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Expanding the Strain-Promoted 1,3-Dipolar Cycloaddition Arsenal for a More Selective Bioorthogonal Labeling in Living Cells

Zoisha S. Chinoy^[a, b] and Frédéric Friscourt^{*[a, b]}

Abstract: The advent of bioorthogonal chemistry, more importantly the strain-promoted 1,3-dipolar cycloaddition of cyclooctynes with azides to give stable triazoles, has opened new avenues for the study of biomolecules in their native environment. While much effort has been focused on improving the kinetics of these reactions, very little attention has been given to their bioselectivity. In this review, we will take you on our journey that led us to develop new cyclooctyne probes with enhanced physical attributes, includ-

ing water solubility, cell-surface labeling selectivity and fluorogenic capabilities. We then went on to expand the bioorthogonal chemistry toolbox by focusing on the development of new chemical reporters. Here you will read about our work investigating the use of other 1,3-dipoles, including nitrile oxides and diazo reagents, for the labeling of biomolecules, and more recently highly biostable sydnones that can be exploited to selectively influence glycan-processing enzymes.

1. Introduction

In the early 2000s, the Bertozzi group elegantly showed that cell-surface glycans could be tagged by Staudinger reaction, using unnatural azido-sugars and a phosphine probe.^[1] This seminal work demonstrated that traditional chemical transformations could be tuned to fit cellular demands and with it laid the foundation of what is now called bioorthogonal chemistry.^[2] Since then, bioorthogonal chemical reactions have emerged as cornerstone technologies for the study of biomolecules, including nucleic acids, proteins, glycans and lipids, in living cells.^[3] Bioorthogonal reactions must adhere by a set of stringent criteria: (i) mutual selective chemical reactivity of the two reagents (reporter and probe), (ii) physiological compatibility (presence of water and oxygen), (iii) inertness towards biological functionalities (such as amino acids residues), and (iv) fast reaction rates, which intrinsically limit the number of possible reactions.

Copper-catalyzed alkyne-azide cycloadditions have changed the way we make and investigate small organic molecules and biomacromolecules,^[4] however the cytotoxicity of copper(I) species has limited the utilization of this click reaction to bioconjugation or fixed cells experiments. Bertozzi and co-workers further refined the click reaction for biological applications in living systems, by exploiting highly strained cyclooctynes to react efficiently with azido-biomolecules without the need for a metal catalyst (Figure 1A).^[5] Strain-promoted alkyne-azide cycloadditions (SPAAC) have proven to be central to bioorthogonal chemical reactions, enabling the imaging of a variety of biomolecules in their native environment, notably in living cells and animals.^[6] However, it became quickly apparent that the first generation cyclooctyne (OCT, Figure 1B), due to poor kinetics, was not suited for the

labeling of biological targets present in relatively low abundance.

To address this issue, the Bertozzi group has, for example, appended a geminal difluoro-group at the propargylic position of the cyclooctyne ring (DIFO, Figure 1C), hence lowering the LUMO of the reagent and consequently increasing the rate of cycloadditions by 60-fold as compared to OCT.^[7] Alternatively, Boons and co-workers fused two benzene rings to the cyclooctyne framework (DIBO, Figure 1C), which, in turn, significantly increased the strain of the alkyne functionality, leading to faster SPAAC reactions.^[8] Several cyclooctynes have been described that exhibit even faster rates of cycloaddition by introducing more sp²-hybridized ring-atoms. For example, the groups of van Delft,^[9] Popik^[10] and Feringa^[11] independently reported a more reactive DIBO derivative by adding a nitrogen atom within the cyclooctyne ring. The cycloaddition reaction between aza-dibenzocyclooctyne (DI-BAC or ADIBO, Figure 1C) and benzyl azide was found to be 5-times faster as compared to DIBO. To decrease the steric hindrance generated by the aromatic rings at the propargylic position, van Delft and co-workers designed a cyclooctyne

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Review

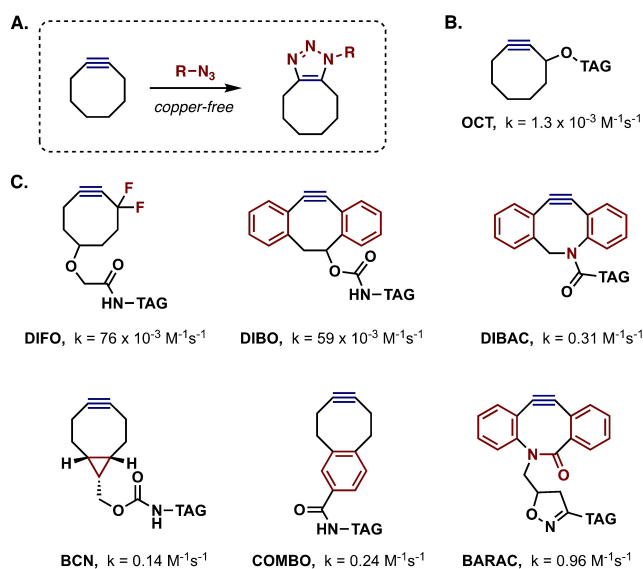


Figure 1. (A) Strain-promoted alkyne-azide cycloaddition reaction (SPAAC). (B) The first generation cyclooctyne, OCT. (C) Representative cyclooctynes with improved kinetics. k represents the SPAAC second-order rate constant for each cyclooctyne. TAG represents an unspecified biophysical probe, including, for instance, biotin or fluorescent dyes.

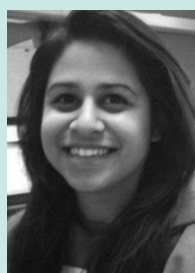
that displayed a 5,6-fused-cyclopropane to the cyclooctyne ring (BCN, Figure 1C).^[12] Following a similar concept, Kele and co-workers reported a monobenzylated cyclooctyne (COMBO, Figure 1C).^[13] These two cyclooctynes reacted fast with azides, exhibiting second-order rate constants over $0.1 \text{ M}^{-1} \text{ s}^{-1}$. Finally, further enhancement of the SPAAC rate was achieved by using BARAC, a dibenzo-fused lactam cyclooctyne (Figure 1C).^[14] Due to its high reactivity, very low concentrations of a BARAC-fluorescein conjugate ($5 \mu\text{M}$)

could be employed to image cell-surface azido-glycans in living cells. Recent theoretical studies have highlighted a clear correlation between the alkyne strain and the rate of the cycloaddition with azides.^[15] Consequently, in order to further enhance the reactivity of cycloalkyne-based probes through ring strain, Bertozzi and co-workers have prepared two cycloheptynes, namely tetramethylthiacycloheptyne (TMTH)^[16] and a dibenzoselenacycloheptyne.^[17] While both cycloheptynes showed improved kinetics towards azides, they unfortunately also exhibited significant stability issues (TMTH showed fast degradation in water and the seleno-derivative could not be isolated), limiting considerably their potential biological applications. These studies clearly highlight the challenging task of finding a good balance between reactivity and stability when designing the next generation of cycloalkyne-based probes.

Although the development of novel cyclooctynes for optimizing SPAAC kinetics has its importance, our group has focused on preparing novel bioorthogonal reagents with enhanced biological properties. Here we recount our efforts in developing water soluble cyclooctynes that are plasma membrane impermeable for the selective labeling of cell-surface azido-biomolecules, as well as cyclooctynes with fluorogenic capabilities for highly selective bioimaging. This account also highlights our recent advances in investigating the use of other 1,3-dipoles with cyclooctynes for chemical variety of the generated cycloadducts as well as controlling the biosynthesis of labeled biomolecules.

2. New Cyclooctynes with Enhanced Properties for a More Selective Labeling of Biomolecules

Chemical tailoring of the cyclooctyne structure has led to the identification of the dibenzocyclooctyne framework as a key



Zoeisha S. Chinoy received her Ph.D. in Chemistry, in 2014, under the supervision of Prof. Geert-Jan Boons at the Complex Carbohydrate Research Center at the University of Georgia, USA. During her Ph.D., she developed the chemoenzymatic synthesis of complex N-glycans, and the synthesis of SKP1 glycopeptides. Following her Ph.D., she moved to France as a postdoctoral fellow to work under the expertise of Prof. Sébastien Lecommandoux in the field of polymer science, at the University of Bordeaux. In 2017, she joined the group of Dr. Frédéric Friscourt at the Institut Européen de Chimie et Biologie in Bordeaux, where her work focuses on the chemoenzymatic preparation of labeled glycans for functional glycomics.



Frédéric Friscourt completed his Ph.D. in 2009 on asymmetric organometallic and organic catalysis under the supervision of Prof. Pavel Kočovský at the University of Glasgow, UK. He then transitioned to the field of Chemical Biology during his postdoctoral fellowship in the laboratory of Prof. Geert-Jan Boons at the Complex Carbohydrate Research Center (GA, USA), where he developed novel chemical probes for imaging the glycome in living cells. In 2014, he obtained a Junior Chair position in Chemical Biology from the University of Bordeaux, France and a co-affiliated position at the European Institute of Chemistry and Biology in Bordeaux as a group leader. He was recently promoted to Associate Professor in Chemical Biology at the University of Bordeaux (2021). His research focuses on using organic chemistry to develop novel tools that can probe the biosynthesis and biological influence of glycans in living systems.

scaffold for high reactivity with organic azides (Figure 1C). However, increasing the aromatic nature of the cyclooctyne probes has also significantly increased their hydrophobicity, which not only limits their water solubility but can also promote their sequestration in biological membranes, thereby reducing bioavailability and increasing non-specific signal. We sought to address this problem by preparing a highly polar cyclooctyne. Since sulfate esters of phenols are found in nature as post-translational modifications of tyrosine,^[18] we hypothesized that *O*-sulfated-dibenzocyclooctyne (S-DIBO, Figure 2A) would be well-suited for biological studies.^[19]

We initiated this work by synthesizing the 8-membered ring in one step via a convenient Friedel-Crafts alkylation between the 1,2-bis(3-methoxyphenyl)ethylacrylamide compound **1** and tetrachlorocyclopropene in the presence of aluminum trichloride (Figure 2A).^[19] It is worth noting that the resulting dibenzocyclopropenone **2** is a caged cyclooctyne that can easily be revealed upon decarbonylation under mild photo-irradiation,^[20] highlighting the simplicity of the synthetic approach. The methoxy functionalities were then converted into the desired *O*-sulfates in a two-step process involving their initial cleavage with boron tribromide, followed by *O*-sulfation of the resulting phenoxy groups with sulfur trioxide complexed with pyridine. The acrylamide moiety of sulfated masked cyclooctyne **3** was then exploited to introduce a variety of tags, such as a biotin moiety, via a thio-Michael addition. Finally, photo-decaging at 350 nm generated the

desired sulfated-dibenzocyclooctyne S-DIBO in overall good yield. As expected, S-DIBO was fully soluble in water and aqueous buffers and was found to be highly stable for extended periods, both as an isolated solid or in solution.

We further studied the applicability of S-DIBO for live-cell imaging (Figure 2B). Using the metabolic oligosaccharide engineering strategy, azides were first introduced into complex glycans of fibroblast cells by feeding peracetylated *N*-azidoacetyl mannosamine (Ac₄ManNAz), a metabolic precursor of sialoconjugates.^[21] Using an elegant fluorescence confocal microscopy study and the use of a polyclonal antibody to mark the Golgi apparatus, we demonstrated that the introduction of the negatively charged sulfate groups on S-DIBO prevented the probe from crossing the plasma membrane, making it selective for the labeling of only the extracellular glycoconjugates in living cells (Figure 2B).^[19] The ability of S-DIBO to not only be fully water soluble but also to selectively label cell-surface molecules puts it in a special category among cyclooctyne probes, opening new biological avenues for SPAAC labeling.

Selective labeling is an important feature of bioorthogonal reactions. However, due to their intrinsic high reactivity, certain cyclooctynes have been shown to react to some degree with biological thiols,^[22] leading to unwanted non-specific background signal. In this context, the recent development of fluorogenic bioorthogonal reagents, which only become fluorescent upon the desired chemical reactions or target binding, have recently been developed for maximizing signal over background, expanding significantly our chemical biology toolbox.^[23] Although numerous fluorogenic azido-probes have been developed in recent years,^[24] fluorogenic cyclooctynes are scarce.

Inspired by the coumarin quenching ability of terminal alkynes,^[25] the groups of Bertozzi and Wong have generated the fluorogenic copper-free click reagents CoumBARAC and CoumOCT, respectively, by incorporating a cyclooctyne ring into the coumarin dye (Figure 3A).^[26] While switching-off known fluorescent dyes with the cyclooctyne moiety clearly simplifies the design of the fluorogenic probes, potential degradation of the quenching moiety may activate the probes, leading to unspecific fluorescence.^[27] On the other hand, fluorogenic reagents that chemically assemble the fluorophore during the bioorthogonal reaction step are consequently more reliable constructs for fluorescent bioimaging. In this context, we recently developed a fluorogenic probe based on the dibenzylcyclooctyne framework (FI-DIBO, Figure 3A) for copper-free imaging of azido-tagged biomolecules.^[28] We prepared FI-DIBO in only five steps with an excellent overall yield of 35%, a rare feat for the synthesis of cyclooctyne probes. More importantly, upon cycloaddition of FI-DIBO with a variety of azides, including aromatic-, triethylene glycol-, amino acid- and carbohydrate-derivatives, the corresponding triazole products exhibited a strong fluorescence emission around 490 nm, when excited at 370 nm. The cycloadducts also displayed a large Stokes shift (over 100 nm), with a moderate fluorescence quantum yield ($\Phi_F =$

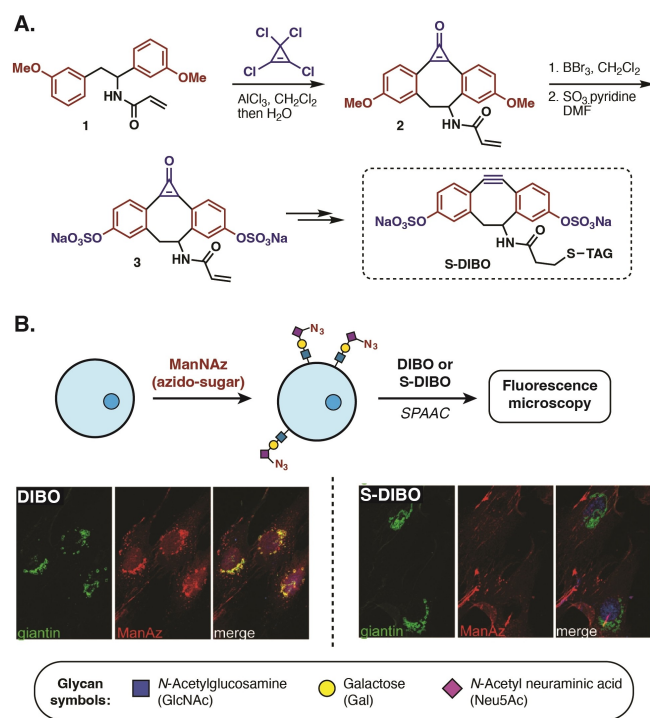


Figure 2. (A) Synthesis of S-DIBO. (B) Cell-surface glycan engineering with *N*-azidoacetyl mannosamine (ManNAz) of living fibroblast cells and their detection using DIBO (left) or S-DIBO (right) probe (red); Giantin: Golgi marker (green), DAPI: Nucleus marker (bleu).

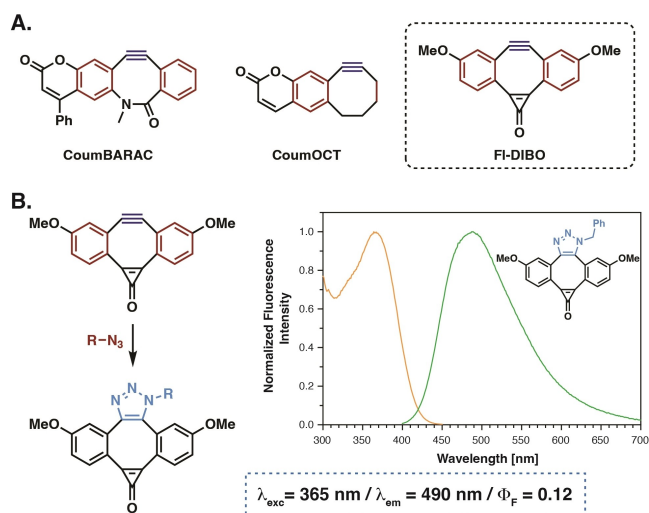


Figure 3. (A) Representative fluorogenic cyclooctynes. (B) Fluorogenic reaction between FI-DIBO and azides and the corresponding excitation (orange trace) and emission (green trace) fluorescence spectra for the benzyl triazole derivative.

0.12) but good molar extinction coefficient, making them more than 1000-fold brighter compared to the starting cyclooctyne (Figure 3B). We then demonstrated the biological suitability of our fluorogenic cyclooctyne (FI-DIBO) by performing highly specific fluorescent labeling of azido-modified proteins, such as bovine serum albumin (BSA), in no-wash conditions.

3. Employing Various 1,3-Dipoles for Modulating the Fluorescence Properties of FI-DIBO Cycloadducts

While our fluorogenic cyclooctyne probe FI-DIBO showed great promise for the specific labeling of azide-containing biomolecules in no-wash conditions,^[28] the limited fluorescence properties of the generated triazoles, notably their moderate fluorescence quantum yields, significantly hampered its application for live-cell imaging. Unfortunately, our first attempts on improving the fluorescence properties of the cycloadducts by varying the chemical structure of the azides were unsuccessful. Therefore, we hypothesized that changing the nature of the heterocycle formed upon strain-promoted cycloaddition between FI-DIBO and other 1,3-dipoles could influence more significantly the photophysical properties of the cycloadducts. We and others had already established that, in addition to azides, nitrones, nitrile oxides, and diazo derivatives could react readily with strained alkynes.^[29]

By screening a series of 1,3-dipoles, we found that the cycloaddition of FI-DIBO with nitrones and nitrile oxides led to cycloadducts exhibiting quenched fluorescence or low quantum yield, respectively.^[30] These results validated our hypothesis that the type of heterocycle generated upon cyclo-

addition is key for switching-on FI-DIBO. In contrast to nitrones and nitrile oxides, mono-substituted diazo reagents produced 1*H*-pyrazole derivatives that displayed a 10,000-fold increase in brightness over FI-DIBO, mostly due to an exceptional increase in their fluorescence quantum yields (Φ_F up to 0.32) and large coefficients of absorption (ϵ_{abs} up to $13,000 \text{ M}^{-1} \text{ cm}^{-1}$) (Figure 4A).^[30] We further applied this novel fluorogenic system for the in-gel fluorescent visualization of diazo-tagged proteins without the need for probe washout.

To our surprise, 3*H*-pyrazoles, obtained from disubstituted diazo reagents, were consistently non-fluorescent. Time-dependent density functional theory (TD-DFT) calculations performed by the Fahrni group suggested that the fluorescence quenching of 3*H*-pyrazoles was the result of the presence of non-emissive energetically low-lying (n, π^*) states (Figure 4B).^[30]

The high reactivity of diazo compounds toward metals and their stability being strongly dependent on the electronic character of their substituents make their synthesis, isolation and biological utilization greatly challenging.^[31] In contrast, sydnone (1,2,3-oxadiazolium-5-olate compounds) are highly

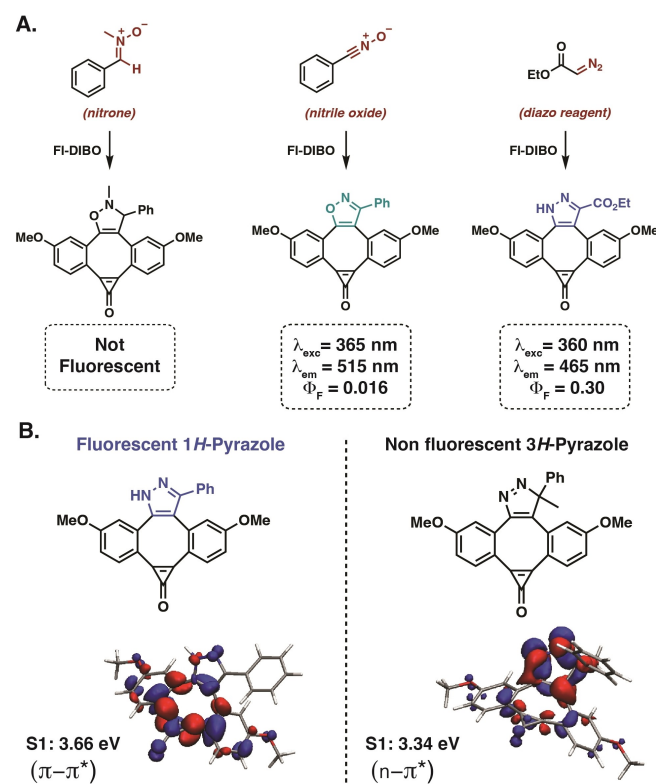


Figure 4. (A) Fluorogenic strain-promoted cycloadditions of FI-DIBO with nitrones, nitrile oxides and diazo reagents. (B) Lowest excited state (S1) of 1*H*-pyrazole and 3*H*-pyrazole, determined by TD-DFT calculations at the CAM-B3LYP level of theory. The dual color plots illustrate the total electron density differences between S1 and the corresponding ground state (decreasing intensity shown in blue, increasing density in red).

stable mesoionic 1,3-dipoles,^[32] which have been shown to undergo metal-free strain-promoted cycloadditions with various cyclooctynes.^[33] Therefore, we envisaged that sydrones, which also generate 1*H*-pyrazoles upon [3 + 2] cycloadditions with alkynes, although of a different substitution pattern, could be viable replacements of diazo reagents for highly fluorogenic copper-free click ligations with FI-DIBO. In addition, sydrones have the attractive feature that their structures are modulable, unlike azides and monosubstituted diazo reagents, at two different positions (3,4-substitutions of the mesoionic ring).

Accordingly, we chemically prepared an array of 3,4-disubstituted sydrones having diverse steric and electronic features.^[34] The pyrazole cycloadducts, generated from reacting the FI-DIBO probe with the synthesized sydrones, were found to be highly fluorescent with compelling photo-physical properties, including excellent fluorescence enhancement (up to 240-fold), high quantum yields (Φ_F up to 0.48) and large Stokes shift (over 100 nm) with emissions ranging from 450 to 480 nm (Figure 5A).^[34] Furthermore, upon chemically tuning the mesoionic ring, we identified 4-chlorosydnone as the most reactive sydnone with FI-DIBO, having a second-order rate

constant of $4.7 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, a rate twice as fast as the previously reported reaction between FI-DIBO and ethyl diazoacetate but four times slower than azides. Interestingly, we observed much closer reactivity pattern between 4-chlorosydnone and azides with other dibenzocyclooctynes, including DIBO ($k_{\text{SydCl}} = 0.063$ vs $k_{\text{azide}} = 0.059 \text{ M}^{-1} \text{ s}^{-1}$) and DIBAC ($k_{\text{SydCl}} = 0.30$ vs $k_{\text{azide}} = 0.31 \text{ M}^{-1} \text{ s}^{-1}$) and even faster reaction rate with unsubstituted cyclooctyne such as BCN ($k_{\text{SydCl}} = 0.57$ vs $k_{\text{azide}} = 0.14 \text{ M}^{-1} \text{ s}^{-1}$).^[35]

Finally, the good stability and reactivity of 4-chlorosydrones with FI-DIBO allowed us to employ them as chemical reporters for the challenging detection of modified-proteins in complex cellular extracts, with exquisite specificity in no-wash conditions, by in-gel fluorescence imaging (Figure 5B).

All together, we established that the fluorogenic properties of FI-DIBO can be tuned in respect to the 1,3-dipole partner employed, making it an ideal probe not only for chemical biology but also for future applications including the screening of chemical reactivity, the sensitive detection of material assembly, and the sensing of various analytes.

4. Exploiting Sydnone 1,3-Dipoles as Chemical Reporters for Controlling the Activity of Glycan Biosynthetic Enzymes

Complex glycans coat the surfaces of eukaryotic cells and participate in a variety of physiological and pathological processes.^[36] The metabolic oligosaccharide engineering (MOE) strategy, popularized by the Bertozzi group, has become an essential technology for labeling glycans in living cells and organisms.^[1,37] In this approach, unnatural azido-sugars are incorporated into cell-surface glycans by hijacking the cell's biosynthetic machinery. Subsequently, the azide chemical reporter is reacted with a chemical probe using efficient and complementary bioorthogonal chemistry, enabling covalent attachment of fluorophores onto glycoconjugates for imaging. This strategy implies that the unnatural sugar must traverse biosynthetic pathways, thus requiring the chemical reporter to be structurally minimally invasive.^[38] Despite the many attractive features of employing azido-sugars for the MOE, and notably its inconspicuousness, we recently showed that azides can be reduced into amines in biological systems by the action of endogenous thiols, potentially leading to the formation of undesired metabolites.^[39] Having recently identified sydrones as highly stable chemical reporters for the imaging of modified-proteins in complex cellular extracts,^[34] as well as for the postsynthetic labeling of oligonucleotides in fixed cells,^[35] we envisaged to utilize sydrones instead of azides for imaging complex glycans in living mammalian cells.^[39] We focused our attention on the labeling of sialoconjugates since sialic acids are usually found at the nonreducing end of complex glycans and consequently are ideally placed for interacting with the cell's exogenous environment.

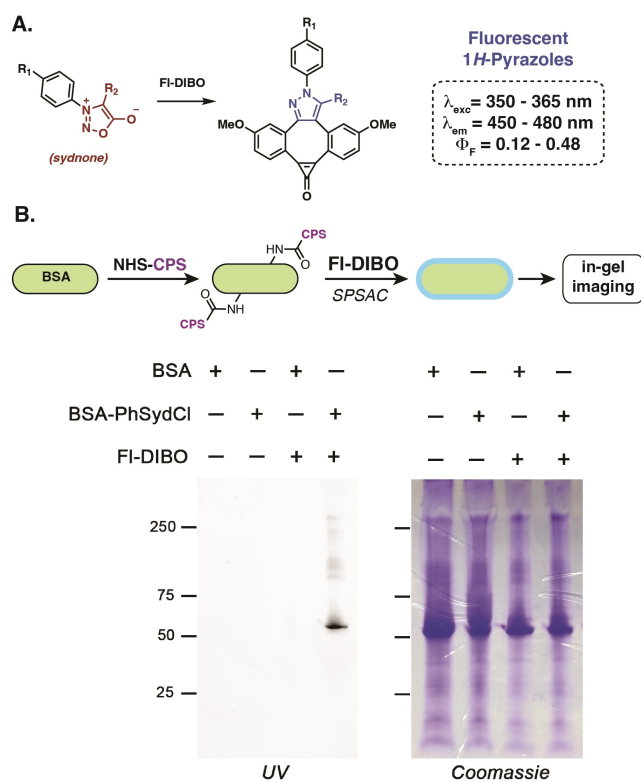


Figure 5. (A) Fluorogenic strain-promoted sydnone-alkyne cycloadditions (SPSAC) between FI-DIBO and sydrones, and the fluorescence properties of the generated pyrazoles. (B) In-gel visualization of sydnone-modified BSA (BSA-PhSydCl) or native BSA incubated with FI-DIBO (100 μM) or no reagent (–) for 3 h in U2OS cell extracts. NHS: *N*-Hydroxysuccinimide; CPS: 4-Chloro-3-phenylsydnone (PhSydCl).

A primary goal of this study was to identify whether sydnones, being structurally bigger than azides, would perturb the recognition of the unnatural sialic acid by biosynthetic enzymes. We initiated this work by chemo-enzymatically synthesizing two unnatural sialic acids, modified either at their 5- or 9-position with a chlorosydnone (Figure 6A). Next, U2OS cells were incubated with both sydnone-fitted sialic acids (Neu5SydCl or Neu9NSydCl) and Neu5Ac, the natural substrate for control. Labeled cells were then reacted with the cyclooctyne BCN-biotin conjugate and stained with Streptavidin-AlexaFluor568 for analysis by confocal fluorescence microscopy (Figure 6B). Introduction of the sydnone moiety onto sialic acid demonstrated that the chemical reporter significantly altered the metabolic fate of the studied sugar. While Neu5SydCl was not metabolically incorporated into cell-surface sialoconjugates, Neu9NSydCl was well tolerated by the glyco-biosynthetic machinery of the cell.^[39] By employ-

ing an *in vitro* enzymatic-¹H-NMR coupled assay, we identified CMP-sialic acid synthetase, the enzyme responsible for the activation of sialic acid into its cytidine monophosphate-analogue, as the enzymatic roadblock for the biosynthesis of sialoglycans with the Neu5SydCl precursor.^[39]

Furthermore, having established that the presence of the sydnone reporter on sialic acid could have a dramatic impact on enzymes substrate recognition, we sought to examine the promiscuity of mammalian sialyltransferases involved in the biosynthesis of *N*-sialoproteins towards Neu9NSydCl. Sialylation of *N*-glycoproteins is mainly controlled by two enzymes, ST6GAL1 and ST3GAL4 that adds a sialic acid residue to the termini of *N*-glycans in an α 2,6- and α 2,3-linkage, respectively. Detailed kinetics analysis of both enzymes, in conjunction with enzymatic sialylation of a glycoprotein and its in-gel visualization using BCN-FITC conjugate (Figure 6C), revealed that while the presence of the azido-reporter does not impact the activity of both enzymes (fully undiscriminated), Neu9NSydCl was found to be a good substrate for ST6GAL1 but not for ST3GAL4, which consequently led to the selective incorporation of the reporter into linkage specific α 2,6-*N*-linked sialoproteins, an achievement that was long-awaited by the glycobiology community.^[39]

We next turned our attention to exploiting the impacted recognition of sydnone-modified sugars by glycan-processing enzymes in a pathological context. Bacteria rely on neuraminidases (sialidases or sialic acid hydrolases) to remove sialic acid from host cell-surface glycans, a crucial event of a multifaceted approach to colonize and invade the targeted host cells.^[40] Consequently, diminishing the activity of bacterial neuraminidases should significantly lower the virulence of bacteria. To study the impact of chemical reporters and their position on sialic acid towards the activity of bacterial neuraminidases, we first chemo-enzymatically prepared a small library of ten sialic acid-containing trisaccharides 4–13 displaying an azide or a chlorosydnone at the 5- or 9-position of the sialic acid (Figure 7A).^[41] We then studied the removal of the unnatural sialic acid residue from the trisaccharides upon incubation with a variety of pathogenic bacterial neuraminidases, including *Vibrio cholerae*, *Clostridium perfringens*, *Streptococcus pneumoniae*, and *Salmonella typhimurium*. From this *in vitro* assay, we identified that Neu5SydCl-modified trisaccharides were fully resistant to all neuraminidases studied whereas the azido-substrates were all processed by the enzymes, albeit at a lower rate than their natural analogues.

To validate the above *in vitro* study into a more relevant biological system, we modified U2OS cell-surface sialosides with unnatural sialic acids displaying an azide, or a sydnone at its 5-position. We then treated the glyco-engineered cells with bacterial neuraminidases and detected the presence or the removal of the unnatural sialic acids by strain-promoted cycloaddition with a biotinylated cyclooctyne and fluorescence microscopy (Figure 7B).^[41] Gratifyingly, while the azido cell-surface glycans were fully removed, the sydnone modified sialosides remained intact, highlighting that Neu5SydCl can

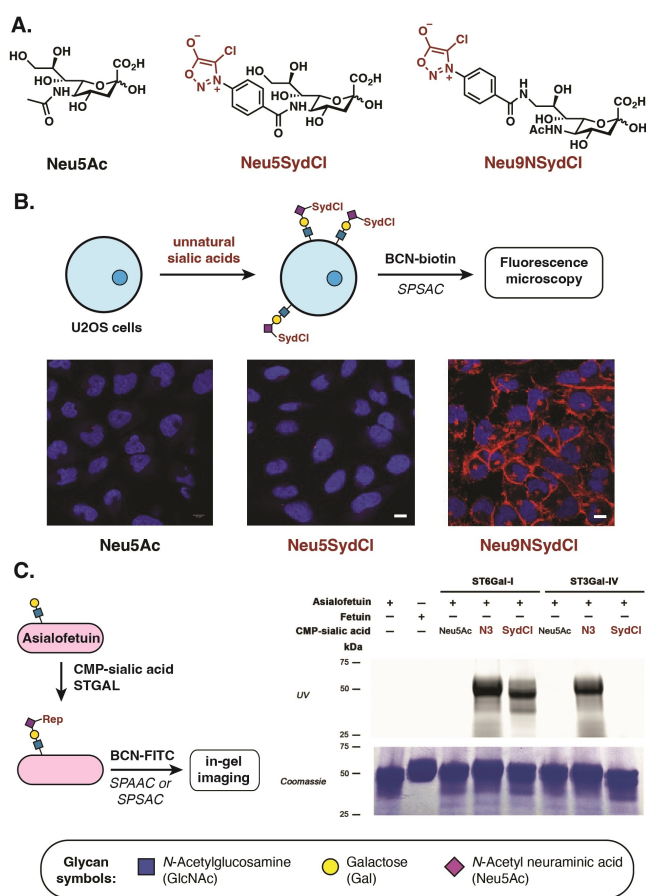


Figure 6. (A) Sialic acids derivatives. (B) Cell-surface glycan engineering, with various sialic acids, of living U2OS cells and their detection using BCN-biotin conjugate and stained with Streptavidin-AlexaFluor568 (red), DAPI: Nucleus marker (bleu), scale bar: 10 μ m. (C) In-gel imaging of the transfer of various CMP-sialic acids (Neu5Ac, Neu9N₃ and Neu9NSydCl) to asialofetuin by ST6GAL1 or ST3GAL4. The presence of the chemical reporter (Rep) was detected by SPAAC or SPSAC with BCN-FITC conjugate (UV channel). Coomassie Blue stain revealed total protein content.

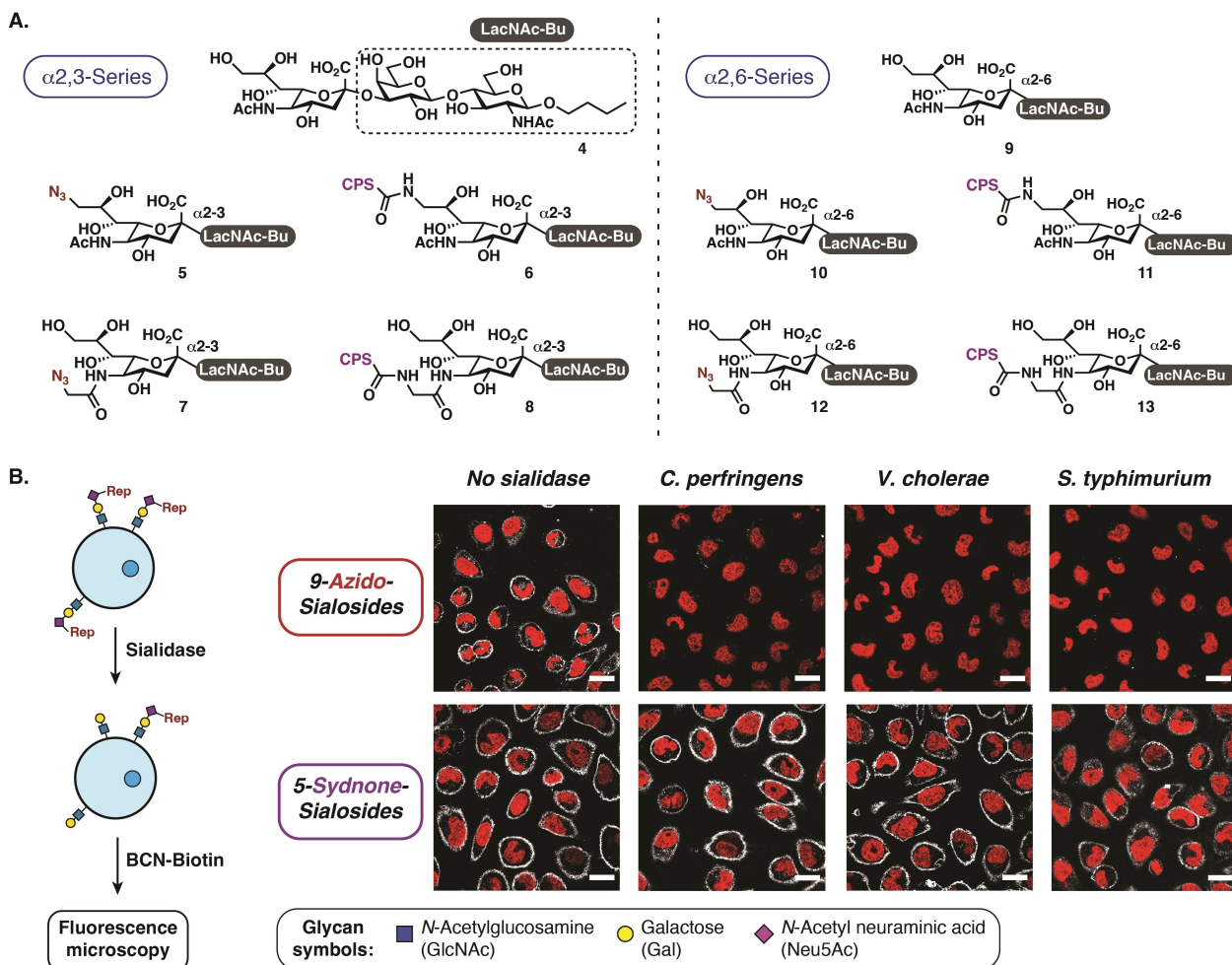


Figure 7. (A) Natural and reporter-modified sialosides. CPS: 4-Chloro-3-phenylsydnone. (B) Glyco-engineered U2OS cells with a 9-azido- or a 5-syndone-sialic acid treated with *C. perfringens*, *V. cholerae*, *S. typhimurium*, or no sialidase. The presence of the reporter was determined by reaction with BCN-biotin conjugate and stained with Streptavidin-AlexaFluor568 (gray), DAPI: Nucleus stain (red), scale bar: 25 μm .

provide mammalian cells with some level of protection against bacterial neuraminidases.

Although bioorthogonal chemical reporters are presumed to be biologically noninvasive, these studies highlight that they are actually not innocent bystanders. Consequently, it becomes critical to systematically investigate the metabolic fate of biomolecules modified with bioorthogonal chemical reporters.

5. Sydnone as Novel Copper-Free Clickable Handles

Both copper-catalyzed and strain-promoted cycloadditions between azides and alkynes have revolutionized, over the past decade, our way of generating and studying small organic molecules, complex materials, and biomacromolecules.^[4c] However, we recently showed that while azides are sensitive to thiol-reduction, sydnone is chemically robust.^[39] To

further expand the click chemistry toolbox, we sought to incorporate a sydnone moiety, as a novel clickable handle, for the replacement of azides where potential reduction can be an issue, or chemically advantageous.

Azides have proven to be efficient quenchers of known fluorophores such as coumarin,^[42] BODIPY,^[43] fluorescein,^[44] and rhodamine,^[45] leading to the development of an array of fluorogenic probes. The fluorogenic azido-reagents can be turned-on with varying degrees of fluorescence enhancement upon cycloadditions with linear and strained alkynes, allowing the tracking of biomolecules in living cells. However, reduction of the azido-moiety into amine by endogenous thiols may activate the fluorogenic probe leading to unspecific fluorescence.^[27] To address this issue, we aimed to develop novel fluorogenic clickable reagents based on sydnone.^[46]

We hypothesized that the change in electron density initiated from the conversion of a sydnone moiety, appropriately placed on a fluorophore scaffold, into a pyrazole ring upon click reaction should significantly alter the fluorescence

properties of the chromophore, generating a novel fluorogenic system. Accordingly, we synthesized, in overall good yields, coumarins **14** and **15** having a sydnone moiety at their 3-position. Gratifyingly, both probes were efficiently quenched in solution. Upon reaction with the cyclooctyne BCN, both fluorogenic sydnones generated strongly fluorescent pyrazoles, exhibiting a fluorescence emission around 430 nm, large Stokes shifts (~85 nm), and high fluorescence quantum yields (Φ_F up to 0.66), implying notable fluorescence enhancements up to 132-fold (Figure 8A). Both fluorogenic sydnones were elegantly applied, in a biochemical context, for the highly specific labeling of proteins in no-wash conditions (Figure 8B).^[46] To note, these novel coumarin sydnones effectively complement the fluorogenic bioorthogonal SPSAC toolbox, as they elegantly provide a new level of versatility, since either the cyclooctyne (FI-DIBO, see Figure 5) or now the sydnone can be selected, for efficient metal-free fluorogenic labeling.

In addition to the development of bioorthogonal chemical probes and reporters, our lab is also interested in unraveling the functional role of complex glycans in living systems. Protein-*O*-glycosylation has been shown to be essential for many biological processes.^[47] However, our knowledge of *O*-glycans is still in its infancy, largely because of a lack of effective technologies for *O*-glycomics.^[48] Having previously demonstrated that sydnones are highly stable under reducing conditions, we envisaged that, unlike azides, sydnones could be incorporated as an aglycon into *O*-glycans, early-on in their synthesis since it should be compatible with carbohydrate chemistry and enzymatic glycosylations. The sydnone-aglycon could then be exploited, as a clickable handle, for the late functionalization of glycans, ideal for *O*-glycomics applications.

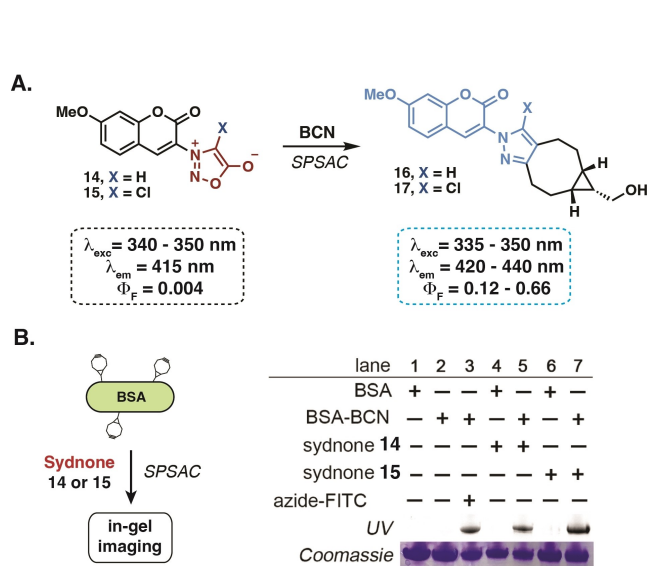


Figure 8. (A) Fluorogenic strain-promoted cycloaddition between BCN and sydnone-modified coumarins **14** and **15**. (B) In-gel visualization of labeled BSA-BCN conjugate with fluorogenic sydnone-modified coumarins **14** and **15**. The fluorescent azido-FITC is used as a positive control.

We began our investigation by synthesizing the key monosaccharide GalN₃- α 1-*O*-linker-sydnone **18** in good yield (up to 71%) and acceptable α -selectivity, a critical feature of *O*-glycans.^[49] We then converted the GalN₃- α 1-*O*-linker-sydnone into the desired *O*-GalNAc derivative **19** via azide reduction, followed by *N*-acetylation and removal of the *O*-acetyl protecting groups, highlighting that sydnones are fully compatible with reactions routinely employed in carbohydrate chemistry (Figure 9A). Next, we extended the *O*-GalNAc **19** into more complex mucin-type *O*-glycan structures **20–21** by enzymatic oligosaccharide synthesis, exploiting a variety of human glycosyltransferases (Figure 9B). Finally, we conveniently immobilized the sydnone-tagged *O*-glycans on microtiter plates using the biotinylated glycans **22–24** generated via bioorthogonal strain-promoted sydnone-alkyne click reaction. The resulting *O*-glycan-array was then utilized for the high-throughput screening of *O*-glycan-lectin interactions (Figure 9C).^[49]

All together, we established that sydnones represent an excellent alternative to azides as clickable handles, especially when azide reduction may lead to undesired complications.

6. Summary and Future Perspectives

Metal-free 1,3-dipolar cycloadditions with strained alkynes, pioneered by the Bertozzi group two decades ago, have become the cornerstone of bioorthogonal chemical ligations, enabling the study of biomolecules in living systems. Despite the significant improvement in reaction rates of strain-

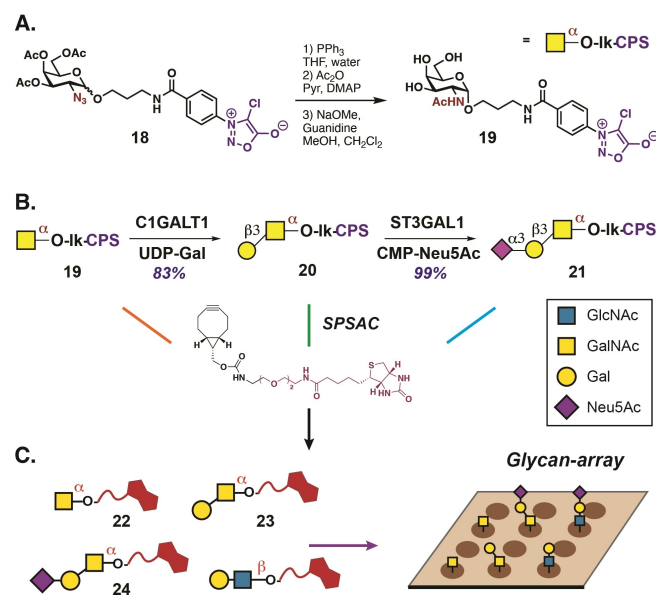


Figure 9. (A) Chemical synthesis of sydnone-modified α 1-*O*-GalNAc **19**. (B) Enzymatic extension of **19** into more complex *O*-glycans **20–21** and their biotinylation with BCN-biotin conjugate. (C) *O*-Glycan array preparation from the biotinylated glycans **22–24**.

promoted alkyne-azide cycloadditions (SPAAC), SPAAC is not without limitation. In a quest to improve the specificity of biological labeling upon SPAAC, we have targeted two approaches. First, we focused on developing novel cyclooctynes with enhanced physical attributes, including plasma membrane impermeability, for the selective labeling of cell-surface biomolecules, and fluorogenic capabilities. In parallel, we have investigated the use of other 1,3-dipoles as novel chemical reporters for the labeling of biomolecules, including nitrile oxides, diazo reagents and more recently highly biostable sydnone. Research from our group and others over the past decade, have significantly expanded the 1,3-dipolar bioorthogonal click reaction arsenal, allowing for a more selective visualization of biomolecules in their native environment.

Future attention needs to focus on investigating the metabolic fate of chemical reporters both in living cells and animals as unnatural functionalities can significantly influence biological processes. In this respect, we and others recently demonstrated that some monosaccharides modified with a chemical reporter were not recognized by various metabolic enzymes.^[39,41,50] These studies would not only provide a better fundamental understanding of physiological processes but would also open new avenues for controlling pathological enzymes activity in our search for novel therapeutic strategies.

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