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Analysis of bisulfite via a nitro derivative of Cyanine-3 (NCy3) in the microfluidic channel

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Abstract

NCy3, a derivative of Cyanine 3 with a nitro substituent, showed a high reactivity to bisulfite in aqueous media, instantly leading to ratiometric change of absorption spectra and significant fluorescence quenching. Applied in the microfluidic channel, **NCy3** functionalize as a sensitive approach for quantitative detection of bisulfite, particularly for samples with a small volume.

Keywords

Cyanine dye; nitration; bisulfite sensor; microfluidic channel

1. Introduction

preservative and antimicrobial reagent against oxidation and spoilage during food processing and wine fermentation [1], and is also added to dried fruit and potato products for long term storage [2]. Based on the report from the World Health Organization (WHO), the daily intake limit of sulfite is 0.7mg/Kg [3]. High doses of sulfide shows a highly biological toxicity, causing disease and allergic reaction [4]. Recent studies reveal that bisulfite is correlated to lung cancer, cardiovascular disease and many neurological disorders [5]. However, bisulfite also exists naturally in the human body. In living cells, sulfite and bisulfite can be produced in aqueous media at physiological condition by endogenous sulfur dioxide (SO₂), which is generated from the oxidation of H₂S or sulfur containing amino acids by reactive oxygen species (ROS) [6]. Thus, the cellular level of bisulfite is able to collaterally reflect the local reducing environment in living cells [7]. Therefore, developing an efficient approach for rapid detection of sulfite is highly desired [8].

In past years, the fluorescence chemosensor has attracted a substantial amount of attention as a simple tool used to detect small molecules *in vitro* and *in vivo* due to high selectivity, sensitivity and real-time measurement [9]. Copious fluorescence approaches have been developed using different photophysical mechanisms (e.g., photoinduced electron transfer (PET), internal charge transfer (ICT), and (föster resonance energy transfer (FRET)) for

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quantitative measurement of various bio-important molecules [10]. However, the sensitivity and selectivity are always a challenge for fluorescence sensing approaches, particularly in biosamples [11].

Microfluidic devices offer scientists many benefits compared to larger scale systems. One benefit of using microchannels is the small volume needed to analyze a sample. Depending on the size of the channel and the number of channels, only femtoliters of the solution are required, which is cost-effective and beneficial for experiments with limited samples [12]. Additionally, microfluidics lends well to multiplexing, so a scientist can run multiple tests on a chip. Finally, microfluidics has a faster reaction time, portability, and enhanced sensitivity compared to large-scale systems [13]. Many systems already use microfluidics such as sequencing, detection of metabolites, physical DNA mapping, 3D cell culture, and optofluidics due to the aforementioned benefits [12–15].

In our group, we recently developed a reaction-based fluorescence sensor based on Cyanine 3 dye, which was able to rapidly react with HSO_3^- based on a nucleophilic addition mechanism. In the presence of HSO_3^- , **NCy3** showed a ratiometric change of absorption spectra and a strong fluorescence quenching within 3 min at 25 °C, indicating a high sensitivity and selectivity. **NCy3** was also applied to a microfluidic device to measure the fluorescence intensity of samples with a small volume, in which a similar result was obtained, indicating **NCy3** could be used as an approach for quantitative detection of sulfite with a high affinity and selectivity in the aqueous media.

2. Methods

2.1 Apparatus

All reagents used for synthesis and measurements were purchased from Sigma-Aldrich (MO, USA), Fisher Scientific (USA), TCI (USA), Alfa Aesar (USA) and Acros Organics (USA) in analytical grade and were used as received, unless otherwise stated. Absorbance spectra were collected by Cary Series Uv-vis Spectrophotometer (Agilent Technologies). Fluorescence measurements were all performed by using a FluoroMax-4 Spectrofluorometer (Horiba Jobin Yvon, USA). All of fluorescence spectra were recorded in a 1 cm quartz cuvette. The excitation and emission slits were set at 2 nm. ^1H and ^{13}C NMR spectra were recorded on (^1H 300MHz, ^{13}C 75MHz) Bruker 300 Ultra-Shield spectrometer at room temperature. The HRMS data was collected in the Nebraska Center of Mass Spectrometry at University of Nebraska-Lincoln by using GCT Mass Spectrometer (Water, USA).

2.2 Synthesis

2,3,3-trimethyl-5-nitro-3H-indole (**1**). 2,3,3-trimethylindole (3.18 g, 0.02 mol) was added into concentrated H_2SO_4 (75 mL) containing HNO_3 (1.70 g, 0.02 mol) within 1 hr and was incubated for 0.5 hr at 0 °C [16]. After reaction, the mixture was poured into ice (1000 mL) to collect the precipitate by suction filtration. The solid was recrystallized in CH_2Cl_2 to yield an orange crystal as the product (3.5 g, 91%). ^1H NMR (300 MHz, CDCl_3) δ : 1.4 (s,

6H), 2.4 (s, 3H), 7.6 (d, $J=8.3$ Hz, 1H), 8.2 (s, 1H), 8.3 (d, $J=8.2$ Hz, 1H). ^{13}C NMR (75 MHz, CDCl_3) δ : 16.1, 22.9, 54.6, 117.3, 120.3, 124.8, 145.8, 147.0, 159.3, 194.3.

1,2,3,3-tetramethyl-5-nitro-3H-indol-1-ium (**2**). A mixture of **1** (0.40 g, 0.0020 mol) and CH_3I (0.33g, 0.0024 mol) were heated to 90 °C in CCl_4 (4 mL) for 15 hours in a sealed reaction tube (50 mL). After cooling to room temperature, the reaction mixture was filtrated to yield a brown yellow solid as the pure product without any further purification (0.41 g, 95%). ^1H -NMR (400 MHz, CDCl_3) δ : 1.4 (s, 6H), 3.2 (s, 3H), 4.2 (d, $J=10.5$ Hz, 2H), 6.5 (d, $J=6.7$ Hz, 1H), 8.0 (s, 1H), 8.2 (d, $J=8.2$ Hz, 1H). ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ : 29.4, 29.6, 43.5, 80.2, 105.5, 118.5, 126.7, 138.5, 139.6, 152.1, 161.3.

1,3,3-trimethyl-5-nitro-2-((E)-3-((E)-1,3,3-trimethyl-5-nitroindolin-2-ylidene)prop-1-en-1-yl)-5,6,7,7a-tetrahydro-3H-indol-1-ium (**NCy3**). Compound **2** (0.218 g, 0.001 mol) and triethyl orthoformate (0.593 g, 0.004 mol) were heated in acetic acid/acetic anhydride (1:1, 5mL) at 100 °C for 12 hrs. After cooling to room temperature, the reaction mixture was purified by column chromatography (silica, 220–400 mesh). Ethyl acetate and methanol (4:1) were used as the elution solvents. A dard red solid (**NCy3**) was obtained as the product (0.170 g, 52%). ^1H -NMR (400 MHz, $\text{DMSO}-D_6$) δ : 1.8 (s, 12H), 3.7 (s, 6H), 6.7 (d, $J=13.5$ Hz, 2H), 7.7 (d, $J=8.4$ Hz, 2H), 7.7 (t, $J=5.7$ Hz, 2H), 8.3–8.4 (m, 3H), 8.6 (s, 2H). ^{13}C -NMR (75 MHz, $\text{DMSO}-D_6$) δ : 27.4, 32.7, 49.6, 106.2, 112.6, 118.8, 126.1, 142.4, 145.0, 148.3, 152.2, 177.3. TOF EI^+ : M^+ m/z 447.2027 (calcd.), 446.5980(found).

2.3 Microfluidic Methods

Master and Replica Fabrication—SU8 microchannel masters were fabricated by photolithography by spincoating SU8 2005 (5.0 μm , MicroChem) onto a silicon wafer and exposing it with UV-light. Replicas of the masters were created using soft lithography. PDMS (Sylgard 184) was mixed (10:1 ratio of pre-polymer to Platinum catalyst) for 15 minutes with a hand held mixer (Kitchen Aid 9 speed mixer), poured onto the silicon master, and placed in a 65°C oven overnight. The PDMS replica was removed from the master and then made hydrophilic with oxygen plasma treatment (Diener Zepto System, 36s O_2 plasma, 0.50 mbar pressure, 15% power) and stored in distilled water until ready to use.

Imaging of Dye in Microchannels—The PDMS device was mounted onto coverslips and attached to a holder with candle wax. Dye (1 μL , 1×10^{-3} M) and 3 μL distilled water or 3 μL HSO_3^- (1×10^{-3} M) was mixed and loaded into microchannels via capillary action. The dynamics of the dye in the presence of water or HSO_3^- was imaged using a CoolSNAP camera and coupled to a Nikon Eclipse TE2000-S microscope. A Brightline 4040B filter set was used with X-Cite 120 Fluorescence Illumination light source to excite the dye and was captured with the NIS Elements D capture software. ImageJ was used to analyze images to compile the 2D montage and determined the fluorescence intensity for each image.⁵

3. Results and discussion

The absorption and emission spectra of **NCy3** were collected in the different media, in which the maximum absorption and emission were observed in the range of 564–578 nm and 581–601 nm respectively (Table 1). **NCy3** showed the highest quantum yield (0.190) in

DMSO and the lowest quantum yield (0.108) in ethyl acetate. Based on the Consideration of solubility and photophysical properties, we chose a mixture of DMSO and H₂O (5:5, v/v) solution containing 20 mM phosphate buffer at pH 7.4 for **NCy3** to detect HSO₃⁻.

As a nucleophile, HSO₃⁻ may react with **NCy3** via a nucleophilic addition reaction, which interrupts the conjugation of **NCy3** and consequently change the photophysical properties. We first investigated the absorption and emission spectra of **NCy3** in the presence of HSO₃⁻ in the DMSO/buffer solution. With addition of HSO₃⁻ to **NCy3**, the absorption spectra rapidly showed a ratiometric signal, a dramatic decrease at 574 nm and an increase at 455 nm, indicating a significant structure change of **NCy3**.

In the absorption spectra, a remarkable blue-shift (119 nm) was detected due to the interruption of π -conjugation. The instant color change, red to light yellow, was also observed by the naked eye. The maximum spectrum change was achieved when the concentration of HSO₃⁻ reached 30 μ M, suggesting the accomplishment of the reaction. In the emission spectra, HSO₃⁻ led to a gradual fluorescence quench at 597 nm, and the maximum quench (92%) was observed with the addition of 30 μ M HSO₃⁻, which was consistent with the change of absorption spectra. The detection limit (3 σ /k) was calculated to be 0.086 nM based fluorescence titration at 597 nm [18].

For the reaction-based sensor, the response time is a critical factor that affects the sensing process. The chemical reaction-based signal significantly improves the sensor selectivity, but limits applications if the reaction rate is too slow. Therefore, the time-dependent spectrum change was investigated after incubation with different amount of HSO₃⁻ (5 μ M, 10 μ M, 20 μ M, and 30 μ M) to **NCy3** at 25 °C in the DMSO/buffer media. A change of absorption at 574 nm and 455 nm were measured as well as the emission at 597 nm. A nucleophilic reaction triggered a spectrum change instantly. After incubating HSO₃⁻ (5 μ M) with **NCy3** at 25°C, both absorption and emission spectra gradually changed over the course of 25 min. However, a high concentration of HSO₃⁻ (30 μ M) led to a rapid spectra change and the maximum change was observed within 5 min, which indicated a high reaction rate based on our data for our reaction-based sensor.

High affinity for a target molecule is another important factor to evaluate a sensor, and is one of the main challenges for sensor designs. To examine the selectivity of **NCy3** towards different molecules, various analytes, including biothiols, reducing reagents, and sulfur-containing ions (30 μ M) were incubated with **NCy3** (1.0 $\times$ 10⁻⁵M) for 5 min in a DMSO/Buffer media at 25 °C. Afterwards, the absorption and emission spectra were measured. As shown in the Figure 3, all of interfering species (HSO₄⁻, S₂O₃²⁻, C₂O₄²⁻, cysteine, glutathione, ascorbic acid, and H₂S) showed just a slight change (i.e., around 10%) in both absorption and the emission spectra. However, during incubation with HSO₃⁻, **NCy3** rapidly displayed a ratiometric signal at 455 nm/575 nm in the absorption spectra as well as a significant quenching at 597 nm in the fluorescence spectra, demonstrating that **NCy3** is very sensitive to HSO₃⁻, allowing **NCy3** to be used as a approach with multiple spectroscopic signal channels for rapid detection of HSO₃⁻. The interruption of the conjugation of **NCy3** trigged by HSO₃⁻ led to an obvious color change from light pink to

yellow, which can be easily observed by naked eye, making the detection process very convenient.

As a nucleophile, H₂S did not show a high reaction rate with **NCy3** at low concentration. However, with increasing concentration and incubation time, **NCy3** gave a significant response to H₂S. As shown in the Figure 4, both the absorption and emission spectra steadily changed within 45 min at 25 °C when the H₂S level was less than 100 μM, indicating a slow reaction process. When increasing the level of H₂S to 150 μM, the spectra displayed a relatively quick change, and plateaued after a 30-min incubation with **NCy3**, suggesting a complete reaction. Based on the previous data, **NCy3** showed a potential to detect H₂S with a high concentration. Besides H₂S, other species (HSO₄⁻, S₂O₃⁻, C₂O₄⁻, Cys, GSH, and ascorbic acid) did not cause significant spectroscopic change even at high concentration and/or longer incubation time with **NCy3**.

According to previous work, the nitro group on the aromatic ring (1,8-naphthalimide) can be reduced by H₂S and consequently lead to photophysical property change, which has been intensively used as a sensing strategy for the detection of H₂S [19]. Thus, **NCy3** was incubated with different reducing reagents (H₂S, GSH, ascorbic acid, and HSO₃⁻) to investigate the reduction of nitro group. However, no reduction reaction was observed in the presence of any these reducing reagents. Only HSO₃⁻ and H₂S led to a nucleophilic addition reaction, and HSO₃⁻ gave a higher reaction rate (Figure 5A). Moreover, the pH effect in the range of pH 3–11 was examined for **NCy3** in different DMSO/buffer media. At low pH range (3.0–8.0), **NCy3** showed a strong fluorescence emission at 597 nm. With an increase of pH, the fluorescence was quenched rapidly. Although the fluorescence intensity of **NCy3** was significantly influenced by pH value, no notable change was observed in the physiological pH range (pH 7.0–8.0) (Figure 5B).

To understand the nucleophilic addition reaction between **NCy3** and nucleophiles (e.g., HSO₃⁻ and H₂S), the Cyanine 3 dye (**Cy3**) without nitro groups was synthesized for a comparison. Compared to **NCy3**, **Cy3** showed a shorter absorption and emission at 546 nm and 565 nm respectively in the DMSO/pH 7.4 buffer (1:1) at 25 °C. Since **NCy3** showed a significant spectroscopic change in the presence of HSO₃⁻ and H₂S in high concentration, the same condition was applied to **Cy3**. However, neither absorption nor emission displayed a notable change. Moreover, high concentration (150 μM) of analytes (HSO₄⁻, S₂O₃²⁻, C₂O₄²⁻, Cys, GSH, ascorbic acid, H₂S, and HSO₃⁻) along with longer incubation time (30 min) with **Cy3** could not produce any significant spectroscopic change, revealing that no nucleophilic addition reaction occurred for **Cy3** (Figure 6). The nucleophilic addition reaction is well established for α,β-unsaturated compounds. As an electron-withdrawing group, the nitro group on **NCy3** played a critical role in significantly boosting the addition reaction. Moreover, the HRMS data indicated that a reduction reaction followed the nucleophilic addition reaction in the present of HSO₃⁻, which thoroughly explained the significant spectra change for absorption and emission.

Because **NCy3** was ultra-sensitive, ratiometric, instant, and multiple channel signal sensing abilities, **NCy3** was applied to develop a microfluidic device for analysis of HSO₃⁻ in very small volume samples. A PDMS device was mounted onto a coverslip and attached to a 3D

printed PLA holder with candle wax [20]. Then, dye (1 μL , 1.0×10^{-3} M) and 3 μL distilled water or 3 μL HSO_3^- (1.0×10^{-3} M) was mixed and loaded into microchannels via capillary action. The dynamics of the dye in the presence of water or HSO_3^- was imaged using a CoolSNAP camera and coupled to a Nikon Eclipse TE2000-S microscope. As shown in Figure 7, with the incubation of HSO_3^- with **NCy3** in the microchannel, a fluorescence quenching was observed within 10 min, indicating that **NCy3** was able to produce a reliable signal in a microfluidic device for detection of HSO_3^- . As a negative control, addition of H_2O did not lead to a significant change in fluorescence.

4. Conclusions

In summary, a fluorescent probe (**NCy3**) was synthesized for detection of HSO_3^- via a nucleophilic addition reaction in aqueous media. **NCy3** demonstrated a rapid ratiometric sensing for HSO_3^- with high selectivity and sensitivity. This probe provided multiple signals, including absorption spectra, emission spectra, and colorimetric signal, to quantitatively measure HSO_3^- . In addition, **NCy3** was successfully employed in developing a microfluidic device to measure trace amounts of HSO_3^- in samples with a small volume, which could be very useful for biosamples or environmental monitoring.

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Figure 1:

The spectra change of NCy3 ($1.0 \times 10^{-5} \text{ M}$) with addition of HSO_3^- (0 – 65 μM) in DMSO/pH 7.4 buffer (1:1) at 25 °C. (A) The absorption decreased at 574 nm and increased at 455 nm; (B) The fluorescence emission at 594 nm was gradually quenched ($\lambda_{\text{ex}} = 574 \text{ nm}$).

Figure 2:

The time-dependent spectrum change: (A) absorption at 574 nm, (B) absorption at 455 nm, and (C) emission at 594 nm of **NCy3** (1.0×10^{-5} M) with different amount of HSO_3^- (5 μM, 10 μM, 20 μM, and 30 μM) in DMSO/pH 7.4 buffer (1:1) at 25 °C.

Figure 3:

The absorption responses (A) and fluorescence emission responses (B) of NCy3 ($1.0 \times 10^{-5}\text{M}$) toward HSO_4^- , $\text{S}_2\text{O}_3^{2-}$, $\text{C}_2\text{O}_4^{2-}$, Cys, GSH, ascorbic acid, H_2S and HSO_3^- ($30\text{ }\mu\text{M}$) after Incubation for 5 min in DMSO/pH 7.4 buffer (1:1) at $25\text{ }^\circ\text{C}$ ($\lambda_{\text{ex}} = 574\text{ nm}$).

Figure 4:

The spectra change of NCy3 ($1.0 \times 10^{-5} \text{M}$) incubating with different amount of H₂S (10 μM, 50 μM, 100 μM, and 150 μM) in DMSO/pH 7.4 buffer (1:1) at 25 °C in 45 min: (A) the absorption change at 574 nm, (B) the absorption change at 455 nm, and (C) the fluorescence change at 594 nm ($\lambda_{\text{ex}}=574 \text{ nm}$).

Figure 5:
 (A) NCy3 reacted with H₂S and HSO₃⁻ based on a nucleophilic addition mechanism. (B)
 The fluorescence intensity change of NCy3 at 594 nm in different media with a pH 3.0–11.0
 at 25 °C in (λ_{ex}=574 nm).

Figure 6:

After incubation **Cy3** ($1.0 \times 10^{-5} \text{M}$) with HSO_4^- , $\text{S}_2\text{O}_3^{2-}$, $\text{C}_2\text{O}_4^{2-}$, Cys, GSH, ascorbic acid, H_2S and HSO_3^- ($150 \mu\text{M}$) for 30 min in DMSO/pH 7.4 buffer (1:1) at 25°C ($\lambda_{\text{ex}} = 574 \text{ nm}$), (A) no nucleophilic addition reaction observed; (B) absorption spectra and (C) fluorescence spectra did not show a significant change

Figure 7:

(A) Intensity of the dye with water (red circles) or HSO_3^- (black squares) in $55 \mu\text{m}$ wide $\times$ $5.0 \mu\text{m}$ high microchannels was normalized and plotted against time. (B) and (C) Dynamics of the dye in the presence of water (B) or HSO_3^- (C) was imaged in microchannels for 10 minutes and compiled into a movie. A 2D slice was taken from each image at the same location and compiled to create the 2D montage (1 min per slice). The distance (x) of the slices of the image was in μm .

Table 1:The photophysical properties of **NCy3** [17].

Solvent	λ_{ab} (nm)	λ_{em} (nm)	ϵ (cm ⁻¹ M ⁻¹)	Φ
DMSO	578	601	27,800	0.190
Acetone	566	583	32,600	0.140
MeOH	564	581	15,200	0.148
THF	578	595	22,900	0.156
EtOAc	576	592	24,700	0.108
CH ₂ Cl ₂	567	581	34,900	0.160