preparations and larger sample sizes. Similarly, we found strong synergy between these measurements and other commonly used measurements for ^{13}C -MFA. It is important to note that glycogen and RNA measurements allow significant improvements in the precision of exchange fluxes that are often unobservable with other commonly used measurements (Crown et al., 2015). Given the convenience of measuring glycogen and RNA labeling and the high information content of these measurements for resolving metabolic fluxes, we believe that the approach presented in this study will be broadly applied in future ^{13}C -MFA studies.

ACKNOWLEDGEMENTS

This work was supported by the NSF CAREER Award (CBET-1054120). CPL was also supported the University of Delaware Graduate Fellows Award. The adapted CHO cells were kindly provided by Kelvin H. Lee (University of Delaware).

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FIGURE LEGENDS

Figure 1. GC-MS analysis of glycogen and RNA labeling. A) The biopolymers glycogen and RNA are first broken down into the respective sugar monomers glucose and ribose by acid hydrolysis. The sugars are then subjected to aldonitrile propionate derivatization for subsequent GC-MS analysis. Two fragments of each species are measured to provide positional labeling information. B) Total ion chromatogram from GC-MS analysis of sugars from hydrolyzed *E. coli*, and C) CHO cells. Peaks corresponding to different sugar monomers are clearly resolved.

Figure 2. ^{13}C metabolic flux analysis of *E. coli* metabolism using amino acid, glycogen and RNA labeling data from a $[1,6-^{13}\text{C}]$ glucose tracer experiment. A) Estimated flux map for *E. coli* central carbon metabolism. Fluxes were determined by simultaneously fitting amino acid (AA), glycogen (Glg), and RNA labeling data. White arrows represent outflux to biomass. B) Comparison of flux confidence intervals obtained from ^{13}C -MFA using only amino acid labeling data, and amino acid labeling data combined with glycogen and RNA data. The 68% and 95% flux confidence intervals are shown for eight representative metabolic fluxes in central carbon metabolism: phosphoglucose isomerase (v_2 ; PGI); oxidative pentose phosphate pathway (v_9 ; oxPPP); Entner-Doudoroff pathway (v_{18} ; ED); transketolase (v_{14} ; TKT); transaldolase (v_{16} ; TAL); citrate synthase (v_{21} ; TCA); PEP carboxylase (v_{33} ; PPC); glyoxylate shunt (v_{29} ; Glyox).

Figure 3. ^{13}C metabolic flux analysis of upper metabolism of *E. coli* using phenylalanine, glycogen and RNA labeling data from three parallel labeling experiments. A) Estimated flux map for *E. coli* determined by simultaneously fitting phenylalanine (Phe), glycogen (Glg), and

RNA labeling data. White arrows represent outflux to biomass. B) Comparison of flux confidence intervals obtained from ^{13}C -MFA using only phenylalanine labeling data, only glycogen and RNA labeling data, and all three metabolites. The 68% and 95% flux confidence intervals are shown for five representative metabolic fluxes: phosphoglucose isomerase (v_2 ; PGI); oxidative pentose phosphate pathway (v_9 ; oxPPP); Entner-Doudoroff pathway (v_{18} ; ED); transketolase (v_{14} ; TKT); transaldolase (v_{16} ; TAL). C) Comparison of confidence intervals for exchange fluxes estimated using different data sets. Note that in both (B) and (C), TKT and TAL refer to the terminal half reactions of transketolase and transaldolase involving fructose 6-phosphate (F6P).

Figure 4. ^{13}C metabolic flux analysis of upper metabolism for *E. coli* strain $\Delta ptsG$ that co-utilizes glucose and xylose. A) Estimated flux map determined by simultaneously fitting phenylalanine (Phe), glycogen (Glg), and RNA labeling data. White arrows represent outflux to biomass. B) Comparison of flux confidence intervals obtained from ^{13}C -MFA using only phenylalanine labeling data, only glycogen and RNA labeling data, and all three metabolites. The 68% and 95% flux confidence intervals are shown for six key metabolic fluxes: relative glucose uptake (v_1 ; Gluc); phosphoglucose isomerase (v_2 ; PGI); oxidative pentose phosphate pathway (v_9 ; oxPPP); Entner-Doudoroff pathway (v_{18} ; ED); transketolase (v_{14} ; TKT); transaldolase (v_{16} ; TAL).

Figure 5. ^{13}C metabolic flux analysis of upper metabolism of CHO cells using 3PG, PEP, glycogen and RNA labeling data from three parallel labeling experiments. A) Estimated flux map determined by simultaneously fitting 3PG, PEP, glycogen (Glg), and RNA labeling data.

White arrows represent outflux to biomass. B) Comparison of flux confidence intervals obtained from ^{13}C -MFA using only 3PG and PEP labeling data, only glycogen and RNA labeling data, and all four metabolites. The 68% and 95% flux confidence intervals are shown for four representative metabolic fluxes: phosphoglucose isomerase (v_2 ; PGI); oxidative pentose phosphate pathway (v_8 ; oxPPP); transketolase (v_{13} ; TKT); and transaldolase (v_{15} ; TAL). C) Comparison of confidence intervals for exchange fluxes estimated using different data sets. Note that in both (B) and (C), TKT and TAL refer to the terminal half reactions of transketolase and transaldolase involving fructose 6-phosphate (F6P).

Figure 6. Determining turnover rates of glycogen and RNA in CHO cells. A) Time profile of viable cell density of CHO cells in suspension culture. B) Comparison of the growth rate of CHO cells (determined by cell counting) and the apparent labeling rates of glycogen (Glg) and RNA (determined from estimated G-values). The differences between the growth rate of CHO cells and the apparent labeling rates are attributable to turnover of glycogen and RNA. Error bars represent mean $\pm$ SD ($n=3$, biological replicates from parallel labeling experiments).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B