A Selective, Ratiometric Fluorescent Sensor for Pb²⁺

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The development of ratiometric[1,2] fluorescent probes for Pb²⁺ that are sensitive to the concentrations of free Pb²⁺ in vivo and that are selective for Pb²⁺ would provide an unparalleled breakthrough in elucidating the toxicology of lead. Currently, only the *total* amount of Pb²⁺ in cells can be measured (e.g. using ICP or atomic absorption spectroscopies). Methods are desperately needed that would allow the concentration of *bioavailable* lead to be determined as a function of time, total blood lead level, cell type, and subcellular location. Toward this end, we report the first ratiometric fluorescent probe that is selective for Pb²⁺. This probe consists of a fluorescent dye (dimethylamino)-naphthalene-1-sulfonamide (dansyl or dns) conjugated to the amino terminus of a tetrapeptide (ECEE). Dansyl was specifically selected because its fluorescence emission is particularly sensitive to the microenvironment in which the fluorophore resides.[3−5] The probe was designed so that upon binding of Pb²⁺, the polarity of the environment surrounding the dansyl fluorophore changes, causing a concomitant shift in the fluorescence emission spectrum of the probe and an increase in the intensity at the emission maximum. By contrast, the fluorescence emission of a similar peptide-based Cu²⁺ probe recently reported by Imperiali and co-workers is quenched upon binding of analyte.[6] The Pb²⁺ probe reported herein is also unique because it is reversible and because it responds selectively to Pb²⁺ over other common metal ions. The generality of the design concept and the modular nature of the probe provide a framework for developing a second generation of probes that have a broad range of affinities for Pb²⁺.

The two primary challenges in making a fluorescent probe for Pb²⁺ are (1) to create a probe that is ratiometric[1] and (2) to create a probe that responds selectively to Pb²⁺ over other metal ions that compete with lead for binding sites in vivo.[7] A *ratiometric* probe allows a calibration curve to be determined in vitro that is independent of the sample conditions (e.g. concentration of the probe, sample thickness, intensity of illumination) and hence can be used to quantitate microscopy studies in vivo.[2] Most of the commercially available calcium dyes are ratiometric by virtue of the fact that the degree of conjugation within the dye becomes disrupted upon addition of calcium, resulting in a concomitant shift in the fluorescence emission maximum.[2,4,7] Although some of these dyes will also respond to lead,[8] they are not generally useful as lead sensors due to the interference from Ca²⁺, which is typically present in much higher quantities than Pb²⁺ in cells.

Here, we have employed an entirely different sensing strategy, in which the detection part of the probe is a separate module from the metal-binding part of the probe. Specifically, the detecting module is a fluorescent dye, dansyl (dns), that exhibits a fluorescence emission spectrum that is extremely sensitive to the polarity of the local environment.[3−6] The dye is conjugated to a tetrapeptide (ECEE) that acts as the lead-binding module. Amino acids were chosen as the units within the lead-binding module because they can be readily varied to optimize the metal-binding properties of future generations of ligands. The specific amino acids within the first generation ligand (glutamate, E, and cysteine, C) were chosen because their side chains contain functional groups (carboxylates and thiols) that are known to coordinate lead under biologically relevant conditions.[9−12] The probe was designed based on the idea that binding of Pb²⁺ would result in a conformational change in the peptide, which would cause the dansyl fluorophore to move to a new environment with a different polarity and consequently cause a shift in the emission spectrum. To test whether this was in fact the mode of sensing, we conducted analogous studies on a control molecule (dns-GGGG), which contains the same dansyl fluorophore and a tetrapeptide (GGGG) that has no functional groups on the amino acid side chains.

The ECEE and GGGG peptides were synthesized on Wang resin using standard Fmoc/OPfp chemistry.[13,14] Dansyl chloride was conjugated to the amino terminus of the peptides prior to cleavage off the resin. Metal binding to the reduced, purified probe was monitored using fluorescence spectroscopy. Fluorescence spectra were obtained by exciting into the dansyl fluorophore absorption at 337 nm and scanning the emission from 400 to 800 nm. Upon addition of Pb²⁺, the emission maximum of dns-ECEE shifts from 557 to 510 nm and increases in intensity (Figure 1). A plot of the ratio of the fluorescence emission intensity at 510 nm to the fluorescence emission intensity 557 nm (I_{510}/I_{557}) yields a calibration curve (Figure 1, inset) that gives an $EC_{50}^{Pb^{2+}}$ for the probe of ∼120 μM.[15] This blue shift in the emission spectrum most likely results from the fluorophore moving to a less polar environment upon metal binding.[3] Alternatively, it is possible that metal binding to the peptide results in deprotonation of the sulfonamide linker between the peptide and the dansyl fluorophore.[16,17] To distinguish between these two mechanisms of detection, future libraries of probes will include other conjugation schemes and fluorophores. However, addition of Pb²⁺ to the control molecule dns-GGGG, which is incapable of binding metal, results in no shift in the I_{510}/I_{557} ratio curve (Figure 1, inset).

Control experiments were conducted to test the reversibility and selectivity of the dns-ECEE probe. First, to test the reversibility of the probe, EDTA (200 μM) was added to a solution of 20 μM dns-ECEE with 200 μM Pb²⁺; addition of EDTA restores the original, metal-free spectrum.[14] This reversibility is critical if the probe is to be used to study metal distribution and equilibria in vivo. Second, to test the selectivity of the probe, we monitored the effect of Ca²⁺, Zn²⁺, and Cd²⁺ upon the emission spectrum of the probe. The emission ratio (I_{510}/I_{557}) of dns-ECEE does not change upon addition of Ca²⁺, Zn²⁺, or Cd²⁺. The selectivity of dns-ECEE for Pb²⁺ over Ca²⁺ and Zn²⁺ is particularly important because Pb²⁺ targets both Ca²⁺- and Zn²⁺-binding sites in vivo,[10,18−20] suggesting that these ions would be likely competitors for a Pb²⁺ probe in cells. These properties, reversibility and selectivity, are critical for imaging applications in cells.

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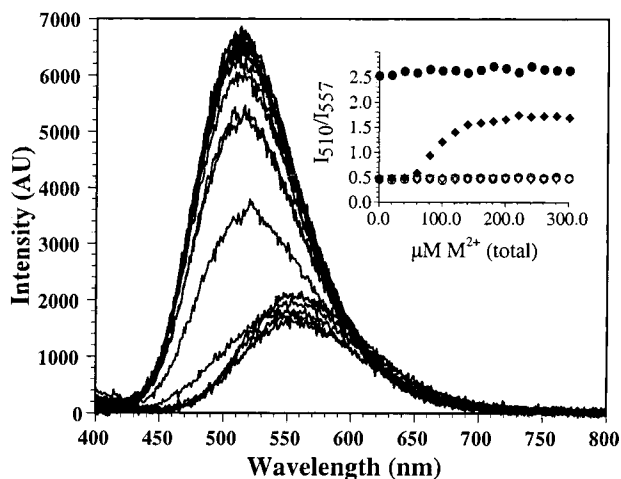(17) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385−426.

Figure 1. The fluorescence emission spectrum of the probe dns-ECEE shifts upon addition of Pb^{2+}. A calibration curve ◆ for fluorescence emission of dns-ECEE (inset) yields an $EC_{50}^{Pb^{2+}}$ of ~120 μM.[15] By contrast, addition of buffer (+), Zn^{2+} (○), Ca^{2+} (◇) or Cd^{2+} (▽) to 20 μM dns-ECEE does not cause a shift in the emission maximum of the probe. Likewise, addition of Pb^{2+} (●) to a control probe (20 μM dns-GGGG) that cannot bind metal does not result in a change in I_{510}/I_{557}. Initial conditions: 20 μM solution of dns-ECEE in 100 mM HEPES pH 7.1. Fluorescence emission intensities are given for excitation at 337 nm.

To test the metal-binding properties of a number of probes simultaneously, an efficient screening method is needed. Toward this end, the peptides dns-ECEE and dns-GGGG were synthesized on amino functionalized polyethylene-glycol dimethyl acrylamide copolymer (PEGA) resin[6,21] and screened in 96-well tissue culture plates using a fluorescence reader. While on the resin, the deprotected and reduced probes were incubated for at least 3 h in 300 or 600 μM solutions of Pb^{2+}, Ca^{2+}, Cd^{2+}, or Zn^{2+}.[14] In addition, competition experiments were conducted in which the probes were exposed to 300 or 600 μM solutions of Pb^{2+} in the presence of equimolar Ca^{2+} or Zn^{2+}. The resins were screened on a Wallac Victor 2 multilabel counter, with excitation at 355 nm and emission measured at 510 and 560 nm.[22]

Critically, dns-ECEE responds to Pb^{2+} in the presence of either equimolar Zn^{2+} *or* equimolar Ca^{2+} (Figure 2). Taken together with the solution-phase studies,[14] these results indicate that the probe is highly selective for Pb^{2+} and that neither Zn^{2+} nor Ca^{2+} interferes with the response of the probe. Although the response of the probes on resin is not always quantitatively identical with that exhibited by the free probe in solution, the metal-binding trends and selectivities are *qualitatively* similar.[23] These results suggest that this methodology can be used to rapidly screen large libraries of related probes for their responses to different metal ions. Potential probes identified by such screening would then need to be studied more extensively and quantitatively in solution to determine whether they are appropriate for imaging applications in vivo.

Future studies will address the need for ratiometric probes that bind Pb^{2+} significantly tighter, and that have better bioavailability, stability, and fluorescence properties. The amount of "free" or *bioavailable* lead in human cells is estimated to be on the order
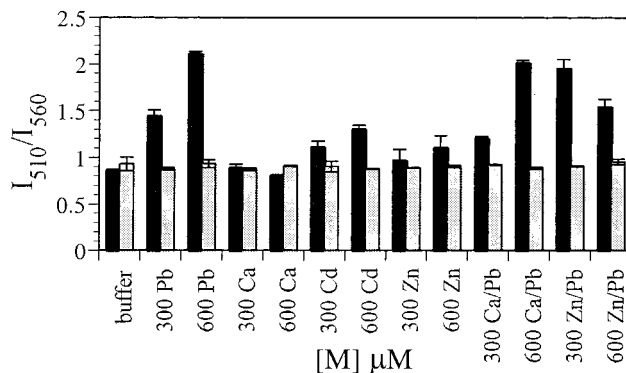
of picomolar to nanomolar, even though *total* blood lead levels are typically on the order of 0.1–2.5 μM (2–50 μg/dL).[24,25] Therefore, to be useful for imaging applications in vivo, any future probes would need to bind lead several orders of magnitude more tightly than the first generation probe reported herein. Furthermore, issues of bioavailabiltiy and biostability are critically important if a probe is to be useful for imaging applications. Fortunately, major advances in these areas (e.g., incorporation of uptake sequences and non-natural amino acids and other residues into probes) have been reported recently.[26–28] Other fluorescent dyes will also be screened to test the generality of the approach and to obtain probes with fluorescent properties that are more suitable for in vivo applications.

The system presented herein lays the foundation for these studies because the modular nature of the probe and the ability to screen probes efficiently on a solid support make this system well suited for examining large libraries of probes. Because the detection scheme is entirely ligand based and is not dependent upon the spectroscopic properties of the metal ion, the design and detection strategy are general enough to be readily extended to the development of fluorescent probes for a variety of different metal ions.

Supporting Information Available: Preparative procedures and analytical data for dns-ECEE and dns-GGGG; Figure illustrating solution-phase competition and EDTA reversibility experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Figure 2. The responses of dns-ECEE (black) and dns-GGGG (gray) to different metal solutions (*x*-axis markers) can be rapidly screened on resin. Excitation is at 355 nm and emission is monitored at 510 and 560 nm. The I_{510}/I_{560} shows that dns-ECEE exhibits the greatest response to Pb^{2+}, less of a response to Cd^{2+} and Zn^{2+}, and *no* response to Ca^{2+}. The presence of Ca^{2+} or Zn^{2+} does not interfere with the response to Pb^{2+}. The control molecule dns-GGGG, which is incapable of binding metal, does not respond to any of the metal solutions. Measurements were made in triplicate on two independently prepared samples; the error bars reflect the distribution observed for these measurements.

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(23) These qualitative differences may arise from several sources. The PEGA resin provides another microenvironment for the fluorophore and may compete with the probe for metal binding. In addition, slightly different wavelengths were monitored due to limitations in what filters are available for the Victor 2 reader.

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