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Detection of Cellular Sialic Acid Content Using Nitrobenzoxadiazole Carbonyl-Reactive Chromophores

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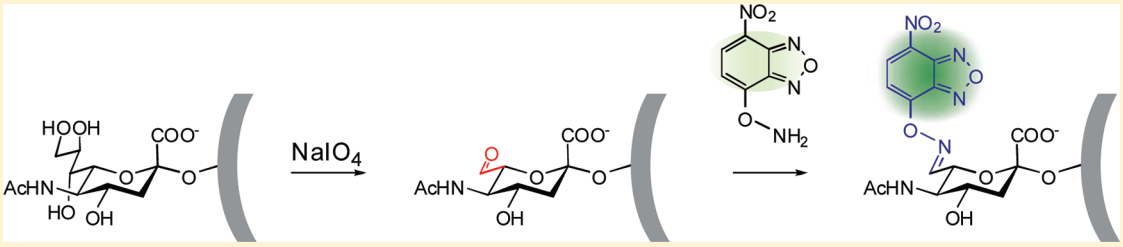
Supporting Information



ABSTRACT: The selective ligation of hydrazine and amino-oxy compounds with carbonyls has gained popularity as a detection strategy with the recognition of aniline catalysis as a way to accelerate the labeling reaction in water. Aldehydes are a convenient functional group choice since there are few native aldehydes found at the cell surface. Aldehydes can be selectively introduced into sialic acid containing glycoproteins by treatment with dilute sodium periodate. Thus, the combination of periodate oxidation with aniline-catalyzed ligation (PAL) has become a viable method for detection of glycoconjugates on live cells. Herein we examine two fluorescent nitrobenzoxadiazole dyes for labeling of glycoproteins and cell surface glycoconjugates. We introduce a novel 4-aminooxy-7-nitro-benz-[2,1,3-*d*]-oxadiazole (NBDAO) (**5**) fluorophore, and offer a comparison to commercial dyes including the known 4-hydrazino-7-nitro-benz-[2,1,3-*d*]-oxadiazole (NBDH) (**2**) and Bodipy FL hydrazide. We confirm specificity for sialic acid moieties and that both dyes are suitable for in vitro and in vivo labeling studies using PAL and fluorescence spectroscopy. The dyes examined here are attractive labeling agents for microscopy, as they can be excited by a 488 nm laser line and can be made in a few synthetic steps. These carbonyl-reactive chromophores provide a one step alternative to avidin−biotin labeling strategies and simplify the detection of sialic acid in cells and glycoproteins.

INTRODUCTION

Sensitive detection of biomolecules by derivatization with fluorescent dyes is a critical technique used in histology, cell biology, and biochemistry.[1−3] Specific detection of conjugates relies on chemoselective ligation of functional groups found on the biomolecule and the label.[4] As a result, strategies to introduce reactive functional groups into biomolecules have been developed. Typical strategies include the metabolic incorporation of synthetic labels and chemical modification of native biomolecules.[4,5] A well-known strategy for labeling of glycoproteins is the use of sodium periodate to oxidize the glycerol side-chain of sialic acid to an aldehyde.[6] While this method is more commonly used in vitro, it has recently been extended to the labeling of glycans in live cells.[7,8] Live cell labeling requires mild conditions and near-quantitative reactions, which has been achieved by performing the oxidation reaction at low temperature, and accelerating the conjugation reaction with an aniline catalyst.[8] This method of periodate oxidation and aniline-catalyzed ligation (PAL) can then be used to label glycoconjugates for microscopy.

Sialic acids are a class of carbohydrates which have important biological functions in embryogenesis, development, and immune response.[9−11] Methods of detecting sialylation include metabolic labeling, lectins, and chemical modification.[12] Metabolic labeling allows the introduction of unique functional groups that provide sensitive detection;[13] however, this strategy can suffer from incomplete label incorporation. Lectins can be large proteins which may suffer from relatively low affinities. Importantly, many lectins or antibodies can perturb cells by cross-linking receptors—limiting their application in live cells.[14,15] Selective chemical modification of sialic acids using PAL presents an attractive alternative.[8] However, previous reports of PAL on cells have relied upon biotin-amino-oxy conjugates which were then detected by fluorescently labeled streptavidin. This two-step method required the use of a 60 kDa tetrameric protein for labeling a single carbohydrate residue (ca. 300 Da).[7,16] Such a bulky label could mask other epitopes on the same cell or macromolecule, and may have reduced labeling capacity due to molecular crowding.

Small molecule fluorophores which contain either hydrazine or amino-oxy functional groups can be applied to bioorthogonal and PAL labeling strategies.[17−19] 7-Nitro-benz-[2,1,3-*d*]-oxadiazoles (NBDs) are a class of small, synthetically accessible, and environmentally sensitive fluorophores which can be easily modified for use with PAL. In general, NBDs possess relatively high fluorescence quantum yields (0.3 for primary amine

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derivatives of NBDCl)[2] and long excitation and emission wavelengths when compared to fluorophores of similar size, such as coumarins.[20] Interestingly, the attachment of a hydrazine functional group to these dyes results in fluorogenic derivatives which allow direct detection of carbonyl derivatives upon formation of the corresponding hydrazone. Reported fluorogenic and fluorescent hydrazino-NBD derivatives include 7-hydrazino-4-nitrobenzo-2-oxa-1,3,-diazole (NBDH),[21] 4-(*N*,*N*-dimethylaminosulfonyl)-7-hydrazino-benz-2,1,3-oxadiazole (DBDH),[22] 4,4-aminosulfonyl-7-hydrazino-2,1,3-benzoxadiazole (ABDH),[22] 4-(*N*,*N*-dimethylaminosulfonyl)-7-*N*-methylhydrazino-benz-2,1,3-oxadiazole (MDBDH),[23] 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-benz-2,1,3-oxadiazole (DAABD-MHz),[24] and *N*-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH).[25] Several of these derivatives are commercially available and have found application in biological assays,[26] as well as the detection of trace carbonyls in air samples such as automobile exhaust.[23]

We considered that small molecule labels compatible with PAL should be of interest for fluorescence microscopy and quantitation of glycoconjugates. To take advantage of the PAL strategy, the fluorophore should contain a nucleophilic group that will react with an aldehyde rapidly in water. Although hydrazines have been explored for this purpose, we chose to investigate an amino-oxy-containing fluorophore.[8] Herein we describe the synthesis of a novel amino-oxy fluorophore, NBDAO (**5**), which has improved sensitivity over existing dyes for selective labeling of sialic acid glycoconjugates on live cells. We contrast the properties of NBDAO to a known carbonyl reactive fluorophore, NBDH (**2**).[21] We first observed labeling of cells with a fluorescence microplate reader. Additionally, NBDAO was used to label a glycoprotein in conjunction with PAL, followed by fluorescence detection in SDS-PAGE. Finally, we compared the performance of NBDAO to commercially available hydrazine dyes NBDH and Bodipy FL hydrazide in fluorescence microscopy and flow cytometry assays. We conclude that these dyes can be used as an effective one-step live-cell labeling strategy for glycoconjugates, and that NBDAO provides the best level of sensitivity among the dyes tested.

METHODS

General Experimental Methods. Reagents were purchased from commercial sources such as Sigma-Aldrich (Oakville, Ont) and used without additional purification. Proton (^{1}H) and carbon (^{13}C) NMR spectra were obtained on Varian 300, 400, 500, or 700 MHz instruments at room temperature as noted. Deuterated solvents were obtained from Cambridge Isotope Laboratories (Andover, MA). Mass spectrometry was performed using an MS50G positive electron impact instrument from Kratos Analytical (Manchester, UK) and a Mariner Biospectrometry positive ion electrospray instrument from Applied Biosystems (Foster City, CA).

Spectroscopy. Absorbance spectra for all compounds were collected at room temperature with a Hewlett-Packard (Palo Alto, CA) model 8453 diode array UV−visible spectrophotometer or Varian (Walnut Creek, CA) Cary 50 spectrophotometer. Fluorescence spectra for all compounds were collected at room temperature with a Photon Technology International model MP1 steady-state fluorimeter. Absorbance and fluorescence measurements were taken using NSG Precision Cells ES quartz cuvettes (190−2000 nm; Farmingdale, NY).

Synthesis of Dyes and Model Conjugates. *4-Hydrazinyl-7-nitrobenz-[2,1,3-d]-oxadiazole (**2**, **NBDH**).* 4-Chloro-7-nitrobenz-[2,1,3-*d*]-oxadiazole (**1**) (100 mg, 0.5 mmol, 1 equiv) was dissolved in chloroform (50 mL). A 1% hydrazine solution (0.77 mL hydrazine in 50 mL methanol) was then added to the solution and allowed to stir at room temperature for 1 h. A yellow-brown precipitate was formed and isolated without further purification (104 mg, quant.) ^{1}H NMR (400 MHz, D_2O): δ 7.04 (d, 1H, ^3J = 10.5 Hz), 6.37 (d, 1H, ^3J = 10.5 Hz); ^{13}C NMR (100 MHz, D_2O): δ 147.6, 145.4, 131.4, 121.9, 120.8, 115.1; IR (microscope): ν = 3378, 3278, 3191, 3051, 2984, 2692, 1618, 1542, 1500, 1465, 1213, 1019, 982, 873, 794 cm^{-1}. ES-HRMS calculated for $C_5H_5N_5O_3Na$: 218.0285, observed: 218.0284.

*4-(2-Benzylidenehydrazinyl)-7-nitrobenz-[2,1,3-d]-oxadiazole (**3**).* 4-Hydrazinyl-7-nitrobenz-[2,1,3-*d*]-oxadiazole (**2**) (25 mg, 0.13 mmol, 1 equiv) was dissolved in methanol (10 mL). Benzaldehyde (0.13 mL, 1.3 mmol, 10 equiv) was added, turning the reaction mixture a red color. The product gradually formed as a dark red-black precipitate and was isolated by vacuum filtration. Additional product was isolated from the supernatant after flash column chromatography (EtOAc/hexanes). The precipitate and column fractions were combined (27 mg, 74%). ^{1}H NMR (400 MHz, $(CD_3)_2CO$): 8.62 (d, 1H, ^3J = 8.8 Hz), 8.59 (s, 1H), 7.92−7.79 (m, 2H), 7.56−7.39 (m, 3H), 7.31 (d, 1H, ^3J = 8.8 Hz); ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ 149.9, 145.2, 144.1, 141.0, 137.0, 134.9, 131.6, 129.8, 128.4, 126.0, 102.7; IR (microscope): ν = 3486, 3307, 3219, 3144, 3060, 2955, 1603, 1581, 1515, 1446, 1406, 1295, 1117, 997 cm^{-1}. ES-HRMS calculated for $C_{13}H_{10}N_5O_3$: 284.0778, observed: 284.0778. R_f = 0.23 (1:3 EtOAc/hexanes).

*Ethyl N-7-nitrobenz-[2,1,3-d]-oxadiazol-4-yloxyacetimidate (**4**).* Ethyl *N*-hydroxyacetimidate (35 mg, 0.34 mmol, 3 equiv) was dissolved in H_2O (2 mL). Sodium carbonate (100 mg, 0.94 mmol, 7.5 equiv) was added and the reaction mixture was allowed to stir for 10 min. 4-Chloro-7-nitrobenz-[2,1,3-*d*]-oxadiazole (**1**) (25 mg, 0.125 mmol, 1 equiv) was then added and allowed to stir at room temperature for approximately 15 min. The resulting brown-yellow precipitate was isolated by vacuum filtration without further purification (35 mg, quant.). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.54 (d, 1H, ^3J = 8.4 Hz), 7.25 (d, 1H, ^3J = 8.4 Hz), 4.27 (q, 2H, ^3J = 6.9 Hz), 2.32 (s, 3H), 1.42 (t, 3H, ^3J = 6.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.2, 154.3, 144.2, 143.9, 134.6, 129.7, 106.2, 64.4, 15.3, 14.5. IR (microscope): ν = 3133, 3090, 2999, 2988, 1933, 1713, 1645, 1623, 1537, 1451, 1379, 1164, 997, 854 cm^{-1}. ES-HRMS calculated for $C_{10}H_{10}N_4O_5Na$: 289.0543, observed: 289.0541. R_f = 0.69 (1:3 EtOAc/hexanes).

*O-(7-Nitrobenzo-[2,1,3-d]-oxadiazol-4-yl)hydroxylamine (**5**, **NBDAO**).* Ethyl *N*-7-nitrobenz-[2,1,3-*d*]-oxadiazol-4-yloxyacetimidate (**4**) (250 mg, 0.94 mmol, 1 equiv) was dissolved in 1:1 TFA/water (25 mL) and allowed to stir at room temperature for 2 h. The TFA was removed in vacuo, and the resulting aqueous solution was concentrated by lyophilization. The product was isolated by flash column chromatography with a dichloromethane mobile phase (94 mg, 55%). ^{1}H NMR (700 MHz, $CDCl_3$): δ 8.56 (d, 1H, ^3J = 8.4 Hz), 7.40 (d, 1H, ^3J = 8.4 Hz), 6.51 (s, 2H). ^{13}C NMR (175 MHz, $CDCl_3$): δ 156.0, 143.9, 143.5, 134.0, 130.1, 105.7; IR (microscope): ν = 3325, 3255, 3167, 3104, 2918, 2851, 1641, 1588, 1537, 1328, 1083, 997, 729 cm^{-1}. ES-HRMS calculated for $C_6H_4N_4O_4Na$: 219.0125, observed: 219.0127. R_f = 0.25 (1:3 EtOAc/hexanes).

*Butan-2-one O-7-nitrobenzo-[2,1,3-d]-oxadiazol-4-yl oxime (**6**).* *O*-(7-Nitrobenz-[2,1,3-*d*]-oxadiazol-4-yl)-hydroxylamine (**5**) (27 mg, 0.14 mmol, 1 equiv) was dissolved

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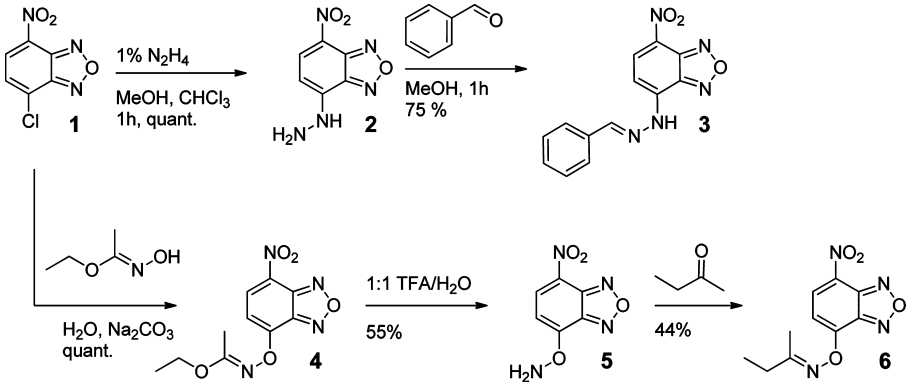
Scheme 1. Synthesis of NBDH (2) and NBDAO (5) Carbonyl Reactive Chromophores and Model Hydrazone and Oxime Ligation Products (3 and 6)



in methylethylketone (MEK) (0.08 mL, 0.83 mmol, 6 equiv). The reaction was allowed to run at room temperature for 2 h. Excess MEK was removed in vacuo and the remaining residue was purified as an inseparable mixture of *E*/*Z* isomers (1.36:1) using flash column chromatography (EtOAc/hexanes) (15 mg, 44%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.56 (d, 1H, ^3J = 9.3 Hz), 7.35 (d, 1H, ^3J = 9.6 Hz), 2.73 (q, 0.90H, ^3J = 7.8 Hz), 2.48 (q, 1.29H, ^3J = 7.5 Hz), 2.25 (s, 1.88H), 2.14 (s, 1.38H), 1.26 (m, 5.23H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.7, 168.8, 153.98, 153.92, 144.0, 143.8, 134.4, 129.8, 106.6, 29.8, 24.0, 19.3, 15.5, 10.4; IR (microscope): ν = 3113, 3092, 2987, 2987, 2919, 2849, 1633, 1533, 1453, 1369, 1327, 887, 854, 733 cm^{-1}. ES-HRMS calculated for $C_{10}H_{11}N_4O_4$: 251.0775, observed: 251.0778. R_f = 0.67 (1:1 EtOAc/hexanes).

Reaction Kinetics. Dye solutions of NBDH (6.88×10^{-5} M) or NBDAO (2.50×10^{-5} M) were prepared in 1 mL aliquots with or without aniline (4.38×10^{-4} M) in buffer (0.1 M sodium acetate, pH 4.7; or 0.1 M HEPES, pH 7.4). An aliquot of MEK (10 μL, 1.11×10^{-4} M) was added and the fluorescence emission of the reaction was monitored for 1500 s (ex. 480 nm; em. 540 nm).

Glycoprotein Labeling. Fetal calf serum fetuin (1 mg/mL, 0.5 mL aliquots) was treated with 1 mL aliquots of neuraminidase from *Clostriduim perfringens* (*C. Welchii*) (0.1 mg/mL) in sodium acetate buffer (0.1 M, pH 5.4) for 0.5 h at 37 °C. Sodium acetate buffer was removed by ultrafiltration (Millipore, Billerica, MA) for 15 min at 1600 g, followed by a water wash (1 mL). Oxidation was performed by treatment with sodium periodate (300 μL, 0.1 M) for 30 min at 4 °C. Excess periodate was removed by ultrafiltration for 15 min at 1600 *g*, followed by a water wash (1 mL). Aliquots of the protein (20 or 10 μL) were then treated with a labeling solution (20 μL) consisting of NBDAO (1.25×10^{-5} M) and aniline (4.38×10^{-4} M) in sodium acetate buffer (0.1 M, pH 4.7) for 0.5−1 h at 4 °C. Labeled proteins were resolved by SDS PAGE (stacking 4%, resolving 12%). The resulting gels were imaged using a FujiFilm Fluorescent Image Analyzer FLA-5000 (Fujifilm medical systems USA Inc., Stamford, CT) with 473 nm excitation and a low band-pass filter. Coomassie staining was used to detect molecular weight standards.

Microplate Assay. HeLa cells were grown in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 to approximately 80% confluency. Cells were trypsinized, and then added to 96 well plates (~2000 cells/well) (tissue culture treated; Corning, Corning, NY). The plate was then incubated for 96 h at 37 °C in a humidified incubator. The growth media was removed and the plates were washed with Dulbecco's phosphate buffered saline (DPBS). Wells were then treated with sodium periodate (80 μL, 1 mM) or DPBS buffer control (80 μL) for 30 min at 4 °C. The solution was removed by inversion, and the plates were washed with DPBS (2 × 100 μL). Control and sample wells were treated with dye for 1 h at 4 °C (NBDH, 2.50×10^{-5} M; NBDAO, 2.5×10^{-5} M; or Bodipy-FL hydrazide, 2.50×10^{-5} M). Dye solutions were prepared in 1 mL aliquots with or without aniline (4.38×10^{-4} M) in DPBS (pH 7.4) or 0.1 M sodium acetate buffer (pH 4.7) as indicated. Fluorescence measurements were taken on a Molecular Devices M2e plate reader (Sunnyvale, CA).

Fluorescence Microscopy. HeLa cells were added to a 6-well plate (~10 000 cells/well) containing treated coverglass (1% (m/v) BSA in TBS), and grown to approximately 60% confluence. The growth media was removed and the wells were washed with DPBS. Sample wells received sodium periodate in DPBS (1 mL, 1 mM) treatment; control wells received DPBS (2 mL); samples were then incubated for 30 min at 4 °C. Cells can optionally be fixed at this point by washing with sodium acetate buffer (0.1 M, pH 4.7; 2 × 2 mL), incubation for 10 min in a 1:1 solution of sodium acetate buffer and methanol, and incubation for 10 min in methanol. Fixing solution is then removed, and the samples are washed with sodium acetate buffer (2 × 2 mL). The double negative control was treated with buffer solution, while control and experimental wells were then treated with dye for 0.5 to 1 h at 4 °C (NBDH, 2.50×10^{-5} M; NBDAO, 2.50×10^{-5} M; or Bodipy-FL hydrazide, 2.50×10^{-5} M). Dye solutions were prepared in 1 mL aliquots with aniline (4.38×10^{-4} M) in sodium acetate buffer (0.1 M, pH 4.7). Each treatment was observed in triplicate. Cells were washed with buffer after staining, and then visualized using a Nikon Eclipse Ti inverted fluorescence microscope with 60× objective (NA 1.49). Images were acquired with a Photometrics QuantEM 512SC camera.

Flow Cytometry. Jurkat cells were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 to approximately 1×10^6 cells/mL. The growth media was removed and the cells were washed with DPBS. Control cell aliquots were incubated in DPBS for 1 h at 37 °C. Sialidase-treated cell aliquots were incubated at 37 °C in the presence of *Clostridium perfringens* neuraminidase (0.4 mg/mL; Sigma-Aldrich, St. Louis, MO). Cells were pelleted by centrifugation (2000 *g* × 4 min) and washed with DPBS. Oxidation-treated aliquots received sodium periodate in

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Table 1. Photophysical Properties of NBDH, NBDAO, Hydrazone, and Oxime Conjugates

compound	solvent	λ max$_{abs}$[a] [nm]	λ max$_{em}$ [nm]	Φ_F (R6G)[b]	ε[c] absorption coefficient [cm^{-1} M^{-1}]	brightness
NBDH (**2**)	aq. pH 7.4	271, 374, 494	na	na	na	na
	aq. pH 5.4	271, 372, 496	552	0.001	14800	10
	aq. pH 4.7	377	na	na	2950	na
	CH_3CN	280, 405, 425	531, 548	0.003	56700	170
	EtOH	334, 470	537	0.280	110500	31547
hydrazone (**3**)	aq. pH 7.4	360, 444	na	na	67900	34
	aq. pH 5.4	356, 440, 495	552	0.006	52100	320
	aq. pH 4.7	379, 506	598	0.004	630	2.5
	CH_3CN	306, 323, 492	555	0.026	98000	2501
	EtOH	307, 327, 493	555	0.038	25600	977
NBDAO (**5**)	aq. pH 4.7	291, 381, 470	552	0.006	8000	46
	CH_3CN	279, 376, 466	541	0.133	14000	1863
	EtOH	275, 376, 472	540	0.054	10200	548
oxime (**6**)	aq. pH 4.7	390, 465	566	0.003	16700	42
	CH_3CN	264, 380, 470	538	0.138	15600	2150
	EtOH	270, 378, 467	545	0.160	7900	1263

[a]Absorbance maxima are listed with the peak used for fluorescence excitation underlined. [b]Fluorescence quantum yield (Φ_F) standard was rhodamine 6G in ethanol measured with excitation at 485 nm. [c]Molar absorption coefficient, ε, measured at 485 nm.

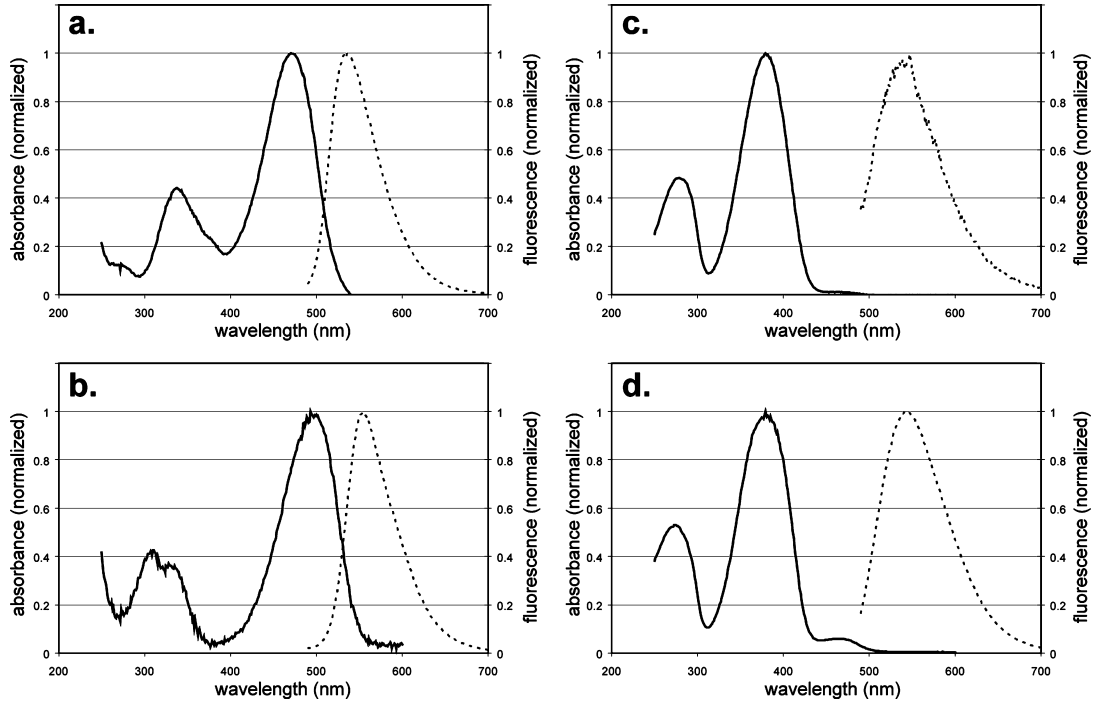

Figure 1. Absorbance and emission spectra in ethanol (excitation 475 nm) of (a) NBDH (**2**), (b) hydrazone (**3**), (c) NBDAO (**5**), and (d) oxime (**6**).

DPBS (1 mL, 1 mM); nonoxidized aliquots received DPBS (1 mL). Samples were then incubated for 30−45 min at 4 °C. Cells were again pelleted by centrifugation (2000 g × 4 min) and washed three times with DPBS. Unlabeled control aliquots were treated with buffer solution, while a set of nonoxidized and oxidized aliquots were treated with dye (100 μL) for 1−1.5 h at 4 °C (NBDAO, 2.50 × 10^{-5} M; Bodipy-FL hydrazide 2.50 × 10^{-5} M (Invitrogen, Carlsbad, CA); NBDH 2.50 × 10^{-5} M). Dye solutions were prepared in 1 mL aliquots with aniline (4.38 × 10^{-4} M) in sodium acetate buffer (0.1 M, pH 4.7). Cells were pelleted by centrifugation (2000 g × 4 min) and washed three times with DPBS before flow cytometry. Flow cytometry was performed on an Accuri C6 cytometer (Ann Arbor, MI) with 488 nm excitation and 533 nm detection. Each treatment was observed in triplicate runs with 10 000 events.

RESULTS AND DISCUSSION

Fluorophore Synthesis. NBDH (**2**) was synthesized from commercially available 7-nitro-4-chlorobenz-2,1,3-oxadiazole (NBDCl) (**1**) in chloroform by reaction with 1% hydrazine as described by Gubitz et al.[21] Alternatively, the NBDH·NH_2NH_2 salt may be prepared from 7-nitro-4-fluorobenz-2,1,3-oxadiazole using the method of Uzu and colleagues.[22] A hydrazone derivative, **3**, was then generated in good yield by conjugation with benzaldehyde in methanol at room temperature (Scheme 1). NBDAO (**5**) was synthesized by an initial S_NAr of NBDCl (**1**) with ethyl *N*-hydroxyaceti-

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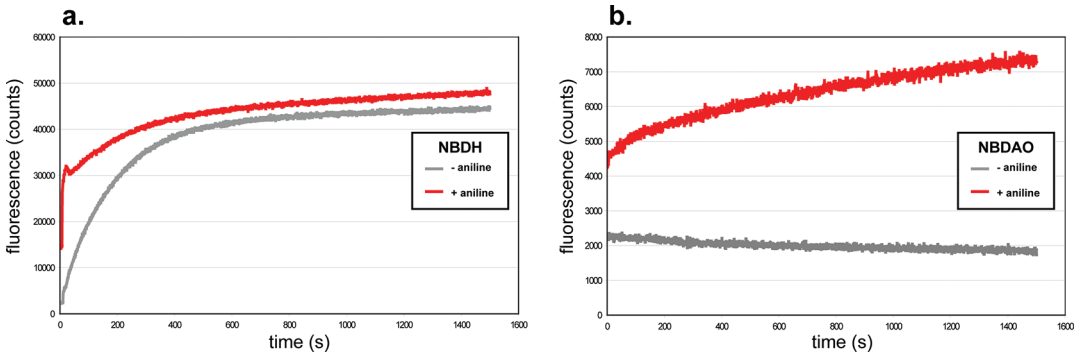

Figure 2. Reaction kinetics of (a) NBDH (**2**) and (b) NBDAO (**5**) with MEK at pH 4.7. The rates of both reactions are enhanced by the presence of aniline in the reaction mixture. The excitation wavelength was 485 nm, and emission was monitored at 540 nm for 1500 s.

midate under basic conditions, to generate the ethyl acetohydroxamate intermediate (**4**).[27] The acetimidate functions as a protected amino-oxy group, and reported deprotection protocols for aryl substrates involve the use of strong acids, such as perchloric acid and hydrofluoric acid.[27,28] We found that the amino-oxy functional group could be unveiled in moderate yield by hydrolysis with aqueous trifluoroacetic acid. Care must be taken at the deprotection step to avoid alkaline workup conditions. In our hands, basic solutions lead to decomposition of the amino-oxy compound, presumably to the corresponding phenolate.[29] Additionally, we found that care should be taken to remove any trace amounts of ketone or aldehyde contaminants (such as acetone) to avoid the rapid formation of the oxime. A representative oxime derivative (**6**) was generated by reaction with neat MEK at room temperature.

Fluorophore Characterization. The NBD dyes (**2** and **5**) and corresponding hydrazone (**3**) and oxime (**6**) conjugates were characterized to determine their absorbance spectra, fluorescence emission, molar absorption coefficients, and fluorescence quantum yields (Φ_F) (Table 1). Quantum yield was determined using a Rhodamine 6G standard in ethanol. Similar to other NBD derivatives, the photophysical properties of these compounds were found to be environmentally sensitive.[3,30] Consistent with previous reports, we confirmed conversion of **2** to **3** was fluorogenic with an 8.5-fold increase in Φ_F and a 15-fold increase in brightness in acetonitrile. Additionally, we observed that NBDH derivatization was fluorogenic in water, particularly at low pH. For example, at pH 5.4 the hydrazone (**3**) has a 9.2-fold increase in Φ_F and 32-fold increase in brightness relative to the starting hydrazine (**2**). However, NBDH and its hydrazone derivative shows less fluorescence in water relative to ethanol and acetonitrile solutions.

The derivatization of NBDAO (**5**) to an oxime (**6**) leads to an increase in the absorption coefficient of the dye; however, there is only a slight shift in the emission wavelength (Figure 1). Both NBDAO and its oxime conjugate (**6**) show a remarkably increased Stokes shift relative to NBDH, which should result in improved sensitivity. Both NBDAO and the oxime derivative have reduced environmental sensitivity when compared to compounds **2** and **3**, respectively, though both dyes (**5** and **6**) exhibited a smaller range of brightness among water, ethanol, and acetonitrile as compared to the hydrazine and hydrazone derivatives (**2** and **3**). The conversion of NBDAO to the oxime appeared to be fluorogenic in certain solvent environments (ethanol), but was relatively static in others. However, we note that several of these values are close to the lower limit of detection, and therefore may not be accurate enough to make this determination. We observed that NBDAO (**5**) underwent degradation to the corresponding phenolate in the presence of alkaline water,[29] which could complicate its use in assays which require high pH. In labeling experiments, we found that this degradation product could be easily removed by performing a wash step after dye treatment.

Reaction Kinetics. The mechanism of oxime or hydrazone formation by NBDAO and NBDH is expected to be accelerated in the presence of aniline, which acts as a nucleophilic catalyst in acidic media.[31,32] Differences in the fluorescent properties of these dyes and their conjugates could be used as a method to observe the rate of the reaction directly. To confirm the role of aniline in these reactions, we observed the kinetics of ligation for NBDH (**2**) or NBDAO (**5**) with MEK in the presence or absence of aniline using a time-based fluorescence assay (Figure 2). As expected, we found that aniline did increase the rate of reaction for both dyes at pH 4.7. In the case of NBDH, the rate acceleration was limited to the early phase of the reaction (<400 s). In the case of the NBDAO dye, the rate acceleration was more substantial, with limited formation of the oxime in the absence of aniline. These findings suggest that NBDAO may be a more selective labeling agent than NBDH when used with aniline catalysis. At pH 7.4, we found that aniline did not increase the rate of reaction for either dye.

Glycoprotein Labeling. To explore the utility of NBDAO for the detection of glycoconjugates, we tested the ability of the dyes to specifically label a known glycoprotein in SDS PAGE. Fetal calf serum fetuin is a commonly used, commercially available sialoprotein.[33,34] Detection of fetuin was performed by treatment with periodate followed by labeling with NBDAO in the presence of aniline (Figure 3). Labeling of a band (~64 kDa) corresponding to the protein was visible by fluorescence imaging. To demonstrate the specificity of this methodology for sialic acid, controls were performed using fetuin which was pretreated with neuraminidase (NEU). As expected, the dye only labeled the glycoprotein when sialic acid was present, and only after periodate treatment—confirming that the labeling chemistry was specific for sialylated protein. We observed only minor residual staining in control samples; and factors that may contribute to background staining could include nonspecific binding[35] and naturally occurring protein oxidation.[36]

Live Cell Labeling. We tested the ability of NBDH (**2**) and NBDAO (**5**) to detect glycans on live cells using PAL. We tested HeLa cells cultured in a microplate which were exposed to periodate, washed, and then treated with one of the

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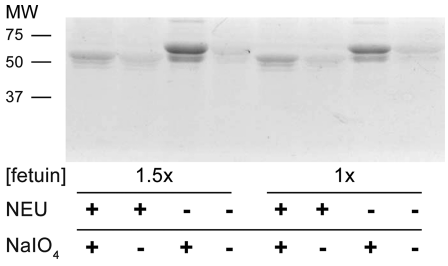

Figure 3. Fluorescent detection of glycoproteins in gel. Labeling of fetal calf serum fetuin by PAL was performed with NBDAO (**5**). Control lanes used protein which was treated with neuraminidase (NEU), and protein not treated with periodate as indicated. Molecular weights are given in kDa.

carbonyl-reactive dyes. Control wells lacked the periodate oxidation step, but were otherwise identical. After labeling, the fluorescence of treated cells was compared to that of the control wells. We found that the mean fluorescence after PAL with both dyes resulted in specific labeling when performed at acidic pH. The NBDH dye gave an increase of 1.3-fold at pH 4.7, and the Bodipy FL hydrazide 3.0-fold, while NBDAO gave a larger increase of 4.3-fold (Figure 4). Labeling by hydrazine

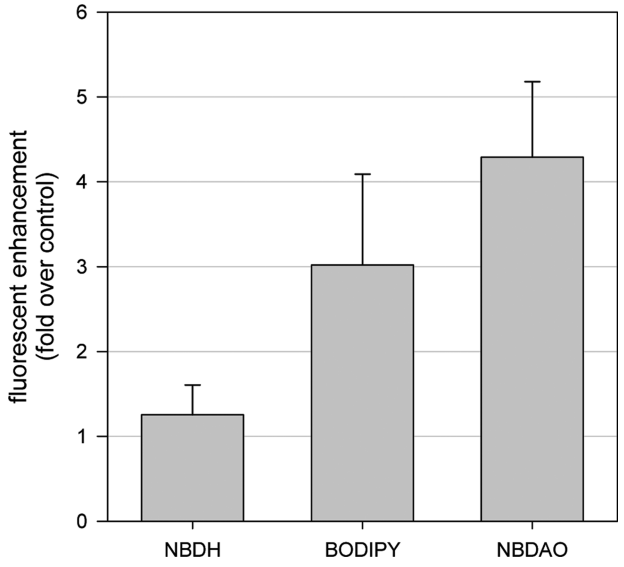

Figure 4. Fluorescence enhancement, expressed as fold enhancement over negative control of 96 well plates after PAL treatment with NBDH (**2**), Bodipy FL hydrazide, or NBDAO (**5**) at pH 4.7. Each point represents at least 8 wells of a representative experiment; error is shown as the standard deviation. All dyes were used at 2.50×10^{-5} M, excitation was at 485 nm, and emission was observed at 538 nm.

and aminooxy dyes were similar to control when the labeling was performed at pH 7.4 (data not shown).

Fluorescence Microscopy. In order to explore the utility of these dyes for imaging applications, we tested their ability to label glycoconjugates in fixed and live cells. HeLa cells were treated with periodate, followed by a solution of dye containing aniline. Both the oxidation and labeling steps were performed at low temperature based on the protocol of Zeng et al.[8] Cells were then imaged using fluorescence microscopy and DIC (Figure 5). To determine the background staining, we included controls that were treated with no periodate and no dye (--), no periodate and dye (-+), and periodate and dye (++). We employed conditions for staining of live and fixed cells for all

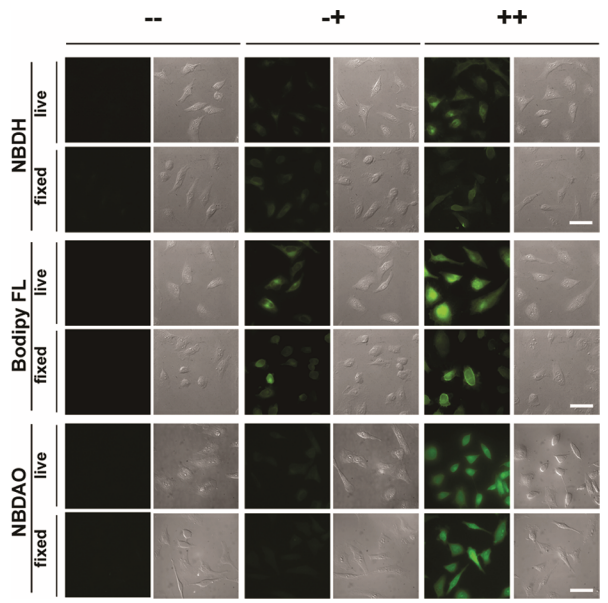

Figure 5. Fluorescence and brightfield images of HeLa cells labeled with NBDH, Bodipy, and NBDAO at pH 4.7. For each dye, cells were observed after one of the following treatments: no periodate and no dye (--); no periodate and dye (-+); and periodate with dye (++). Fluorescence images (488 nm ex/515 nm em) are shown on the left, and DIC images of the same field are shown on the right. Scale bars represent 50 μm. Dye labeling is shown for live and fixed cells (see Materials and Methods).

treatments. We evaluated labeling conditions at pH 4.7, 5.4, 6.1, and 7.4. In our hands, pH 4.7 gave the best results for dye labeling. Fluorescence images of NBDH-treated cells showed staining which was dependent on the use of both periodate and dye. The Bodipy FL hydrazide dye showed higher background than NBDH in samples not treated with periodate (-+), and provided intense labeling of cells after periodate treatement (++). The NBDAO dye provided both low background in samples not treated with periodate (-+), and bright staining of cells treated with periodate (++). Additional controls performed with compound **6** typically showed only background staining (data not shown). Based on these results, we concluded that NBDAO was more suited to fluorescence imaging, and presents a significant improvement over NBDH and Bodipy FL hydrazide. We also note that PAL labeling protocols which exploit small-molecule fluorophores should require fewer steps than affinity-based reagents.[8] The data in Figure 5 suggest that staining by all three dyes in live cells is not restricted to the cell membrane. These results may indicate that periodate oxidation and the dyes used here can penetrate to intracellular compartments. Previous reports have observed labeling of intracellular compartments by NBD-based dyes[37,38] and protein conjugates.[39]

Flow Cytometry. To determine the applicability of small molecule dyes for quantitation of cellular sialic acid content, we examined labeling of Jurkat cells by flow cytometry (Figure 6). Cells were treated with periodate, followed by a solution of dye containing aniline. As with the experiments described above, the oxidation and labeling step was performed at low temperature. To compare the background staining, we included samples that were treated without periodate or dye (--), periodate and no dye (+-), no periodate and dye (-+), and both periodate and dye (++). We tested NBDAO in the assay, and compared it to NBDH and Bodipy FL hydrazide. The NBDAO

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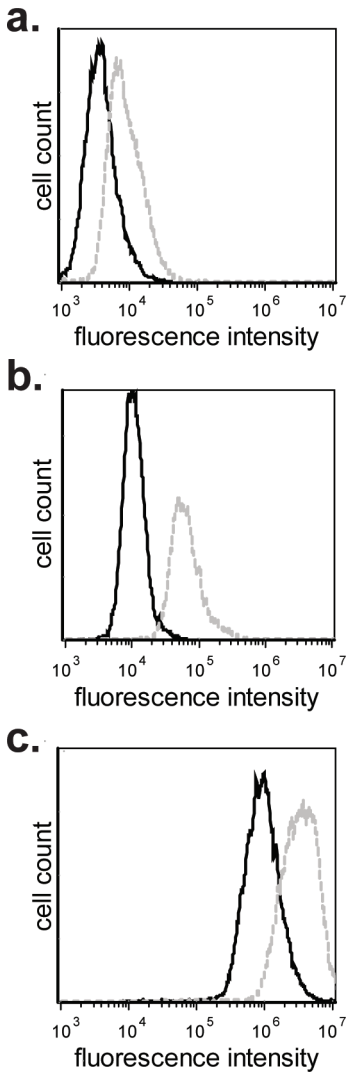

Figure 6. Representative flow cytometry histograms of the relative fluorescence of Jurkat cells treated with a solution containing only the fluorophore (-+, solid black line), or the fluorophore after PAL-treatment of the cells (++, dashed gray line). Dyes shown are (a) NBDH, (b) NBDAO, and (c) Bodipy FL hydrazide.

and NBDH-treated cells showed minor nonspecific background staining (-+) which increased the mean fluorescence intensity (MFI) of cells by approximately 4.5- and 3.0-fold over untreated controls, respectively (see Supporting Information). In contrast, the Bodipy FL hydrazide dye gave a larger increase in background staining (approximately 500-fold). This result likely indicates that the Bodipy fluorophore is more hydrophobic, leading to integration of the dye into the cell membrane. To determine the fidelity of sialic acid detection for each dye, we compared cells which were dye treated (-+) to those which were PAL and fluorophore treated (++). In the case of the NBDAO dye, cells showed an almost 7-fold increase in fluorescence after staining. This was the largest increase we observed, as both commercial dyes (NBDH and Bodipy FL hydrazide) gave only 2−3-fold increases over background. Based on these data we concluded that NBDAO gave the best combination of low background and high signal-to-noise for sialic acid detection. Jurkat cells that were treated with bacterial neuraminidase for one hour, followed by PAL and NBDAO, showed reduced MFI (78 ± 8%) compared to untreated cells, suggesting that this method may be useful to monitor enzymatic changes to sialoproteins in vitro.

Detection strategies for sialic acid on cells and glycoproteins currently include the use of lectins, metabolic labels, or bioconjugation of dyes. In this study, we have examined the utility of small molecule dyes that contain either hydrazine or amino-oxy functional groups to label sialic acid based on the PAL conditions of Zeng et al.[8] In previous studies, labeling of sialic acid by PAL employed a biotin−streptavidin complex; however, here we have explored the utility of small molecule dyes for direct detection of sialoforms using fluorescence. We tested two commercially available dyes, and compared them to the NBDAO dye reported here. Our synthesis of NBDAO (**5**) was carried out in two steps: an S_NAr to form the ethyl acetohydroxamate intermediate (**4**), followed by acidic deprotection.[27] The synthesis of NBDH (**2**) was performed using the method established by Gubitz et al.,[21] and this dye is also commercially available. The kinetics of the reactions between NBDH or NBDAO with MEK were evaluated using a time-based fluorescence assay. Increased reaction rates were observed for both NBDH and NBDAO in the presence of aniline for both reagents at low pH. Comparison of the spectroscopic properties of the dyes and their conjugates initially suggested that the fluorogenic NBDH would have improved signal-to-noise over NBDAO. However, we found that NBDAO was superior to NBDH in the plate assay and microscopy experiments. Importantly, both the NBDH and Bodipy FL hydrazide dyes had poor sensitivity when compared to NBDAO in flow cytometry experiments. These observations may be due to the increased Stokes shift of compound **5** and its oxime conjugates, or the increased stability of the oxime linkage formed between NBDAO and the aldehyde.[40]

■ SUMMARY

We report the synthesis and application of the carbonyl reactive fluorophore, NBDAO, for the selective labeling of sialic acid using PAL. We compared the labeling of NBDAO to two commercially available carbonyl reactive dyes, NBDH and Bodipy FL hydrazide. We found that NBDH and NBDAO dyes can be used to follow the kinetics of hydrazone or oxime formation when combined with an aldehyde substrate. Importantly, when used in conjunction with aniline catalysis these dyes are ideal for labeling of glycoproteins, detection of the sialic acid content of whole cells, and as small-molecule fluorophore stains of glycans on live cells.[41] We demonstrated that these dyes can be used as a simple procedure for sialic acid labeling when combined with periodate oxidation as a method for modifying sialic acids in cells.[8,41] As well, we have shown these dyes can be used as a method for quantitative detection of sialic acid content by flow cytometry. The NBDAO dye showed significant staining of sialic acid over background (6.7-fold), which was superior to the commercially available fluorophores NBDH (2.0-fold) and Bodipy FL hydrazide (2.9-fold). We expect this methodology will prove useful for a variety of applications in glycobiology, particularly in the study of changes to sialic acid content and subcellular location in live cells. These dyes may also prove useful in other carbonyl ligation strategies.[42−45]

■ ASSOCIATED CONTENT

Supporting Information

Absorption and emission spectra of compounds **2**, **3**, **5**, and **6** in water, acetonitrile, and ethanol and flow cytometry histograms.

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Notes

The authors declare no competing financial interest.

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