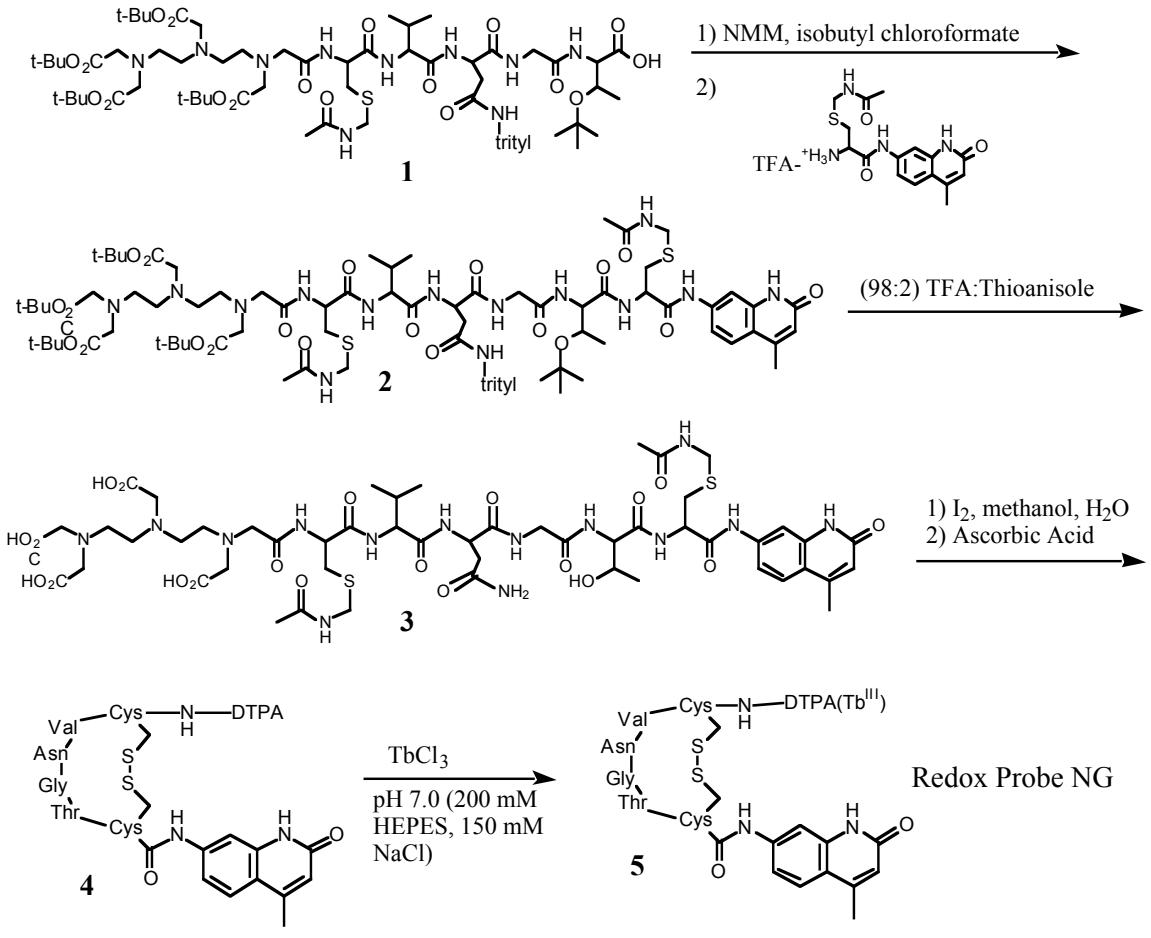
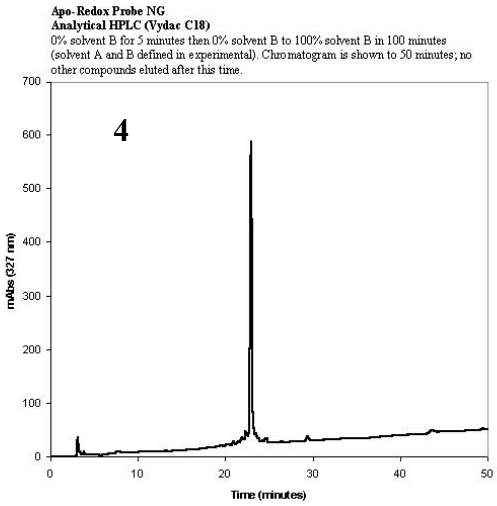
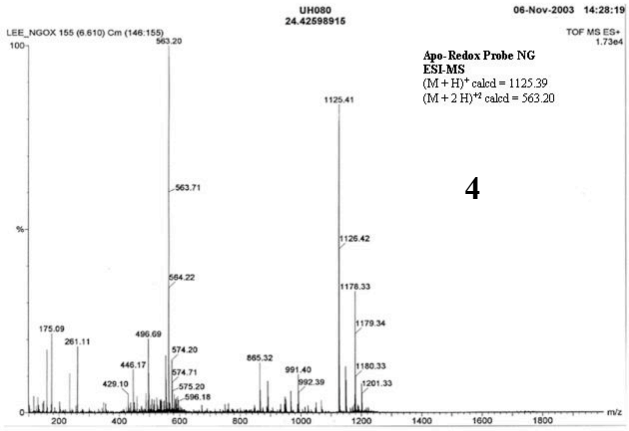
Synthesis of Redox Probe NG (5)*:* Detailed synthetic procedures will be included in a forthcoming publication. Briefly, peptide **1** (prepared manually from 2-chlorotrityl chloride resin and Fmoc-based SPPS) is coupled to the TFA salt of carbostyril(124) Cys(Acm) affording **2**. Compound **2** was t-butyl and trityl deprotected affording compound **3**. Compound **3** was then acetamidomethyl deprotected and oxidized simultaneous to yield the apo-probe, compound **4**. MS (ESI) *m/z* 1125.41 [$(M+H)^+$, calcd for $C_{45}H_{65}N_{12}O_{18}S_2$, 1125.39].

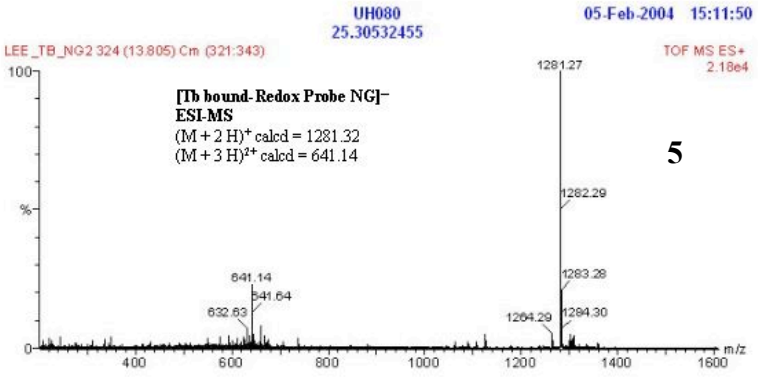
Terbium chelation. Compound **4** (15.8 mg, 14.0 μmol) was dissolved in 15.0 mL of 200 mM HEPES buffer containing 150 mM NaCl at pH 7.0. Then, 280 μL of a 100 mM aqueous $TbCl_3$ solution (28.0 μmol Tb(III)) was added and the resulting solution was stirred for 30 minutes. After which time, the solution was introduced onto a preparatory Vydac C_{18} column and isolated employing a gradient of 0-100% B over 50 minutes (where HPLC solvent A = water and solvent B = methanol). Note: the use of elutants containing TFA resulted in acid-assisted terbium dissociation and were avoided. After lyophilization, compound **5** was isolated as a white powder. (8.9 mg, 7.0 μmol, 50 %): MS (ESI) *m/z* 1281.27 [$(M + 2 H)^+$, calcd for $C_{45}H_{62}N_{12}O_{18}S_2Tb$, = 1281.32]. The analytical HPLC for **4** and mass spectrum for **4** and **5** are shown below.



where DTPA= $-COCH_2N(CH_2CO_2H)CH_2CH_2N(CH_2CO_2H)CH_2CH_2N(CH_2CO_2H)_2$







Probe Reduction Potential Determination: The reduction potential of the probe was determined by allowing fully oxidized probe (14 –20 μM depending on the experiment and quantitated as described below) to equilibrate in a redox buffer composed of varying ratios of oxidized and reduced dithiothreitol (DTT) at pH 7.0 (200 mM HEPES, 150 mM NaCl) according to the following equilibrium (equation 1). The measured equilibrium constant, K_{eq} can be related to the reduction potential via the Nernst equation using the biochemical standard state, pH 7 (Equation 2).

$$P_{red} + DTT_{ox} \leftrightarrow P_{ox} + DTT_{red} \qquad \text{(equation 1)}$$

where :

P_{red} = reduced probe

DTT_{ox} = oxidized dithiothreitol

P_{ox} = oxidized probe

DTT_{red} = reduced dithiothreitol

$$E_p^{o'} = E_{DTT}^{o'} - \frac{RT}{nF}\ln K_{eq} \qquad \text{(equation 2)}$$

where :

$E_p^{o'}$ = probe standard reduction potential

$E_{DTT}^{o'}$ = DTT standard reduction potential = -0.312 V [1]

R = 8.31 J K^{-1} mol^{-1}

T = 298 K

n = 2

F = 9.65 x 10^4 J V^{-1} mol^{-1}

$$K_{eq} = \frac{[P_{ox}][DTT_{red}]}{[P_{red}][DTT_{ox}]}$$

For each $[DTT_{red}]/[DTT_{ox}]$ ratio in Table 1, the concentrations of DTT_{red} and DTT_{ox} at equilibrium was determined by HPLC by comparing the integrated areas of both these species to calibration curves. The concentrations of oxidized and reduced probe were determined by HPLC; these species could not be fully resolved and a curve fitting procedure was used to determine their concentrations. Chromatograms of fully oxidized and fully reduced probe of know concentrations were obtained independently and used as basis spectra, Plot 1. Linear combinations of these basis spectra were used to fit the experimental chromatograms affording $[P_{ox}]$ and $[P_{red}]$ (experimental details and fit chromatograms are given below).

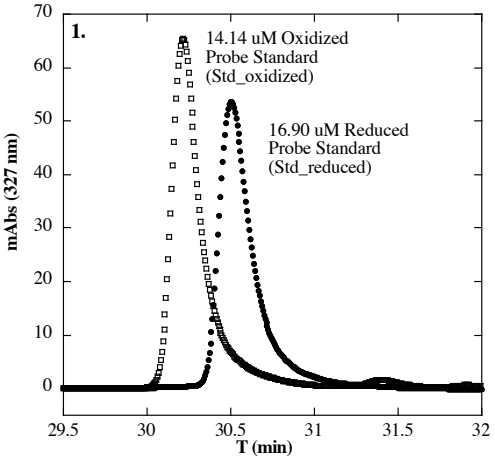
Table 1.

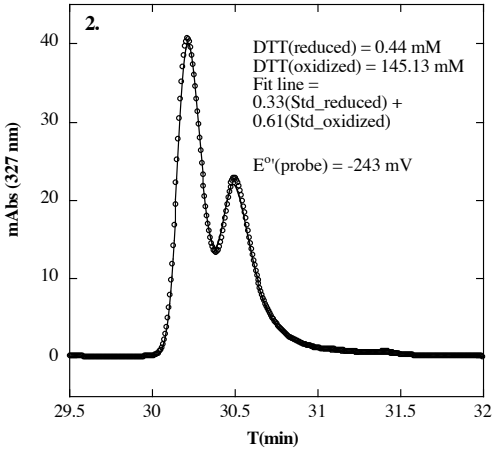
Plot	**$[DTT_{red}]$(mM)**	**$[DTT_{ox}]$(mM)**	**$[DTT_{red}]/[DTT_{ox}]$**	**$[P_{ox}]$ (μM)**	**$[P_{red}]$ (μM)**	**$E^{o'}_p$ (mV)**
2	0.44	145.13	0.003	8.63	5.58	-243
3	0.37	129.71	0.003	9.19	4.56	-246
4	1.13	128.12	0.009	5.09	10.82	-242
5	1.70	127.45	0.013	4.38	14.37	-242
6	1.67	86.45	0.019	3.25	14.87	-242
7	2.35	83.28	0.028	2.26	15.38	-242
8	3.30	84.44	0.039	1.70	16.73	-241

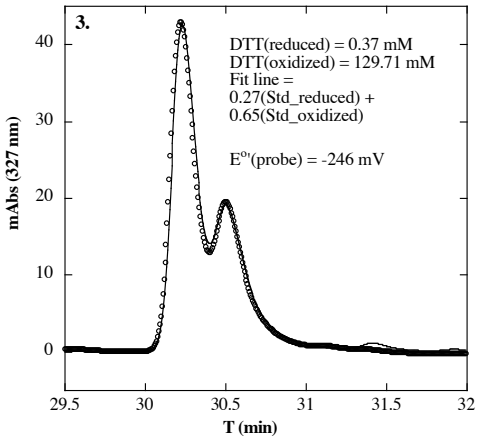
Experimental: Samples were prepared under an argon atmosphere and the buffer (200 mM HEPES, 150 mM NaCl, pH 7.0) was sparged with helium prior to use. For each $[DTT_{red}]/[DTT_{ox}]$ ratio corresponding to plots 2-8, individual samples were prepare from stock solutions of DTT_{red} (100 mM), DTT_{ox} (150 mM) and probe (1.0 mM). An example protocol is given for $[DTT_{red}]/[DTT_{ox}]$ = 0.039 corresponding to plot 8.

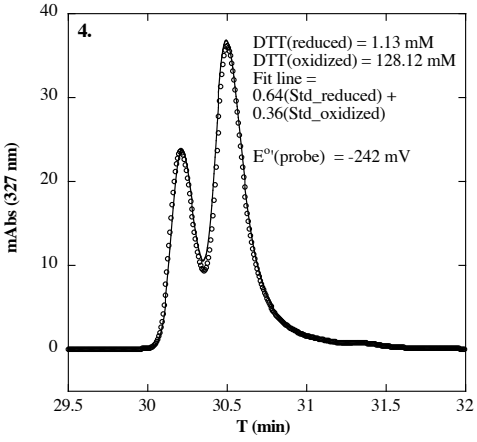
An epandorf tube containing 143 μL of HEPES buffer was charged with 190 μL of DTT_{ox} stock and 7.0 μL of P_{ox} stock and mixed. 130 μL of this solution was removed for HPLC analysis to determine the total probe concentration. To the remaining solution,

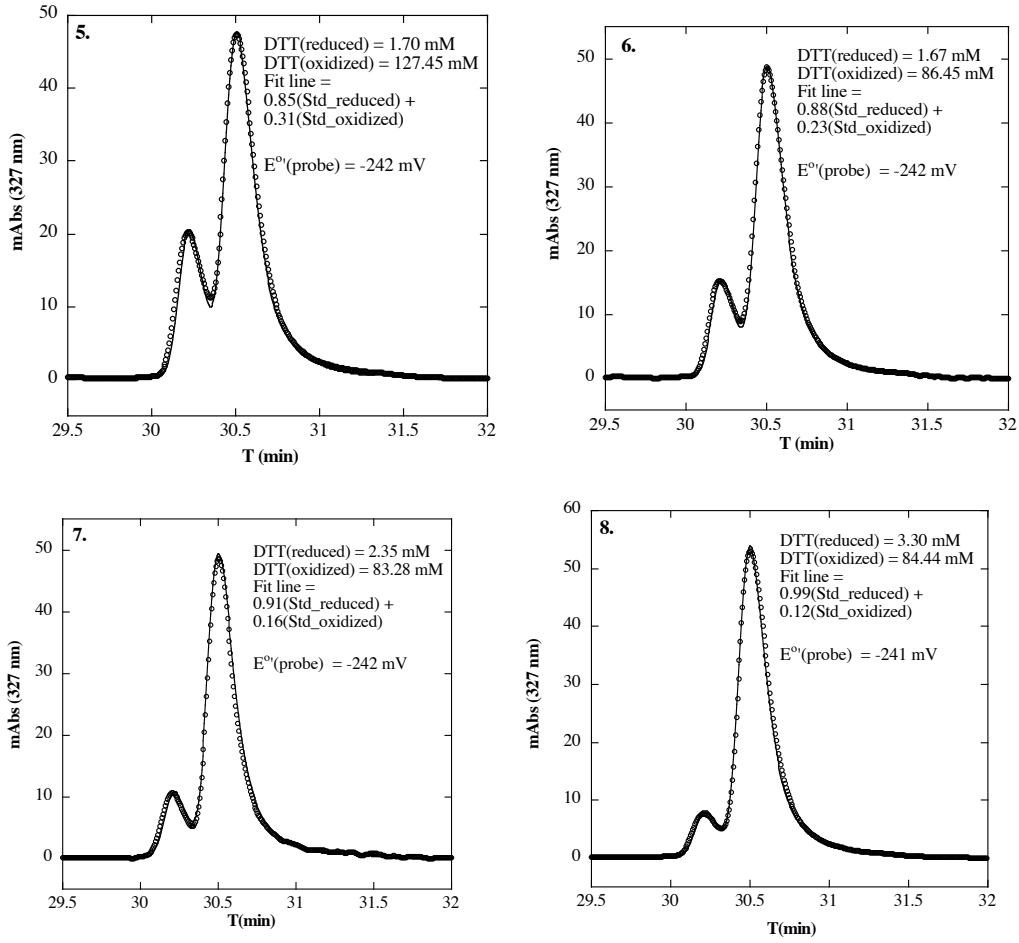
7.0 μL of DTT_{red} stock was added. The resulting solution was mixed and incubated for 5 hours (sufficient time to reach equilibrium as previously determined). The reaction was then quenched with 7.0 μL of neat TFA, resulting in a solution pH change from 7.0 to below 2.0 (inhibiting possible air oxidation). HPLC analysis of the final solution allowed direct measurement of [DTT_{ox}], [DTT_{red}], [P_{ox}] and [P_{red}] as follows. Two chromatograms were collected. First, 50 μL of the final solution was injected onto a Vydac C_{18} column and eluted employing a gradient of 0% solvent B for 5 minutes and then 0-100% solvent B over 100 minutes (HPLC solvent A consisted of 0.1% aqueous TFA and solvent B consisted of 90% acetonitrile, 10% water and 0.1% TFA). DTT_{ox} was monitored at 283 nm and its concentration determined by comparison to a calibration curve. Second, 100 μL of the final solution was injected and eluted using the same gradient. DTT_{red} was monitored at 248 nm and its concentration was determined by comparison to a calibration curve. Oxidized and reduced probe was monitored at 327 nm and their concentrations were determined by curve fitting as described above (fits shown below).











Probe redox state dependent fluorescence: All measurements were performed in an anearobic 1 cm quartz fluorescence cell using an SLM-Amico-Bowman Series 2 luminescence spectrometer. The photomultiplier voltage was set to afford a sensitivity of 3% full scale using the Raman water band (λ_{ex} = 350 nm, λ_{em} = 397 nm, 4 nm excitation and emission slit widths). Spectra collected from samples containing probe were done so using λ_{ex} = 327 nm and excitation and emission slit widths of 1 nm and 4 nm, respectively. All stock and sample solutions were buffered with 200 mM sparged HEPES containing 150 mM NaCl at pH 7.0. The data shown in figure 1 was generated by first collecting the spectrum of a 10 μM solution of fully oxidized probe. This sample was prepared by adding 20 μL of a 1mM probe stock to 1.86 mL of buffer via syringe excluding oxygen. The final probe concentration was determined by UV (ε_{327} = 18, 300 $M^{-1}cm^{-1}$). After the fully oxidized probe's spectrum was collected, 100 μL of a 200 mM GSH stock and 20 μL of a 10 mM GSSG stock were added via syringe to afford a $[GSH]^2/[GSSG]$ ratio = 1.0 M (environmental reduction potential = -0.240V). The resulting sample was mixed by agitation, allowed to come to equilibrium and a spectrum collected. According to equations 3 and 4, this ratio establishes a redox buffer in which

45 % of the total probe exists in its reduced state and 55 % exists in its oxidized state. As a result, a 45 % reduction of fluorescence intensity is expected. Finally, 20 μL of a 50 mM TCEP stock was added to fully reduce the probe and a spectrum collected. All spectra shown in Figure 1 are baseline and concentration corrected. This entire protocol was repeated three times with varying $GSH]^2$/[GSSG] ratios to ensure reproducibility. The data in figure 1 represents one of these experiments.

The equilibrium between oxidized and reduced probe and glutathione can be written as equation 3 and expressed mathematically as equation 4.

$$P_{red} + GSSG \leftrightarrow P_{ox} + 2\,GSH \qquad \text{(equation 3)}$$

$$\frac{[GSH]^2}{[GSSG]} = K_{eq}\frac{[P_{red}]}{[P_{ox}]} \qquad \text{(equation 4)}$$

where :

$$K_{eq} = \exp^{\frac{E^{o'}_{GSH} - E^{o'}_{p}}{RT/nF}}$$

And :

$E^{o'}_{GSH}$ = GSH reduction potential = - 0.240 V[1]

$E^{o'}_{p}$ = probe reduction potential = - 0.243 V

$R = 8.31\ JK^{-1}\,mol^{-1}$

$T = 298\ K$

$n = 2$

$F = 9.65 \times 10^{4}\ JV^{-1}\,mol^{-1}$

$[P_{red}]$ and $[P_{ox}]$ can be expressed as the fraction of total probe that is reduced (f_{red}) and oxidized (f_{ox}), respectively. Therefore,

$$\frac{[P_{red}]}{[P_{ox}]} = \frac{f_{red}}{f_{ox}} = \frac{f_{red}}{1 - f_{red}}$$

And :

$$f_{red} = \frac{F_{obs} - F_{ox}}{F_{red} - F_{ox}}$$

And :

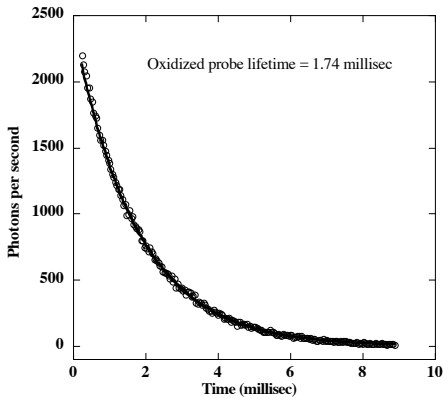
F_{obs} = observed fluorescence

F_{ox} = fluorescence of fully oxidized probe

F_{red} = fluorescence of fully reduced probe

Luminescence lifetime determination: Lifetime measurements were obtained on a Photon Technology International fluorimeter with a xenoflash lamp employing 5 nm

instrumental slit widths. A 20 μM solution of oxidixed probe (200 mM HEPES, 150 mM NaCl, pH 7.0) was excited at 327 nm at a frequency of 100 Hz. The probe concentration was determined by UV spectroscopy (ε_{327} = 18,300 $M^{-1}cm^{-1}$). Luminescence emission was monitored at 545 nm via photon counting with an integration time of 40 μsec. The data shown below was fit to a single lifetime exponential using FeliX 32 analysis (v1.0) software yielding an observed lifetime of 1. 74 (0.01) millisec.



References:

1. Gilbert, H.F., *Thiol/disulfide exchange equilibria and disulfide bond stability*. Methods in Enzymology, 1995. **251** (Biothiols, Part A): p. 8-28.