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Complete List of Authors:	Jaiswal, Mohit; University of Florida, Chemistry; University of Florida, Tran, Trang T.; University of Florida Guo, Jiatong; University of Florida Zhou, Mingwei; University of Florida, Chemistry Garcia Diaz, Josefina ; University of Florida FANUCCI, GAIL; University of Florida, Chemistry; University of Florida Guo, Zhongwu; University of Florida, Chemistry

COMMUNICATION

Enzymatic glycoengineering-based spin labelling of cell surface sialoglycans to enable their analysis by electron paramagnetic resonance (EPR) spectroscopy

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Mohit Jaiswal,[a] Trang T. Tran,[a] Jiatong Guo,[a] Mingwei Zhou,[a] Josefina Garcia Diaz,[a] Gail E. Fanucci,[a]* and Zhongwu Guo[a]*

Abstract. A novel method for spin labelling of sialoglycans on the cell surface is described. C9-Azido sialic acid was linked to glycans on live cells via CSTII-catalysed α2,3-sialylation utilizing azido-sialic acid nucleotide as a sialyl donor, which was followed by attachment of a spin label to the azide via click reaction. It enables the study of cell surface sialoglycans by EPR spectroscopy.

Cells are decorated by a dense layer of carbohydrates, called the cell glycocalyx that plays a vital role in various biological events. Unlike nucleic acids or proteins, glycan biosynthesis is devoid of templates to cause huge structural diversity. Moreover, glycans are conformationally flexible. Although these properties are useful for the biological functions of glycans, *e.g.*, the density, spatial organization and orientation of cell surface glycans can dictate cell recognition,[1-3] the enormous structural diversity and complexity of glycans make structural analysis and other studies of glycans on the cell surface a significant challenge.

To address the issue, we have recently developed an innovative method for spin labelling of glycans on cells based on metabolic glycoengineering (MGE).[4] In this context, cells were treated first with an azide-modified monosaccharide precursor that could be metabolically incorporated in glycans and then with an alkyne-modified nitroxide as the spin label (SL) that was linked to azide-modified glycans through a bioorthogonal click reaction.[5-7] This enabled the study of glycans on cells by electron paramagnetic resonance (EPR) spectroscopy. Distinctive from other analytical techniques, such as fluorescence microscopy, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), EPR spectroscopy can provide insights into the spatial organization, mobility, and dynamics (defined as rate of motion and order parameter) of labelled molecules in complex environments via EPR spectral line shape analyses and simulations.[8-13] As a result, EPR spectroscopic studies of the spin-labelled cells provided for the first time interesting results about the different mobility and environments of *N*-acetylglucosamine (GlcNAc) and sialic acid (Neu5Ac) in the cell glycocalyx. Furthermore, spin labelling can also facilitate conformational studies of glycoproteins by NMR, such as their binding-induced conformational changes.[14]

Although we have validated that MGE is an effective method for cell surface glycan spin-labelling and EPR study of spin-labelled cells can shed lights on the packaging, populations, and local environments of glycans on cells,[4] MGE has its limitations. First, MGE is nonspecific as the unnatural monosaccharide precursor can be used by various biosynthetic pathways and incorporated at different sites and in different linkage forms. Second, the precursor can be converted into other sugars in the cell, *e.g.*, via salvage pathways,[15,16] to result in labelling of not only targeted sugar units but also others. Third, MGE is time-consuming and difficult to control. To address the issues, we report here a new, direct and more specific method for spin labelling of cell surface glycans based on enzymatic glycoengineering (EGE)—modifying glycans on cells through enzyme (*e.g.*, glycosyltransferase, GT)-mediated incorporation of functionalized sugars. EGE has been pioneered and extensively explored by several groups[17-20] and is a powerful tool for profiling glycans by molecular imaging and mass spectrometry.[21-23]

The concept for this novel spin-labelling method is outlined in Figure 1. First, an azide-modified monosaccharide is attached to glycans on cells via EGE, *i.e.*, specific ST-mediated glycosylation using corresponding azido-sugar nucleotide as a glycosyl donor. To further enhance the efficiency of EGE, one can also treat the cell with a glycosidase beforehand to remove the targeted sugar residues on the cell surface to expose more glycosylation sites. Thereafter, a SL is attached to the azide-labelled sugar residues on cells through a bioorthogonal click reaction to facilitate EPR studies. Compared to MGE, an advantage of EGE is its direct and relatively specific glycan modification. For example, the linkage forms and sites of incorporated unnatural sugar residues in the glycans are defined by the enzymes used; there is no significant conversion of unnatural glycosyl donors into other sugars since EGE does not involve monosaccharide metabolism and glycan biosynthesis inside the cell, and enzymatic glycosylations can be controlled and rapidly accomplished.

[a] *Department of Chemistry, University of Florida, 214 Leigh Hall, Gainesville, FL 32611, United States*

Electronic Supplementary Information (ESI) available: Experimental procedures, additional FACS, imaging and EPR results and data. See DOI: 10.1039/x0xx00000x

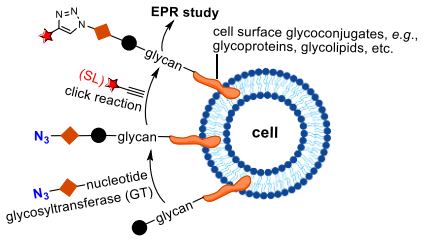

Figure 1. Schematic representation of a novel method for spin labelling of cell surface glycans to introduce azide-modified sugars via EGE and then install SLs via click reaction between the azide-labelled glycans and an alkyne-functionalized SL

To verify the above hypothesis, we investigated the labelling of sialoglycans on live cells using CSTII, a bacterial sialyltransferase (ST) from *Campylobacter Jejuni*,[24] for EGE. An important reason for us to select CSTII is that it is a very active enzyme but has not been explored for cell EGE yet. In addition, like some other STs, CSTII is a promiscuous enzyme, accepting C9- and C5-modified sialic acids as substrate, and thus has been successfully used for enzymatic sialooligosaccharide synthesis.[17,25] Although CSTII is a bifunctional ST with α2,3-ST and α2,8-ST activities to catalyse the transfer of Neu5Ac in cytidine 5'-monophosphate (CMP)-Neu5Ac to the galactose (Gal) or *N*-acetylgalatosamine (GalNAc) 3-*O*-position and Neu5Ac 8-*O*-position, respectively,[26] its α2,8-ST activity is much lower than its α2,3-ST activity.[24] Hence, CSTII-catalysed α2,8-sialylation can be neglected when the enzymatic reaction is performed under controlled condition, *e.g.*, low sialyl donor concentration and short reaction time.[27] Our studies on CSTII proved that under the experimental conditions (0.5 μM Neu5Ac-CMP) utilized for EGE, the α2,8-sialylation products did not emerge until after 45 min of reaction as monitored by MS (Supporting Information).

We aimed to modify cell surface sialoglycans with C9-azido sialic acid (Neu5Ac9Az, Figure 2), since, as reported,[25] CSTII has been verified to accept Neu5Ac9Az-CMP (**1**) as a substrate to attach Neu5Ac9Az to Gal and GalNAc (Supporting Information). Then, a nitroxide radical SL was coupled to the azide-modified glycans on cells through copper-free click reaction, using our previously reported DBCO-SL conjugate **2**[4] (Figure 2). Finally, spin-labelled cells were studied by EPR spectroscopy.

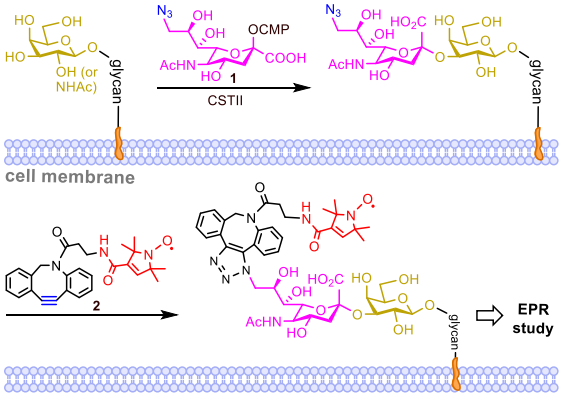

Figure 2. EGE-based spin labelling of cell surface sialoglycans utilizing CSTII and Neu5Ac9Az-CMP (**1**) for cell EGE and DBCO-modified SL **2** for spin labelling

Prior to spin labelling, we probed first if treating cells with CSTII and Neu5Ac9Az-CMP could glycoengineer cell for attachment of a fluorescent label, which is readily observed by flow cytometry (FACS). In this regard, HeLa cells were incubated with CSTII and Neu5Ac9Az-CMP, washed, and treated with DBCO-fluorescein (FAM). In the control group, HeLa cells were treated by the same protocol, except for lacking CSTII. Then, the cells were analysed with FACS. A significant increase in mean fluorescence intensity (MFI) was observed for cells treated with CSTII and Neu5Ac9Az-CMP compared to the control (Figure 3a), and this increase was time-dependent (5-60 min, Figure S1, Supporting Information). In the meantime, we carried out a study in which the cells were exposed to *Arthrobacter ureafaciens* sialidase, which hydrolyses α-linked sialic acids, prior to treatments with CSTII, and then Neu5Ac9Az-CMP and DBCO-FAM. FACS results of the sialidase-treated cells (green, Figure 3B) showed a further increase in MFI compared to that without sialidase treatment (red, Figure 3B). This result was anticipated, as removal of natural Neu5Ac in the glycans by a sialidase would expose more asialylated Gal and GalNAc units for sialylation and labelling. In addition, when the labelled cells were treated with peptide N-glycosidase F (PNGase F), which hydrolyses N-glycans from glycoproteins, a marked decrease in the MFI of these cells was observed (Supporting Information), proving that some of the fluorescence tags were linked to N-glycans on the cell surface, whereas others were attached to O-glycans and glycolipids.

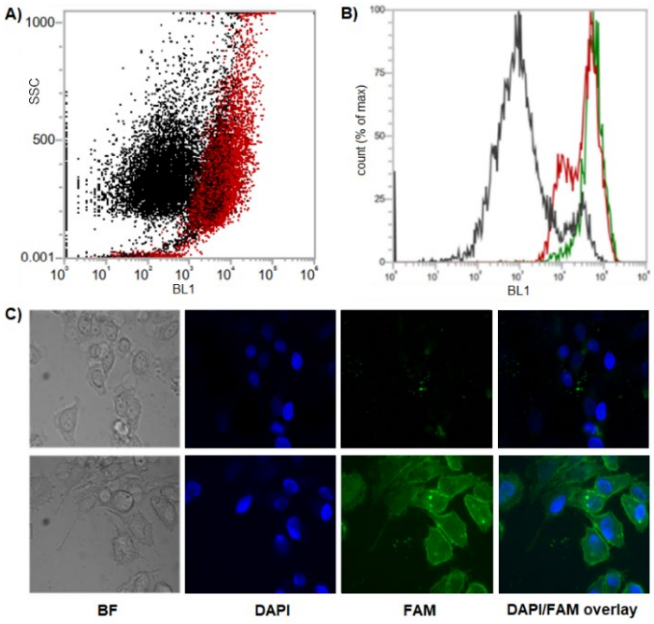

Figure 3. EGE-based fluorescent labelling of cell surface sialoglycans. **A**) FACS results of HeLa cells treated with Neu5Ac9Az-CMP (30 min) and DBCO-FAM without CSTII (black, control) or with CSTII, Neu5Ac9Az-CMP (30 min) and DBCO-FAM (red). **B**) FACS results of HeLa cells treated with Neu5Ac9Az-CMP (60 min) and DBCO-FAM (black, control), or with CSTII and Neu5Ac9Az-CMP (60 min) and DBCO-FAM (red), or with sialidase, and then with CSTII, Neu5Ac9Az-CMP (60 min) and DBCO-FAM (green). **C**) Bright-field (BF) and fluorescence microscopic images of HeLa cells that were pre-treated with sialidase and then with Neu5Ac9Az-CMP and DBCO-FAM (top panel) or with CSTII, Neu5Ac9Az-CMP and DBCO-FAM (bottom panel), and finally stained with 4',6-diamidino-2-phenylindole (DAPI) (for DNA staining).

It is interesting to note that EGE without sialidase pre-treatment yielded two groups of cell populations with different MFIs, but EGE after sialidase pre-treatment led to not only an increase in

MFI for all cells but also more even distribution of fluorescent labels. This may suggest that originally Hela cells have different sialylation levels to result in varied EGE. Fluorescence labelling of cell surface sialoglycans was further validated by microscopy results (Figure 3C). Clearly, these studies have shown that CSTII with Neu5Ac9Az-CMP can be utilized to effectively engineer cell surface sialoglycans for Neu5Ac9Az installation as a molecular handle to enable further functionalization.

After having verified the efficiency of CSTII and Neu5Ac9Az-CMP for cell EGE, we moved on to probe EGE-based spin labelling of cell surface sialoglycans under established conditions. Sialidase pre-treatment was applied prior to EGE, as this procedure could provide more efficient and homogenous labelling of cells. CSTII-catalysed sialylation of cells was kept for only 30 min to avoid α2,8-sialylation. The engineered cells were incubated with **2** at room temperature for 1 h, washed, and finally subjected to EPR analysis to verify spin labelling. Control group cells were treated by the same protocol without CSTII. EPR spectra were acquired using a benchtop X-band CW-EPR spectrometer, with samples prepared and data collected according to our previous report.[4] In each experiment, discrete control EPR spectra were collected for a given batch of cell to account for variations in cell diversity. Hence, the cell count-normalized EPR spectrum of a control was applied only to samples from a given batch of cells. As described previously,[4] the EPR signal from the control group (Figure 4, left) likely resulted from nonspecific partitioning of SL **2** into the cell membrane. Nevertheless, the cell count-normalized, control-subtracted product spectrum (Figure 4, right) clearly revealed a strong EPR signal derived from the CSTII and Neu5Ac9Az-CMP-treated cells, suggesting their robust and excellent labelling with the nitroxide radical.

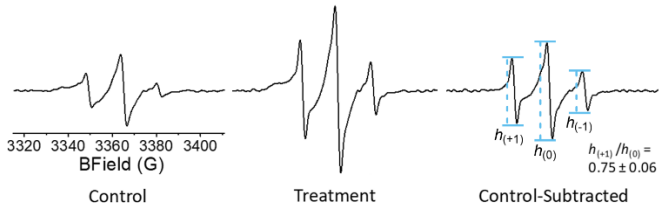

Figure 4. 100 G X-band EPR spectra of sialidase-treated HeLa cell after incubation with Neu5Ac9Az-CMP (without CSTII) and **2** (left, control), or with CSTII, Neu5Ac9Az-CMP and then **2** (middle), as well as cell count-normalized control-subtracted product spectrum (right).

The line shapes for the EPR spectrum of the treated cells (Figure 4) are characteristic for biomolecule-tethered nitroxide radical, in contrast to free **2** in solutions,[4] indicating the attachment of SLs to glycans on cells. In addition, it should be noted that the EPR signals were not from molecules in the cells, as once inside cell, the nitroxide radicals are quickly quenched by a variety of reducing agents.[28-31] As such, the cell count-normalized control-subtracted EPR spectra of engineered cells did reflect SLs linked to the enzymatically engineered sialoglycans.

EPR spectral line shape of a SL reflects its local mobility, which combines the mobility of both the SL and the backbone if the SL is linked to cells or large molecules. The average mobility of a SL can be described by a variety of line shape parameters, such as $h_{(+1)}/h_{(0)}$ and $h_{(-1)}/h_{(0)}$ values. For a simple nitroxide like **2**, its fast isotropic motion in solution produces a spectrum with three sharp peaks of almost equal intensity, resulting in $h_{(+1)}/h_{(0)}$ and $h_{(-1)}/h_{(0)}$ values to be close to 1.0.[4] As motional rates slow down or become restricted in space, EPR peaks will broaden and show diminished intensities for $h_{(+1)}$ and $h_{(-1)}$ transitions, compared to central $h_{(0)}$ transition, leading to lower values.[32] Biomolecule-linked SLs with fast-limit isotropic-like motion have $h_{(+1)}/h_{(0)}$ and $h_{(-1)}/h_{(0)}$ values in the range of 1.0~0.6.[4] The relatively large $h_{(+1)}/h_{(0)}$ value (0.75 ± 0.06) for the EPR signal of engineered and spin-labelled HeLa cells as depicted in Figure 4 implies a high degree of mobility of the SL. This agrees well with the relatively flexible structure of the LS and biological finding that Neu5Ac is typically attached to the non-reducing end of glycans, thereby expected to exhibit high flexibility and mobility.[33-35] This may be related to the important and diversified biological functions of sialic acids and sialoglycans in nature.

Whereas the $h_{(+1)}/h_{(0)}$ parameter gives insight into the average mobility of the SLs and spin-labelled glycans, analysis of the EPR spectrum via spectral simulations using EasySpin software can provide more details about the environments around the SL.[36] Simulation results of the EPR spectrum of above spin-labelled HeLa cells revealed at least two motion components (Figure 5). One is a fast motion component with a correlation time (τ_c) of 0.40 ns and a narrow line width (0.50 ± 0.05 G), and the other is the slower motion component with a τ_c of ~7 ns and a broad line width (1.41 ± 0.05 G). We interpret this difference in mobility to result from varied packing environments around the SL, since the tether for the SL is quite long and contains several flexible bonds; thus, the restriction in SL mobility is likely to arise from the crowded local environments. This hypothesis is supported by cell glycobiology. On the cell surface, some glycans, such as that on glycoproteins and peptidoglycans, are located at the relatively non-crowded outskirt of the cell glycocalyx and thus are in less restricted packing environments to give rise to EPR spectra with high mobility, whereas other glycans, such as that of glycolipids or at the inner core of glycoproteins, are in more crowded environments to give rise to EPR spectra with limited or restricted mobility. Hence, the two motion components of the EPR spectrum in Figure 5 may reflect the average local environments of these different glycans.

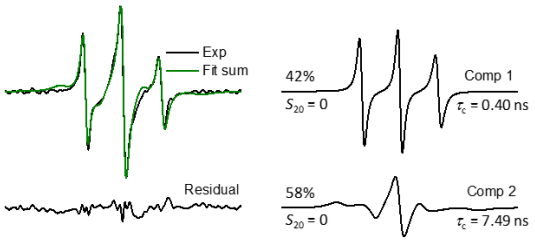

Figure 5. 100 G X-band EPR spectrum (left top, black) of HeLa cell treated with CSTII, Neu5Ac9Az-CMP, and then **2** and simulation spectrum (left top, green), as well as the residual between experimental and simulated spectra (left bottom) and the two component spectra resulted from the fitting (right).

In conclusion, we have validated for the first time that CSTII, an α2,3-ST, in combination with Neu5Ac9Az-CMP can be utilized to effectively engineer Neu5Ac9Az attachment to glycans on live cells, thereby to install a flexible molecular handle for further functionalization. Through this molecular handle, a fluorescent

tag was successfully attached to the glycoengineered cell. More significantly, a nitroxide-based spin was attached to the azide-modified glycans on live cells to enable EPR spectroscopic study. EPR analysis of the spin-labelled cells helped gain some insights into the mobility and packing environments of sialic acids in the cell glycocalyx. In particular, simulations of the obtained EPR spectra revealed two motional components, corresponding to a relatively relaxed and a more crowded local environments, for sialic acid. These results have demonstrated the potential of this spin-labelling technique, combined with EPR spectroscopy, to understand the structure, organization and dynamics of glycans on live cells, which is difficult to achieve by other technologies. Additionally, although only CSTII was explored in this proof-of-principle study, it is envisioned that other STs and GTs can also be used for EGE and spin-labelling of other cell surface glycans and biological systems, such as viruses and model membranes, which are pursued in our labs currently. As a result, this spin-labelling technique should be widely applicable. Finally, detailed studies and comparison of the results using different sugars and enzymes will lead to a better understanding of glycobiology.

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MJ, JG and JGD contributed to molecule synthesis, cell culturing and glycoengineering; TTT and MZ contributed to EPR studies; GEF and ZG were responsible for the design and supervision of this project. All authors contributed to manuscript preparation.

There is no conflict of interest to declare.

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