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Real-Time Bioluminescence Imaging of Glycans on Live Cells

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The totality of glycans produced by cells, referred to as the glycome, is a dynamic indicator of the cell's physiology.[1] The glycome changes as a function of developmental stage, cellular activation, and transformation from a healthy to a pathological state (e.g., cancer).[2] Molecular imaging of the glycome promises to advance our understanding of these processes and their implications in the diagnosis and treatment of disease.[3] The notion of imaging glycans *in vivo* was recently enabled by the bioorthogonal chemical reporter technique.[4] First, a sugar analogue adorned with a bioorthogonal functional group is metabolically incorporated into cellular glycans. In a second step, the modified sugar is chemically reacted with an exogenously added imaging probe bearing complementary functionality.

This method of visualizing glycans was first developed in the context of cultured cells, using azidosugars as metabolic labels and the Staudinger ligation with phosphines as a means to introduce fluorescence imaging probes.[5] Since then, other chemistries have been explored, including Cu-catalyzed cycloaddition of metabolically incorporated alkynyl sugars with azide-functionalized fluorophores (i.e., click chemistry)[6] and strain-promoted cycloaddition of azidosugars with cyclooctyne probes (i.e., Cu-free click chemistry).[7] The suitability of these chemistries for various imaging applications reflects a balance of attributes, including intrinsic kinetic parameters, reagent toxicity, and bioavailability. With respect to kinetics, the Cu-catalyzed azide−alkyne cycloaddition (CuAAC) has a significant advantage over the Staudinger ligation.[8] However, the cytotoxicity of the Cu(I) catalyst disqualifies this chemistry from use with live cells or organisms.[9] Difluorinated cyclooctyne (DIFO) probes have fast kinetics and no observable toxicity; consequently, they were used for the first *in vivo* imaging study of glycans in developing zebrafish.[10] However, in mice, the most common animal model of human disease, DIFO probes appear to have limited bioavailability.[11] Thus, despite their superior kinetic parameters, DIFO probes label cell-surface azidosugars less efficiently than phosphine reagents in this model organism.

To date, phosphines remain the best-performing reagents for tagging azidosugars in mice, but their slow reaction kinetics mandates the use of high concentrations *in vivo*. With fluorescent probes, this situation leads to high background labeling and low signal-to-noise ratios. While fluorogenic "smart" phosphines can partially mitigate the problem,[12] the complexity of such probes, as well as sensitivity issues caused by tissue autofluorescence, has undermined efforts to image glycans in mice using fluorescent phosphine reagents.

Bioluminescence imaging (BLI)[13] obviates many of the intrinsic limitations of fluorescence imaging with phosphine probes. The technique is based on the sensitive detection of visible light produced during enzyme (luciferase)-mediated oxidation of a small molecule substrate (luciferin) when the enzyme is expressed as a reporter. BLI is gaining appreciation for its use in real-time animal imaging, as the total absence of tissue luminescence bestows negligible background, enabling exquisite sensitivity.[14] Many types of luciferase transgenic mice as well as disease specific models are now commercially available. Since BLI is dependent on the presence of luciferase, transfected cells or transgenic animals are required for the experiments. Thus, clinical translation is not possible; however, this technique still represents a powerful tool to answer basic research questions. We reasoned that a phosphine−luciferin conjugate (**1**, Figure 1) designed to release luciferin upon Staudinger ligation would enable sensitive detection of azidosugars with very low background signal, even when the reagent was employed at relatively high concentrations (e.g., in the micromolar range). Further, the intrinsic sensitivity of BLI might enable the use of lower phosphine probe concentrations than were found to be required for fluorescence imaging (e.g., in the nanomolar range). Here we report the synthesis of compound **1** and its use in real-time BLI of azidosugars on live cells.

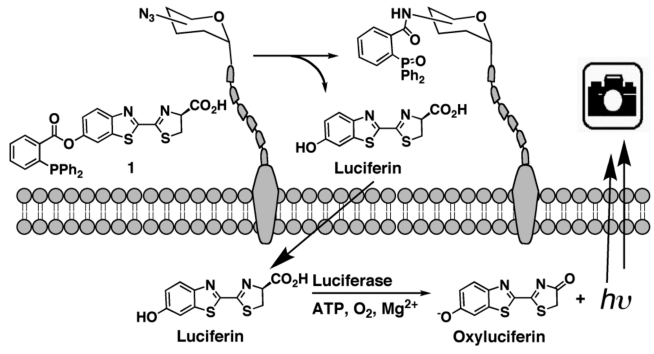
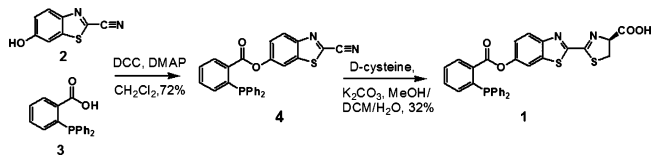


Figure 1. Use of **1** for bioluminescence imaging of cell-surface azidosugars. Compound **1** releases firefly luciferin upon Staudinger ligation with azides. After luciferin diffuses into cells, luciferase-catalyzed conversion of luciferin to oxyluciferin is accompanied by production of a photon of light, which is detected using a CCD camera.

Compound **1** comprises firefly luciferin linked to triphenylphosphine through a carboxy ester that is cleaved during Staudinger ligation with an azide.[15] Compound **1** itself cannot produce bioluminescence due to the esterified phenolic oxygen, a modification that disrupts recognition of luciferin by luciferase.[16] Thus, only luminescence due to firefly luciferin released during the Staudinger ligation is observed. The synthesis of **1** is outlined in Scheme 1 and described in detail in the Supporting Information (SI).

Scheme 1. Synthesis of Phosphine−Luciferin Conjugate **1**



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Ester hydrolysis is a possible competing side reaction that would uncouple luciferin release from the Staudinger ligation, thereby undermining **1**'s design. Thus, we probed the rates of the two processes in model reactions. Using benzyl azide as a substrate in acetonitrile/water (19:1), Staudinger ligation with **1** proceeded with a second-order rate constant of 2.3×10^{-3} $M^{-1}s^{-1}$ (SI, Figure S1), a value similar to those found for previously studied phosphine substrates.[15] In the context of *in vivo* cell-surface labeling, this number translates into reaction times in the range of 1−2 h to achieve conversion of a majority of azides.[17] Hydrolysis of **1** in cell culture media containing physiological glutathione occurred with a half-life of ∼5 days (see SI). Thus, **1** possesses sufficient hydrolytic stability for our envisioned application.

To evaluate **1**'s performance in cell-surface azidosugar imaging, we employed a prostate cancer cell line stably transfected with firefly luciferase (LNCaP-luc). We chose this cell line because it was one of the most robust with respect to azidosugar incorporation.[18] The cells were incubated for 2 days with various concentrations of peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) or peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz) (10, 35, or 50 μM) to allow for metabolic incorporation of *N*-azido sialic acid (SiaNAz)[19] and GalNAz[20] into sialylated and mucin-type O-glycans, respectively. Control cells were incubated in media that did not contain azidosugars. In addition, we incubated cells with peracetylated *N*-azidoacetylglucosamine (Ac_4GlcNAz), which is incorporated into nuclear and cytosolic proteins but not, to any appreciable extent, into cell-surface glycans.[20,21] After several washes to remove all exogenous azidosugars in the media, the cells were incubated with various concentrations of **1** (ranging from 3 nM to 100 μM), and the number of photons produced as a function of time was quantified using a charge-coupled device camera.

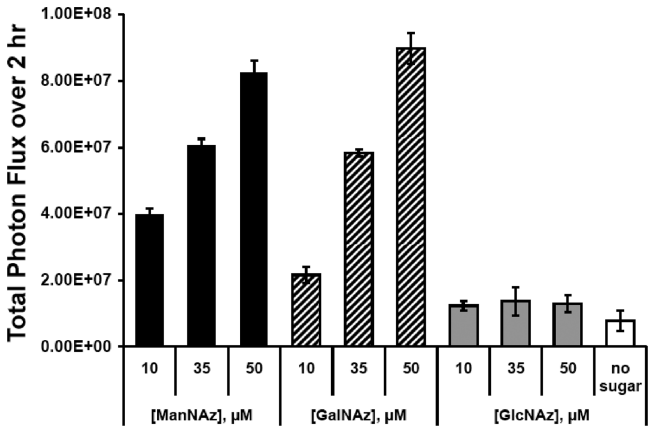

Figure 2. Labeling of cell-surface azidosugars with phosphine−luciferin **1**. LNCaP-luc cells, a prostate cancer cell line stably transfected with luciferase, were incubated for 2 days in the presence of various concentrations of Ac_4ManNAz, Ac_4GalNAz, Ac_4GlcNAz, or media. The cells were washed three times with 200 μL of PBS and then treated with **1** (100 μM) for 120 min. The total bioluminescence was obtained by calculating the area under the curves represented by those in Figure S2 (SI). Error bars represent the standard deviation of the mean for three replicate experiments.

Cells incubated with Ac_4ManNAz or Ac_4GalNAz and **1** showed significantly higher luminescence than control cells lacking azidosugars (Figure 2). Also, the signal was proportional to the concentration of azidosugar (Figure 2) and the probe (Figure 4). Figure 2 shows the total bioluminescence produced by cells treated with each azidosugar. Figure S2 in the SI depicts the real-time signal produced from cells treated with different concentrations of Ac_4ManNAz. Cells treated with Ac_4GlcNAz showed the same background level of bioluminescence as cells treated with no azidosugar. Collectively, these results indicate that the observed signal above background is due to luciferin released during the Staudinger ligation with cell-surface azidosugar.

Given the hydrophobic nature of **1**, we questioned whether the luminescence observed was due to Staudinger ligation at the cell surface, or, following passive diffusion into the cell, reaction with intracellular azido-metabolic intermediates. We therefore treated cells labeled with each azidosugar with sialidase to cleave cell-surface sialic acids.[22] This treatment was expected to reduce the number of azides present on cells incubated with Ac_4ManNAz but have a negligible effect on cells incubated with the other two azidosugars.[17] After reaction with **1** and subsequent imaging, we observed a significant decrease in the number of photons produced from Ac_4ManNAz-labeled cells treated with sialidase as compared to Ac_4ManNAz-labeled cells having no sialidase treatment (Figure 3). The ∼60% reduction in signal observed with sialidase-treated cells agrees well with previous studies using other detection methods.[22] In contrast, the number of photons produced by Ac_4GalNAz- or Ac_4GlcNAz-labeled cells was unaffected by sialidase treatment. These results indicate that the observed luminescence largely reflects Staudinger ligation with cell-surface azidosugars.

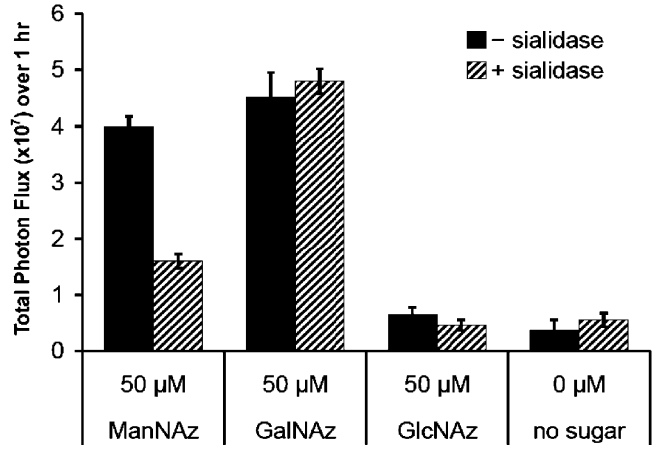

Figure 3. Sialidase treatment reduces labeling seen with **1**. LNCaP-luc cells were incubated for 2 days in the presence of 50 μM Ac_4ManNAz, Ac_4GalNAz, Ac_4GlcNAz, or media. Cells were treated with *Arthrobacter ureafaciens* sialidase (patterned bars) or left untreated (black bars). The cells were then treated with **1** (100 μM) for 1 h, and the total bioluminescence was quantified. Error bars represent the standard deviation of the mean for three replicate experiments.

We noted that cells without azidosugar treatment displayed low levels of bioluminescence upon treatment with **1**. Chemical hydrolysis seemed an unlikely explanation given the time scale of the experiments (1−3 h). We therefore tested the hypothesis that esterases produced by the cells or in residual serum from cell culture media were catalyzing the hydrolysis of **1**. Prior to the addition of **1**, LNCaP-luc cells were treated with sodium fluoride (NaF), phenylmethylsulfonyl fluoride (PMSF), or 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), inhibitors of carboxyesterases, serine esterases, and aryl esterases, respectively.[23] DTNB treatment reduced the luminescence signal ∼3-fold, whereas the other two inhibitors had no significant effect (SI, Figure S3). Thus, aryl esterase activities may contribute to background luminescence.

BLI has the intrinsic potential for very high sensitivity compared to other optical imaging modalities. For **1** to realize this potential, the BLI signal produced by Staudinger ligation with azidosugars must exceed background luminescence derived from ester hydroly-

sis, ideally at relatively low probe concentrations. To evaluate the sensitivity of **1**, we incubated Ac_4ManNAz-treated cells with doses of **1** ranging from 3 nM to 100 μM, and collected photons at time points from 5 to 200 min. Significant azide-specific luminescence above background was observed at all concentrations of **1** (Figure 4) and with as little as 5 min of photon collection (SI, Figure S4). As expected, the signal-to-background ratio increased with probe concentration, with a maximal value of ~10 observed at 50−100 μM. These results contrast markedly with our previous observations using fluorescent-phosphine probes. In those studies, the minimum probe concentrations required to observe specific signal above background labeling were ~10 μM.[5] Therefore, BLI with **1** appears to exceed the sensitivity of its fluorescence counterpart by several orders of magnitude, even when accounting for background luminescence derived from ester hydrolysis.

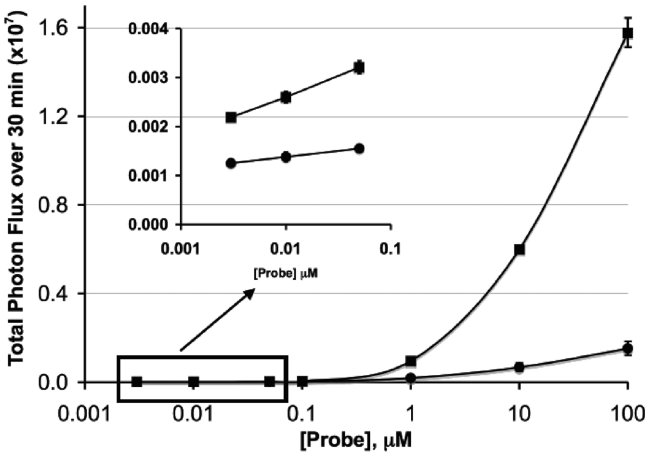

Figure 4. Sensitivity of labeling of SiaNAz with **1**. LNCaP-luc cells were incubated for 2 days in the presence of 50 μM Ac_4ManNAz (■) or media (●). The cells were washed three times with PBS and then treated with **1** for 30 min. For each concentration, the total bioluminescence was quantified over 30 min. Error bars represent the standard deviation of the mean for three replicate experiments.

Looking ahead, **1** is a promising reagent for *in vivo* imaging of azide-labeled glycans. Improvements can be envisioned, the most obvious of which is redesign of the compound to mitigate enzymatic hydrolysis. How relevant this issue will be in the context of live animal imaging remains to be determined. Even in its present form, **1** enables real-time imaging of cell-surface glycans with unprecedented sensitivity using very low probe concentrations. Since other biomolecules can be metabolically labeled with azido precursors[4]—lipids, proteins, and nucleic acids to name a few—BLI with **1** may represent a new platform for real-time imaging of numerous cellular processes. Even though we have only demonstrated its use in visualizing glycans, it can be envisioned that this probe could also be used for these applications.[4,24] Bioluminescence imaging with **1** will thus be a useful tool for answering many fundamental biological questions. Studies with **1** in both healthy mice and cancer models are currently underway.

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Supporting Information Available: Experimental details, spectral data, kinetics, hydrolysis, and total photon flux from various concentrations of **1**, from esterase inhibition, and from hydrolysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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