Rational design of fast and selective near-infrared fluorescent probe for targeted monitoring of endogenous nitric oxide

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1. Materials and instruments.

All reagents and solvents were purchased from commercial sources and were of the highest grade. Solvents were dried according to standard procedures. All reactions were magnetically stirred and monitored by thin-layer chromatography (TLC). Flash chromatography (FC) was performed using silica gel 60 (200–300 mesh). Absorption spectra were taken on Agilent Technologies carry 60 UV-Vis. Fluorescence spectra were taken on F97pro fluorescence spectrometer. $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker AV-400 spectrometer with chemical shifts reported in ppm at room temperature. Mass spectra were measured on a HP 1100 LC-MS spectrometer. The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; br = broad.

2. Preparation of the test solution.

DEA•NONOate was purchased and used without further purification. Deionized water and spectroscopic grade MeCN were used for spectroscopic studies. Hypochlorite and hydrogen peroxide solution was prepared by dilution of commercial NaClO solution and H$_2$O$_2$ solution in deionized water. The aqueous solutions of NaNO$_2$ were freshly prepared and used as nitrite (NO$_2^-$) sources, respectively.$^1$ Various analytes (100 equiv) represented by ONOO$^-$, AA, Cys, GSH, Glu, Pro, Trp, Asa, Arg, which were purchased were added to the solution of probe SiRD (5 μM) in HEPES buffer (50 mM, pH = 7.4, H$_2$O:CH$_3$CN=7:3) respectively. The resulting solution was kept at room temperature for 30 min, and then the fluorescence spectra were recorded.

3. Experimental section.

Synthesis of SiRD

\[
\begin{align*}
\text{BrNH}_2 & \quad + \quad \text{K}_2\text{CO}_3,\text{MeCN} \quad 80^\circ\text{C}, 24\text{h} \\
\text{BrN} & \quad \text{K}_2\text{CO}_3,\text{MeCN} \quad 80^\circ\text{C}, 24\text{h} \\
\text{Br} & \quad \text{HNO}_2,\text{HCl} \quad 50^\circ\text{C} \\
\text{Br} & \quad \text{HCl, NaNO}_2 \quad 50^\circ\text{C} \\
\text{Br} & \quad \text{TiCl}_4, \text{CH}_3\text{CN} \quad 70^\circ\text{C} \\
\text{Br} & \quad \text{NH}_2 \quad \text{SiRD} \\
\end{align*}
\]

3-Bromo-N, N-diethylaniline:

\[
\begin{align*}
\text{BrNH}_2 & \quad + \quad \text{K}_2\text{CO}_3,\text{MeCN} \quad 80^\circ\text{C}, 24\text{h} \\
\text{BrN} & \quad \text{K}_2\text{CO}_3,\text{MeCN} \quad 80^\circ\text{C}, 24\text{h} \\
\text{Br} & \quad \text{HNO}_2,\text{HCl} \quad 50^\circ\text{C} \\
\text{Br} & \quad \text{HCl, NaNO}_2 \quad 50^\circ\text{C} \\
\text{Br} & \quad \text{TiCl}_4, \text{CH}_3\text{CN} \quad 70^\circ\text{C} \\
\text{Br} & \quad \text{NH}_2 \quad \text{SiRD} \\
\end{align*}
\]

3-Bromoaniline (10 g, 0.6 mol), EtI (20 g, 0.13 mol), K$_2$CO$_3$ (8 g, 0.6 mol), and anhydrous MeCN (100 mL) were added into flask. The resulting mixture was heated to 80 °C with rigorous stirring for 11 h before being cooled to room temperature. Solid materials were filtered off using a Celite cake under vacuum and washed with CH$_2$Cl$_2$. The filtrate was evaporated under reduced pressure to give the crude product as a brownish-orange liquid. The residue was purified by column chromatography on silica gel (dichloromethane: petroleum ether= 1: 10) to give 3-Bromo-N,N-diethylaniline (10.3 g, a colorless liquid) in an 86% yield: $^1$H NMR (400 MHz, CDCl$_3$): δ 1.13 (t, 6H, $J = 6.91$ Hz), δ 3.29 (q, 4H, $J = 7.07$ Hz), δ 6.55 (d, 1H, $J = 8.29$ Hz), δ 6.71 (s, 1H), δ 6.76 (s, 2H, $J = 6.29$ Hz), δ 7.01 (t, 1H, $J = 6.29$ Hz).
4, 4’-methylenebis (3-bromo-N, N-diethylaniline) (S1):

![Chemical Structure]

To a solution of compound 3-Bromo-N, N-diethylaniline (2.8 g, 12.5 mmol) in AcOH (80 mL) was added 37% formaldehyde (1.8 g, 63 mmol), and the mixture was stirred at 80 °C for 75 min. After cooling to room temperature, the reaction mixture was carefully neutralized with saturated NaHCO₃ aq. and NaOH aq. and extracted with CH₂Cl₂. The organic layer was washed with brine and dried over Na₂SO₄. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (dichloromethane: petroleum ether = 1: 4) to give pure S1 as a white solid (2.2 g, 76% yield). MS (ESI): Calcd for [M + H]+, 469.0698; Found, 469.0695. [M+H]+; ¹H NMR (400 MHz, CDCl₃): δ 1.12 (t, 12H, J = 6.91 Hz), δ 3.27 (q, 8H, J = 7.07 Hz), δ 3.98 (s, 2H), δ 6.51 (d, 2H, J = 8.29 Hz), δ 6.84 (d, 2H, J = 6.29 Hz).

Si-Xanthone (SiX):

![Chemical Structure]

To a dried flask flushed with argon, compound S1 (1.2 g, 2.5 mmol) and anhydrous THF (20 mL) were added. The solution was cooled to –78 °C, 1.3 M n-BuLi in (5.4 mL, 7 mmol) was added, and the mixture was stirred for 0.5 h. At the same temperature, a solution of SiMe₂Cl₂ (5 mmol) in anhydrous THF (10 mL) was slowly added, and the mixture was slowly warmed to room temperature, then stirred for 6 h. The reaction was quenched by addition of 2 N HCl aq. Then the mixture was neutralized with NaHCO₃, and extracted with CH₂Cl₂. The organic layer was washed with brine and dried over Na₂SO₄. The solvent was removed under reduced pressure and the crude compound was used without further purification.³

To a solution crude compound in acetone (50 mL) at 0 °C was added KMnO₄ (1.2 g, 7 mmol) in small portions over a period of 1 h with stirring. The mixture was stirred for another 1 h at the same temperature, then diluted with CH₂Cl₂ (50 mL), filtered through paper filter and evaporated to dryness.² The residue was purified by column chromatography on silica gel (dichloromethane: ethyl acetate = 40: 1) to give pure SiX as a yellow solid (0.18 g, 19% yield). And the product was further recrystallized form dichloromethane/petroleum ether to give SiX as yellow crystals. MS
(ESI): Calcd for [M + H]⁺, 381.2362, Found, 381.2368. ¹H NMR (400 MHz, CDCl₃): δ 0.45 (s, 6H), δ 1.23 (t, 12H, J = 7.08 Hz), δ 3.43 (q, 8H, J = 7.08 Hz), δ 6.74 (s, 2H), δ 6.79 (d, 2H, J = 9.00 Hz), δ 8.35 (d, 2H, J = 8.98 Hz); ¹³C NMR (400 MHz, CDCl₃): δ -0.1, 13.6, 44.9, 113.6, 115.1, 129.1, 132.3, 140.3, 150.0, 184.4.

**SiRD:**

![SiRD](image)

The mixture of SiX (0.1 g, 0.3 mmol) in CH₃CN (10 mL) was stirred at 0 °C under N₂ for 10 min then Tf₂O (200 L, 1.2 mmol) was added dropwise over 1 min. The reaction mixture was stirred for 10 min then o-phenylenediamine (0.324 g, 3 mmol) was added. The mixture was stirred overnight at 25 °C (If the reaction was incomplete, another portion of Tf₂O and nucleophile was added). The solvents were removed under reduced pressure and the residue was purified by flash chromatography (CH₂Cl₂/MeOH = 60/1) to afford the pure product SiRD (70 mg). Yield: 55.1%

MS (ESI): m/z calcd for C₂₉H₃₉N₄Si [M]⁺: 471.29; found: 471.2946. ¹H NMR (400 Hz, CDCl₃) 7.72 (d, J = 9.6 Hz, 2H), 7.12 (m, 1H), 7.03 (dd, J₁ = 7.8 Hz, J₂ = 1.8 Hz, 2H), 6.75 (m, 3H), 6.60 (s, 1H), 6.55 (s, 1H), 3.39 (q, J = 7.2 Hz, 8H), 1.16 (t, J = 7.2 Hz, 12H), 0.52 (s, 6H). ¹³C NMR (400 Hz, CDCl₃): δ 173.3, 150.7, 140.4, 139.2, 133.0, 130.5, 130.1, 126.5, 124.9, 122.8, 120.9, 119.2, 114.1, 69.0, 45.6, 44.7, 30.9, 29.6, 14.6.

**Reference**

4. Supplementary Spectra and chart

**Figure S1.** Proposed reaction mechanism and HRMS chart of resulting compounds.

**Figure S2.** Fluorescence spectra of SiRD (5 μM) (λ<sub>ex</sub> = 680 nm) in the presence of different amount of DEA·NONOate (0 – 10 μM), and the corresponding linear relationship between the fluorescent intensity and DEA·NONOate concentrations. Conditions: HEPES buffers (50 mM, pH = 7.4, H<sub>2</sub>O:CH<sub>3</sub>CN = 7:3).
Figure S3. Fluorescence intensity of SiRD in the absence and presence of 100 equiv DEA·NONOate at various pH values ($\lambda_{em} = 710$ nm). Conditions: HEPES buffers (50 mM, pH = 7.4, H$_2$O:CH$_3$CN = 7:3).

Figure S4. Absorption (A) and emission spectra (B) of SiRD (5 μM) upon addition of Cys (500 μM) in the presence of 100 equiv DEA·NONOate ($\lambda_{ex} = 480$ nm). Conditions: HEPES buffers (50 mM, pH = 7.4, H$_2$O:CH$_3$CN = 7:3).

Figure S5. Fluorescence spectra of SiRD (5 μM) with the addition of DEA·NONOate (500 μM) in the presence of Cys (500 μM) ($\lambda_{ex} = 480$ nm). Conditions: HEPES buffers (50 mM, pH = 7.4, H$_2$O:CH$_3$CN = 7:3).
**Figure S6.** Proposed reaction mechanisms of compound 1 in the presence of Cys and HRMS chart of compound 3.

**Figure S7.** Fluorescence spectra of SiRD (5 μM) with the addition of different amount of Cys (0 – 400 μM) in the presence of DEA·NONOate (100 μM), and the corresponding linear relationship between the fluorescent intensity and Cys concentrations ($\lambda_{ex} = 480$ nm). Conditions: HEPES buffers (50 mM, pH = 7.4, H$_2$O:CH$_3$CN = 7:3).

**Figure S8.** Absorption (A) and emission spectra (B) of SiRD (5 μM) upon addition of GSH (500 μM) in the presence of 100 equiv DEA·NONOate ($\lambda_{ex} = 680$ nm) for 30 min in a mixed HEPES buffer solution (50 mM, pH = 7.4, H$_2$O:CH$_3$CN = 7:3).
**Figure S9.** Percentage of viable Hela cells after treatment with indicated concentrations of SiRD after 12 hours.

**Figure S10.** Fluorescent imaging of exogenous NO with the SiRD probe (2 μM) in Hela cells. (A-D) Images from cell pre-incubated by the SiRD probe for 30 min; (E-H) stained by DEA·NONOate (200 μM) for 30 min; (I-L) Fluorescent images of cells staining by the SiRD incubated with NEM (200 μM) for 30 min, then added DEA·NONOate (200 μM).

**Figure S11.** (A-C), (E-F) Fluorescence images of Hela cells costained by probe (2 μM, 30 min), Mito Tracker Green FM (0.2 μM, 30 min) and DEA·NONOate (200 μM, 30 min) in sequence. Emission signals were collected at 500–530 nm for green channel (excited at 490 nm), and collected at 550–670 nm for red channel (excited at 480 nm). D and G are the Pearson’s co-localization coefficient located in the mitochondria.
5. $^1$H NMR, $^{13}$C NMR and HRMS charts.

Figure S12. $^1$H NMR chart of compound 3-Bromo-N, N-diethylaniline (CDCl$_3$, 400 MHz).

Figure S13. $^1$H NMR chart of compound S1 (CDCl$_3$, 400 MHz).
Figure S14. HRMS chart of compound S1.

Figure S15. $^1$H NMR chart of compound SiX (CDCl$_3$, 400 MHz).
Figure S16. $^{13}$C NMR chart of compound SiX (CDCl$_3$, 400 MHz).

Figure S17. HRMS chart of compound SiX.
Figure S18. $^1$H NMR chart of compound SiRD (CDCl$_3$, 400 MHz).

Figure S19. $^{13}$C NMR chart of compound SiRD (CDCl$_3$, 400 MHz).
Figure S20. HRMS chart of compound SiRD.