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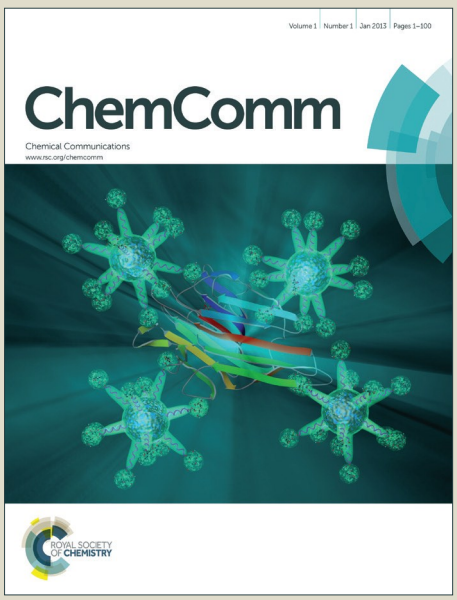

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COMMUNICATION

Sequential Bioorthogonal Dual Strategy: ManNAl and SiaNAl as distinct tools to unravel sialic acids metabolic pathways††

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Recent methodological developments in metabolic oligosaccharide engineering (MOE) pave the way for tremendous advances in glycobiology. Herein, we propose a Sequential Bioorthogonal Dual Strategy (SBDS) combining the use of two unprotected alkyne-tagged monosaccharide reporters (ManNAl and SiaNAl) with the bioligation of fluorescent probes by copper-catalysed azide-alkyne cycloaddition (CuAAC). With SBDS, we are able to shed light on trafficking and cellular uptake mechanisms of sialic acid. Using their corresponding analogues, we visualized that SiaNAl enters via endocytosis, whereas its biosynthetic intermediate ManNAl uptake is mediated by a yet unknown but specific plasma membrane transporter. Sialin, a lysosomal protein, is shown to be crucial for the export of exogenous sialic acid from lysosomes to the cytosol. Metabolic labeling with alkyne-tagged derivatives of *N*-acetylneuraminic acid (Neu5Ac) or *N*-acetylmannosamine (ManNAc) could thus be used to follow endocytosis in physiological vs pathological conditions.

With the advent of bioorthogonal click chemistry, the emergence of metabolic oligosaccharide engineering (MOE) has opened a completely new field of investigation in the past few years.[1] Indeed, a traditional limitation in the field of glycosciences used to be the molecular imaging of mono-, oligo- or polysaccharides in terms of subcellular localization and quantification. Metabolic inclusion of a synthetically modified monosaccharide reporter in living cells or organisms, followed by bioorthogonal ligation of a probe, now provides a very elegant strategy to visualize and quantify various glycoconjugate families.[2-6] Several methodologies have specifically been developed for the study of sialylated glycoconjugates.[7-12] Sialic acids are a diverse family of monosaccharides that consistently occupy the non-reducing termini of glycan chains in glycoproteins and glycolipids.[13-14] The sialic acid metabolism occurs in different compartments of the cell. Its synthesis starts in the cytosol from UDP-GlcNAc, which is epimerized and phosphorylated into ManNAc-6P by UDP-GlcNAc 2-epimerase (GNE/MNK). ManNAc-6P is then condensed with PEP by the Neu5Ac-9-Phosphate synthase (NANS) to give rise to Neu5Ac-9P. After dephosphorylation by *N*-acetylneuraminic acid phosphatase (NANP), the synthesis then continues in the nucleus where the Neu5Ac is activated into CMP-Neu5Ac by the CMP-Neu5Ac synthase (CSS). Once activated, the CMP-Neu5Ac is exported from the nucleus into the cytosol then to the Golgi apparatus via the SLC35A1 transporter, before its incorporation into glycoconjugates via sialyltransferases.[15]

However, biological insights into the metabolism of glycoconjugates have yet to emerge from such chemical reporter strategies. To our knowledge, all reported studies but two [16-17] have so far taken an interest in observing glycoconjugates incorporated at the cellular membrane exclusively.

The available chemical toolbox for such investigations mainly consists of peracetylated saccharide derivatives bearing a reporter group, typically an azide or a terminal alkyne. As those unnatural monosaccharides are presumed not to be membrane permeable when unprotected, they have so far been systematically peracetylated in order to increase their hydrophobicity and thus facilitate their cellular entry via passive diffusion.[18] While powerful, these tools do not allow the fine study of cellular uptake and metabolic compartmentation.

In order to highlight such processes, we have successfully developed a Sequential Bioorthogonal Dual Strategy (SBDS) based on the distinct metabolic incorporation of two unprotected monosaccharide reporters, namely *N*-(4-

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†† This study was funded by grants from Université de Lille1 (Bonus Qualité Recherche) to C.B. and Y.G., from Agence Nationale de la Recherche (ANR-SOLV_CDG), from ERANet for Research Programmes on Rare Diseases Joint Transnational Call 2011 (ERARE 11-135 (EURO-CDG)) to F.F., and from the Ministère de l'Enseignement Supérieur to P.A.G. and S.P..

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

pentynoyl)neuraminic acid (SiaNAl) and *N*-(4-pentynoyl) mannosamine (ManNAl) (Fig. 1). The visualization of tagged glycoconjugates was then obtained by chemical ligation of an azido probe via copper-catalyzed azide-alkyne cycloaddition (CuAAC),[19] and imaging by confocal fluorescence microscopy.

In order to study the dynamics of incorporation of our chemical reporters into glycoconjugates, we have successfully used SBDS with the two key players ManNAl and SiaNAl after optimization of their synthesis and of our previously described staining methodology.[20]

Human fibroblast cells from healthy individuals were metabolically labeled at different time points (2, 5 and 7h, respectively) with SiaNAl or ManNAl. Figure 1 shows the distinctive staining patterns observed as a function of labeling time and alkyne-tagged monosaccharide. When comparing the incorporation of SiaNAl with that of ManNAl, two pools of labeled sialylated glycoconjugates can easily be differentiated: one is perfectly localized in the Golgi compartment while the other is localized in early endosomes (respectively shown by co-localizations with the Golgi marker TGN46 and the early endosomal marker Eea1, Fig. S1).

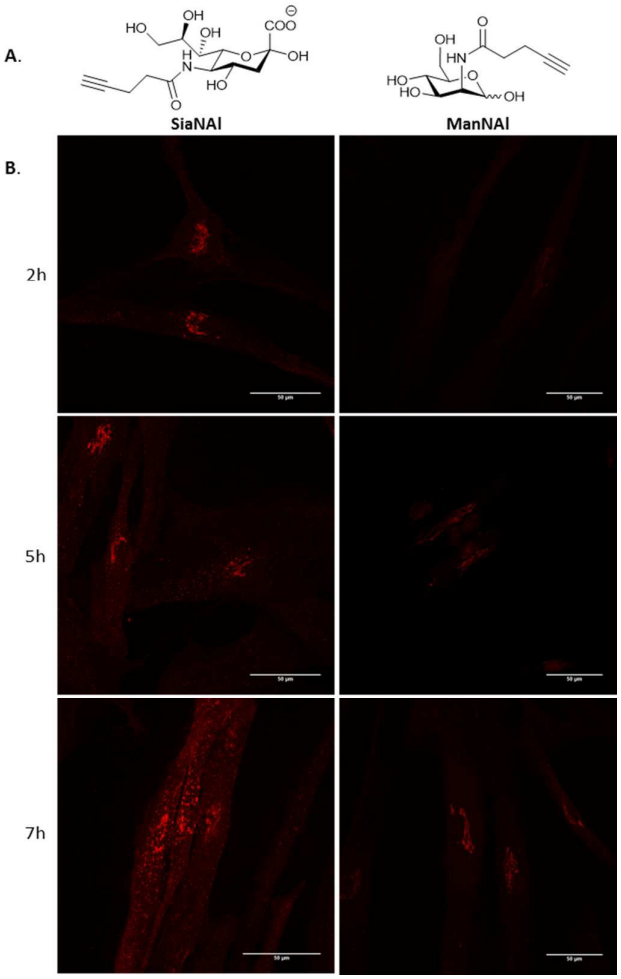

Figure 1. **A.** Structure of the two chemical reporters, SiaNAl and ManNAl. **B.** Fibroblasts from healthy individuals were metabolically labelled with 500 µM of SiaNAl or ManNAl for 2, 5, and 7 h respectively. They were then stained with AzidoFluor545 fluorescent probe (alkyne tagged sialic acid linked to glycoconjugates in red). Staining was visualized using confocal microscopy. Scale bar: 50 µm

Depending on the used chemical tool (ManNAl or SiaNAl), the dynamics of incorporation into glycoconjugates are strikingly different in accordance with the known sialic acids metabolic pathway.

While sialylated glycoconjugates can be observed in the Golgi region after only 3h of cellular SiaNAl metabolic labeling, the same Golgi pattern is only observed with ManNAl after 7h of labeling.

In order to confirm the sialin-mediated uptake pathway proposed by Bardor *et al.*,[21] we applied our SBDS in sialin deficient patients' cells. Indeed, sialin (Q9NRA2) is an anion transporter known to be a membrane protein that exports sialic acids out of lysosomes into the cytosol.[22] To highlight the potential role of sialin in cellular SiaNAl/ManNAl incorporation, control and sialin-deficient patients' cells were metabolically labeled for 8h with either SiaNAl or ManNAl. After incorporation of ManNAl, labeled sialylated glycoconjugates were mainly observed in the perinuclear Golgi like region of both control and sialin deficient patients' cells (Fig. 2). In contrast, the sialin deficient cells metabolically labeled with SiaNAl displayed no staining. The sialin deficient patients' cells have the capacity to transform ManNAl into CMP-SiaNAl, which can then be incorporated into the newly synthesized glycoconjugates. These result show the inability of SiaNAl to reach the cytosol and be converted to CMP-SiaNAl in sialin deficient cells. In support of the pathway suggested previously for Neu5Gc,[21] the present study not only confirms the crucial role of sialin in SiaNAl metabolism but also the potential of this metabolic labeling methodology to decipher deficiencies in sialic acid pathways.

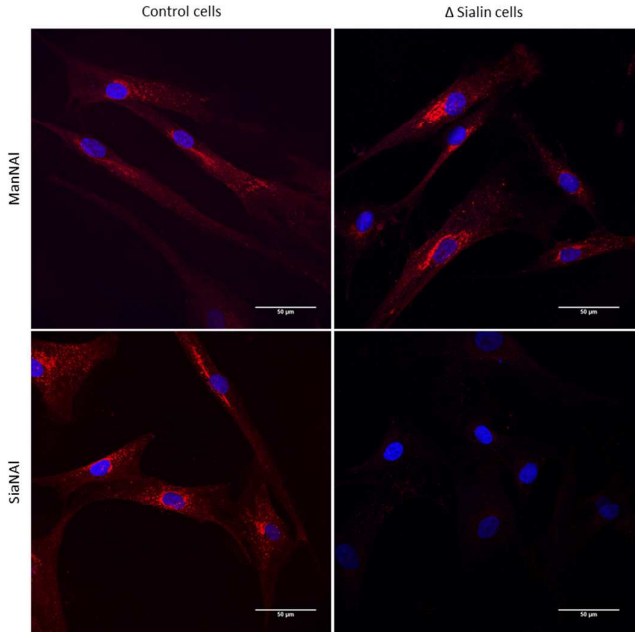

Figure 2. Fibroblasts from healthy individuals and Sialin deficient patients were either metabolically labelled with 500 %M of ManNAl or SiaNAl for 8 h and stained with AzidoFluor 545 fluorescent probe. Cells were then examined using confocal microscopy. Scale bar: 50 µm.

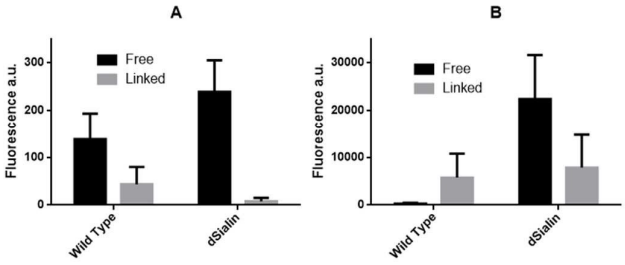

Figure 3. Quantification by HPLC after DMB derivatization (A) SiaNAl, both free and linked to glycoconjugates into Sialin deficient patients cells versus control fibroblast cells. (B) Free and linked Neu5Ac into ΔSialin cells versus control fibroblast cells. Bars represent arithmetic means +/- SEM of two independent DMB experiments.

As no staining was visible in sialin deficient cells after SiaNAl labeling, we wanted to discriminate between an endocytosis or a lysosomal export defect of sialic acids mediated by sialin. Control and sialin deficient cells were labeled with SiaNAl for 8h and the amount of free and linked SiaNAl was quantified by reverse phase fluorescence HPLC after DMB (1,2-diamino-4,5-methylenedioxybenzene dihydrochloride) derivatization (see SI). As shown in Fig. 3, a similar amount of total SiaNAl is observed in sialin deficient cells and control cells. However, while 15% of the observed SiaNAl is incorporated in glycoconjugates in control cells, SiaNAl is exclusively observed as the unbound free monosaccharide in sialin deficient cells. This result demonstrates that SiaNal entry into sialin deficient cells is not prevented. Once in the cell, SiaNAl is blocked into cellular compartments (most likely endosomes and lysosomes), preventing its downstream metabolic incorporation into glycoconjugates. Interestingly, a 60-fold increase in the amount of unbound Neu5Ac is found in sialin deficient cells compared to control cells. Opposite to the aforementioned SiaNAl results, the amount of linked Neu5Ac is unchanged, which indicates that the biosynthetic pathway of sialoglycoconjugates is not affected. Altogether, these data clearly demonstrate that sialin deficiency does not prevent SiaNAl entry, likely achieved via endocytosis, but its export from lysosomes into cytosol.

Subsequently, we investigated the specificity of ManNAl cellular entry mechanism in healthy fibroblasts, as no membrane transporters have been reported for ManNAc to date. Competitive uptake experiments were performed with the relevant mannose-like carbohydrates whose transporters are known[23] (Fig. S2). As expected, the incorporation level into glycoconjugates indicated that *N*-acetylmannosamine strongly compete with ManNAl for uptake. In contrast, mannosamine and mannose did not. Interestingly we showed that in contrast to ManNAl and SiaNAl, no signal is observed when cells were metabolically labelled with unprotected GalNAl and GlcNAl, then arguing for a specific ManNAl entry mechanism (data not shown). Although we cannot completely exclude a passive diffusion through the plasma membrane for ManNAl, our results re-enforce the presence of a specific ManNAc transporter at the plasma membrane as previously suggested by Varki's group[21].

In summary, the present report based on the use of unprotected monosaccharides demonstrates the power of these two different metabolic strategies. SiaNAl and ManNAl are shown to be suitable tools to track sialylated glycoconjugate metabolism and particularly to dissect the sialic acids metabolic routes. Indeed, our data bring evidences that both synthetic monosaccharides are up-taken by differential cellular mechanisms.

Then, we demonstrated that this Sequential Bioorthogonal Dual Strategy is suitable to elucidate fundamental intracellular glycobiological questions. In this instance, we confirmed that sialin is crucial to the export of exogenous sialic acids from lysosomes to cytosol. SiaNAl could be a major tool to identify lysosomal diseases or endocytosis deficiencies. Such labeling strategies could also prove valuable in the search of therapeutic molecules to restore sialin functions. Complementary to SiaNAl metabolic labeling, ManNAl is an alternative tool to visualize the ManNAc downstream pathway. For example, in case of GNE deficiency,[24] this methodology could simply be used to assess treatment efficacy.[25]

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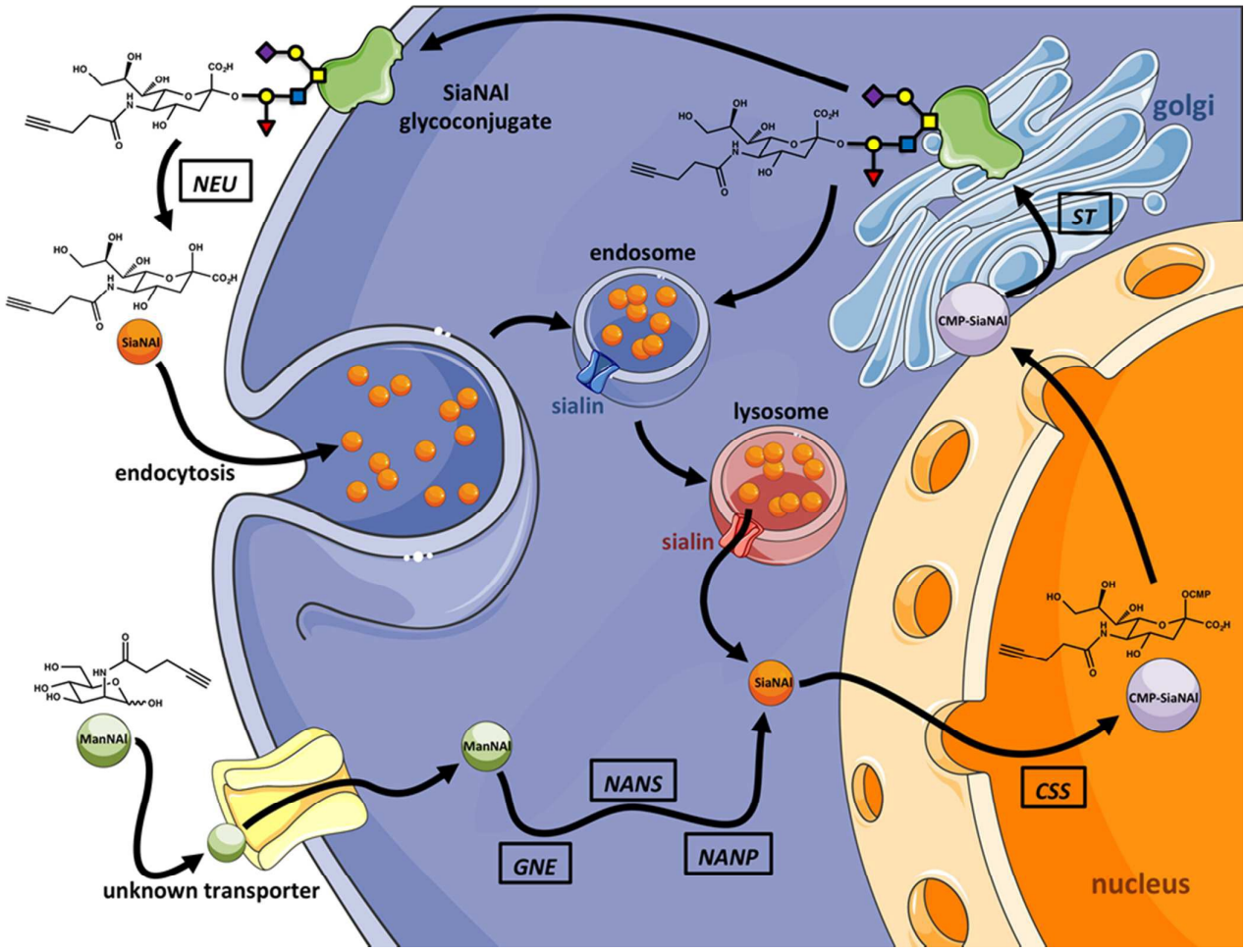

Figure 4. A working hypothesis of cellular uptake and metabolization of ManNAl and SiaNAl. SiaNAl enters *via* endocytosis and is recycled from lysosomes *via* Sialin. Once inside the cytosol, SiaNAl is converted into CMP-SiaNAl in the nucleus lumen and used at the Golgi level by the sialyltransferases. The newly sialylated glycoconjugates are then exported either to the plasma membrane or at the early endosomes. We hypothesize that ManNAl enters *via* a facilitate transporter uncharacterized yet. The cytosolic ManNAl is converted into SiaNAl by the different enzymes (GNE, NANS, NANP). In sialin deficient patients and after ManNAl labelling. In conclusion, SiaNAl labelling can be extremely to identify endosomal/lysosomal dysfunctions while ManNAl labelling allow the sialylated glycoconjugates monitoring.