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De Novo Designed Peptidic Redox Potential Probe: Linking Sensitized Emission to Disulfide Bond Formation

Kyung Lee, Valerie Dzubeck, Lauren Latshaw, and Joel P. Schneider*

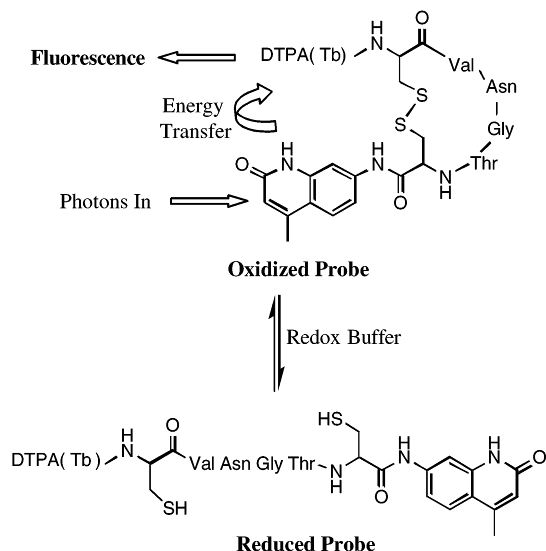
University of Delaware, Department of Chemistry and Biochemistry, Newark, Delaware 19716-2522

Received May 8, 2004; E-mail: schneijp@udel.edu

Probes that can actively measure changes in the redox state of a given environment via fluorescence would circumvent limitations of current approaches such as the use of specialized equipment[1−4] or, in the case of making in vivo measurements, irreversible cellular damage.[5,6] Herein, we present the design and utility of a peptidic probe capable of accurately measuring environmental redox potential via sensitized emission. This probe is characterized by long-lived luminescence (millisecond), nanomolar detection limits, and a probe reduction potential of −0.243 V suggesting that it may be useful for biological applications.

Recently, large fluorescent probes based on green fluorescent protein[7] and a yellow variant of green fluorescent protein[8] were reported. Although potentially useful, DNA manipulation is required for their production. We have designed a small probe, available via simple chemical synthesis, whose oxidation state is coupled to its ability to luminesce, Scheme 1. Luminescence occurs by

Scheme 1



sensitized emission, a process that entails the excitation of a chromophore that subsequently transfers its excitation energy to a lanthanide metal ion; the newly excited lanthanide then relaxes via emission.[9] The efficiency of energy transfer from the chromophore to the metal ion is distant dependent. When oxidized, the probe adopts a conformation in which the chromophore is in close spatial proximity to a terbium chelate allowing sensitized emission to occur. When the probe is reduced, it adopts random coil conformation that spatially separates the chromophore and the terbium chelate, limiting the sensitized emission process. The fluorescence difference between the probe's oxidized and reduced states can be exploited to actively measure environmental redox potential.

This probe is a chemically modified hexapeptide designed to adopt a disulfide bonded β-hairpin under oxidizing conditions. The

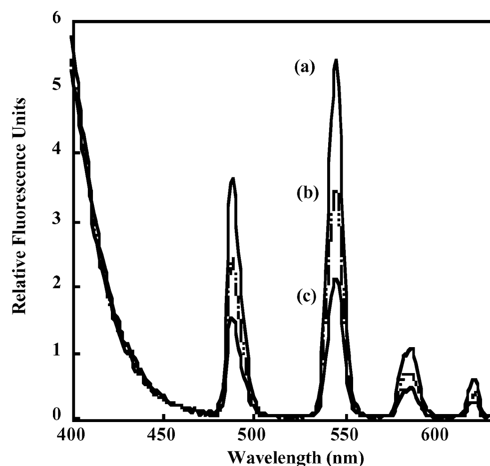


Figure 1. Emission of 10 μM probe at pH 7 (200 mM HEPES, 150 mM NaCl, λ_{em} 327 nm) (a) fully oxidized, (b) upon addition of glutathione redox buffer, and (c) fully reduced after addition of excess TCEP.

tetrapeptide sequence -Val-Asn-Gly-Thr- is incorporated at positions AA_2−AA_5 and has a known moderate propensity to adopt a β-turn structure.[10] Two redox active cysteine residues are incorporated at positions AA_1 and AA_6 based on Sternberg's structural analysis of small disulfide rich proteins which shows that disulfide bonds formed across antiparallel β-strands occur exclusively from cysteines located at "wide pair" hydrogen bonding positions.[11] This design criterion ensures that disulfide bond formation occurs intramolecularly and is of proper geometry conducive to β-hairpin formation. The N-terminus is functionalized with the highly stable ($K_d = 2 \times 10^{-23}$ M) eight-coordinate diethylenetriaminepentaacetic acid chelate of terbium, DTPA-Tb.[12] The C-terminus incorporates a highly absorbent carbostyril chromophore. The ability of the carbostyril/DTPA(Tb) pair to undergo efficient sensitized emission is well established.[13] These design elements culminate in a probe capable of adopting two conformations at equilibrium under redox buffered conditions, a highly fluorescent, disulfide-bonded β-hairpin (oxidized state) and a minimally fluorescent random coil (reduced state).

Figure 1 shows the emission of an aqueous solution of a fully oxidized probe at pH 7 (trace a). Excitation of the carbostyril at 327 nm results in terbium-based luminescence from the expected ${}^5D_4 \rightarrow {}^7F_j$ ($j = 0-6$) transitions with the most intense band resulting from the ${}^5D_4 \rightarrow {}^7F_5$ transition.[14]

Luminescence at this wavelength is well red-shifted and bright; although micromolar concentrations were used in Figure 1 for convenience, low nanomolar detection limits are easily realized using conventional fluorimeters without optimization. In addition, this probe offers a luminescent lifetime of 1.7 ms (see Supporting Information) making time-resolved measurements possible. Fluorescence of the fully reduced probe (trace c) is minimal resulting in a 3.4-fold difference between oxidized and reduced states, a large

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enough difference to allow accurate measurement of changes in environmental reduction potential.

The useful range of environmental redox potentials that can be accurately measured by any given probe is dependent on the reduction potential of the probe itself. The reduction potential of the peptidic probe, determined by HPLC experiments (see Supporting Information) employing dithiothreitol-based redox buffers, is −0.243 V. Interestingly, the estimated cytoplasmic potential of many cell types is near −0.240 V[15,16] suggesting that this probe may be useful for making cellular measurements. However, utility in intact cells necessitate efficient probe uptake, proteolytic resistance, and targeted cellular trafficking, factors that are under current investigation.

Preceding these requirements, the basic criterion of any probe is its ability to accurately measure change in redox poise. This was demonstrated in a simple cell-free experiment in which glutathione[17] (GSH, γ-Glu-CysH-Gly) and its corresponding disulfide (GSSG) were added anaerobically to a buffered solution of fully oxidized probe at pH 7 resulting in a solution redox poise of −0.240 V. Based on the probe's reduction potential, a 45% reduction of emission intensity (with respect to the reduced probe's intensity) should be observed. Figure 1 (trace b) shows the resulting spectrum, and the expected decrease in luminescence intensity is indeed observed. Not all of the carbostyril's excitation energy is transferred to the terbium as depicted in Scheme 1. A large fraction is lost as carbostyril-based fluorescence which is clearly seen at 400−450 nm. Interestingly, the carbostyril-based fluorescence is nearly invariant with respect to the probe's oxidation state, suggesting that this peptide may be employed as a two-color probe, e.g., carbostyril fluorescence can be used to calculate total probe concentration and terbium luminescence can be used to calculate the fraction of reduced probe; both values are needed to determine redox poise according to eq 1.

$$E'_{\mathrm{p}} = E^{\circ'}_{\mathrm{p}} - \frac{RT}{nF}\ln\frac{[\mathrm{P_{red}}]}{[\mathrm{P_{ox}}]} \qquad (1)$$

$$\text{and}\quad f_{\mathrm{red}} = [\mathrm{P_{red}}][\mathrm{P_{ox}}]^{-1}/[\mathrm{P_{red}}][\mathrm{P_{ox}}]^{-1} + 1$$

This equation relates the fraction of reduced probe to environmental redox potential (E'_{p}) where $R = 8.31$ J K^{-1} mol^{-1}, $T = 37$ °C (310 K), n = number of reducing electrons (2), $F = 9.65 \times 104$ J V^{-1} mol^{-1}, [P_{red}] and [P_{ox}] = concentration of reduced and oxidized probes, and f_{red} = mole fraction of reduced probe. Equation 1 can be used to generate the plot in Figure 2 which shows the theoretical range of environmental potentials that can be measured accurately by this probe. This probe will be most sensitive to redox changes between −0.2 and −0.3 V. It is also evident that measuring potentials outside this range will be problematic, since only very small changes in fluorescence are expected even when large changes in potential occur (e.g., at the two plateau regions in Figure 2).

However, a key feature of the probe's design is that the reduction potential of the disulfide bond formation event may be tuned by incorporating amino acid residues with varying turn propensities into the β-hairpin. Therefore, the equilibrium in Scheme 1 can be shifted in favor of either the disulfide bonded β-hairpin conformation or the random coil conformation depending on the choice of peptide sequence.

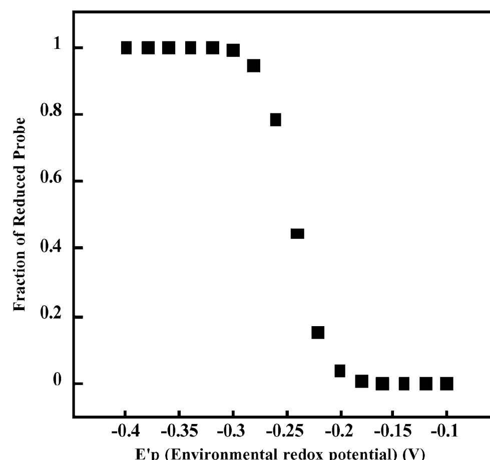

Figure 2. Calculated fraction of reduced probe as a function of redox potential demonstrating the probe's useful range.

For example, a probe that contains residues which strongly favor turn formation should favor disulfide bond formation resulting in a probe characterized by a low (more negative) reduction potential and vice-versa. This tunability, which will be reported soon, affords a family of probes capable of measuring a wider range of environmental redox potentials.

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Supporting Information Available: Experimentals for probe synthesis (HPLC/mass spectroscopy characterization), reduction potential determination, and all fluorescence measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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