Supramolecular metal displacement allows on-fluorescence analysis of manganese(II) in living cells†

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Due to the importance of Mn2+ ions in biological processes, it is of growing interest to develop protocols for analysis of Mn2+ uptake and distribution in cells. A supramolecular metal displacement assay can provide ratiometric fluorescence detection of Mn2+, allowing for quantitative and longitudinal analysis of Mn2+ uptake in living cells.

Manganese functions in both catalytic and structural roles in diverse proteins such as the photosynthetic apparatus and the enzyme superoxide dismutase.1 It plays important roles in gene expression via Mn-binding transcription factors, and metalloregulatory proteins are involved in metal-specific homeostasis.2 Its roles in virulence3 and oxidative stress4 have been reviewed. Additionally, Mn2+ is used widely as a versatile tool for biological studies at the cellular and organ level. Its similarity to calcium and efficient quench of intracellular dyes was to develop an on-fluorescence method for the quantitation of manganese(II) in living cells.

Fluorescent probes have proven highly useful for cell biology studies. Chelating dyes such as Quin-2 and Fura-2 have been used to measure Ca2+ in different cellular compartments.5,6 The membrane permeable dye Mag-fura-2 has been used to quantify ER Ca2+ levels.7 Fluctuation of Ca2+ levels associated with cellular transitions have been measured.8 Several available probes developed for Ca2+ are quenched by Mn2+.9 Some on-fluorescence probes for Mn2+ are available (e.g., Calcium Green, Invitrogen), but they generally show poor selectivity, particularly against Ca2+. We are not aware of the availability of ratiometric fluorescent probes for intracellular Mn2+.

Supramolecular strategies for analyte detection have drawn increasing attention for the solution of a variety of detection, quantification, and imaging problems.10 We reported previously a two-metal, two-dye displacement assay to ratiometrically monitor Cu2+ ions in aqueous solution.11 We applied a similar strategy to the development of systems for naked-eye detection of Mn2+.12 Recently, Lippard and coworkers reported an on/off MRI/fluorescence displacement system.13 In this paper, we utilize our metal-displacement assay to visualize Mn2+ concentrations both in solution and in living cells.

Similarly to the previous method, two commercially available dyes were employed, calcine blue (CB) and fluozin-1 (Fz1). Initially, Cd2+ was chelated by the stronger ligand CB and the formed complex was strongly fluorescent ("on"), while free ligand Fz1 gave weak fluorescence ("off"). Added Mn2+ competes with Cd2+ for CB, and quenches CB. In the new equilibrium state, Cd2+ forms a complex with ligand Fz1 whose fluorescence is consequently turned "on". Solution experiments indicated that the metal displacement assay was compatible for use with Mn2+. With an equimolar solution of Cd2+, CB, and Fz1, strong blue and weak green fluorescence was observed with excitation at 350 and 493 nm, respectively (Fig. 1). As Mn2+ was added, the blue fluorescence diminished and the green fluorescence increased proportionately. Over the concentration range shown, the 433 nm fluorescence was quenched by 12-fold, while that at 518 nm increased by 7-fold. Since complexation of Mn2+ quenched CB and Cd2+ caused Fz1 to show increased fluorescence quantum yield, the observed behavior is consistent with the expectation that Mn2+ competes with Cd2+ for complexation of the stronger chelator (CB), liberating Cd2+ to form a complex with Fz1. Interestingly, a large excess of Mn2+ compared with concentrations of the Cd2+ and the two dye molecules did not result in quenching at 518 nm, indicating that Mn2+ did not compete effectively with Cd2+ for chelation by Fz1 (Fig. 1c). This conclusion was supported by a direct competition experiment (Fig. S4, ESI†) in which addition of 10 μM Mn2+, Cd2+ and Fz1 showed only slightly diminished fluorescence compared to Cd2+ and Fz1 alone, implying a strong preference for Cd2+ . This is a significant...
advantage for this system with Mn$^{2+}$ compared to Cu$^{2+}$, where excess Cu$^{2+}$ quenched green fluorescence.

The system was less sensitive to Mn$^{2+}$ as compared with Cu$^{2+}$ as a result of the relative binding constants of the chelating dyes with Mn$^{2+}$ and Cd$^{2+}$ (5.9 × 10$^6$ vs. 7.1 × 10$^4$ M$^{-1}$ for CB). Lower association of Mn$^{2+}$ compared with Cu$^{2+}$ would be expected as predicted by the classic Irving-Williams series. Association constants of Mn$^{2+}$ and Cd$^{2+}$ were determined by titration; in addition, a direct competition experiment for Mn$^{2+}$ and Cd$^{2+}$ for CB indicated a 7.5-fold preference for Cd$^{2+}$, in contrast to the very strong association of Cu$^{2+}$. Fortunately, with 10 μM concentrations of the two dyes and Cd$^{2+}$, ratio-metric behavior was observed over a range from 5–1000 μM.

Metal ion response screening for the same system was analyzed. A solution containing 10 μM Fz1, 10 μM CB, 10 μM Cd$^{2+}$, 10 μM metal ion was titrated with 50 μM Mn$^{2+}$. The ions Na$^+$, Cs$^+$, Mg$^{2+}$, and Li$^+$ did not interfere with fluorescence (Fig. S1, ESI†). The biologically important metal, Zn$^{2+}$, does compete with Mn$^{2+}$ and could be a potential source of background fluorescence in certain applications. Fluorescence intensity at 493 nm and at 350 nm was studied at several pH values. In the range of pH from 6.1 to 7.6 (Fig. S6, ESI†), Fz1 response is not dependent on pH, and its fluorescence enhancement is well above background, and its fluorescence intensity increases slightly with pH.

The method was next adapted to detect intracellular Mn$^{2+}$. We examined the displacement assay approach in Human Embryonic Kidney cells (HEK 293-F, fast growing variant) and an HEK cell line that stably over-expressed Divalent Metal Transporter 1 (DMT-1) obtained from the Garrick Laboratory.21 Cell permeable acetoxymethyl (AM) esters of CB and Fz1 (CBAM and Fz1AM) were employed, as these neutral derivatives cross the cell membrane and are then hydrolyzed to cell impermeant CB and Fz1 by intracellular esterases within one hour.22,23 An excess of CBAM compared to Fz1AM was required as a result of the reduced take-up of CBAM which has only two AM esters as compared to Fz1AM has three and can therefore cross the cell membrane more easily. In experiments where Mn$^{2+}$ supplementation was used, cells were first incubated with Mn$^{2+}$, followed by the two dyes, and finally the Cd$^{2+}$.

Fig. 1 Reporter metal displacement assay. (a) Reaction scheme. (b) Normalized fluorescence response for the titration of aqueous solution (pH = 7.2) containing 10 μM CB, 10 μM Fz1, 10 μM Cd(ClO$_4$)$_2$, 50 mM HEPES and 100 mM KNO$_3$ with 0, 5, 10, 20, 40, 60, 80, 100 and 1000 μM MnCl$_2$. (c) Normalized fluorescence intensity at 433 nm (CB) and 517.5 nm (Fz1), as a function of Mn$^{2+}$ concentration.

Fig. 2 HEK and DMT-1 cells treated with: 5 μM Mn$^{2+}$, 3.6 μM Fz1AM, 26.6 μM CB-AM and 10 μM Cd$^{2+}$. (a) HEK cells show blue fluorescence but little green fluorescence, suggesting minimal Mn$^{2+}$ present in the cell. (b) DMT-1 cells under the same conditions show lower blue fluorescence with bright green fluorescence indicative of markedly increased Mn$^{2+}$ uptake.

Previous $^{54}$Mn$^{2+}$ uptake experiments performed on HEK cells showed minimal uptake without the expression of the metal transport protein DMT-1.21 Supplementation of HEK and DMT-1 cells with 5 μM Mn$^{2+}$ yielded very little green fluorescence in HEK cells while DMT-1 cells showed strong enhancement of fluorescence in the green channel, and diminished fluorescence in the blue channel (Fig. 2). Anticorrelated green and blue fluorescence was heterogeneously distributed within the field of view (Fig. S3, ESI†). The ability of this method to resolve cellular, and potentially subcellular, localization of Mn$^{2+}$ promises distinct advantages over other techniques. Control experiments were consistent with the operation of the displacement mechanism. In DMT-1 cells treated with only Fz1AM and Mn$^{2+}$, absence of Cd$^{2+}$ gave no fluorescent response (Fig. S8, ESI†). Cells treated with only CBAM enhanced with Cd$^{2+}$ but not Mn$^{2+}$ (Fig. S9, ESI†). Treatment with CBAM and Fz1AM showed quenched CB with Mn$^{2+}$ and enhanced CB with Cd$^{2+}$ (Fig. S10, ESI†). These experiments demonstrate that the fluorescence enhancement is well above background, and that Zn$^{2+}$ does not significantly interfere at the micromolar Mn$^{2+}$ concentrations examined here.

To fully realize the utility of our system for live imaging of cellular Mn$^{2+}$, real-time uptake of Mn$^{2+}$ was analysed in the DMT-1 cells (Fig. 3). Cells were incubated with the standard amount of CBAM, Fz1AM, and Cd$^{2+}$. Mn$^{2+}$ was then added and images were collected every twenty min over a period of 100 min. Fluorescence changes plateaued after 80 min (Fig. S11, ESI†) consistent with $^{54}$Mn assays in cell pellets.21 Blue fluorescence from CB correspondingly decreased over the entire time course.
The intracellular supramolecular assay responded quantitatively to varying concentrations of Mn$^{2+}$ (Fig. 4 and Fig. S12, ESI). Blue and green fluorescence intensities shifted over a range of 0–50 μM extracellular Mn$^{2+}$ in DMT-1 cells. In a multwell experiment, each concentration of Mn$^{2+}$ from 0 to 50 μM gave a decrease in blue fluorescence by a factor of 3 and an increase in green fluorescence by a factor of 11, consistent with a saturable system (Fig. 4). A double reciprocal plot shows a linear relationship as expected for the DMT1 transporter for both green and blue average fluorescence intensities (Fig. S13, ESI). These data again fit very well with previous $^{54}$Mn$^{2+}$ uptake experiments.1

Mn sensitivity was also explored in an additional cell line. The human lung carcinoma A549 cell line26,27 behaved similarly to control HEK cells, giving signal only with high Mn$^{2+}$ exposure (Fig. S14, ESI). Transient transfection with a DMT1 expressing plasmid improved sensitivity to Mn$^{2+}$ by several orders of magnitude in transfected cells (Fig. S15, ESI). These data suggest that, in multiple cell types, Mn$^{2+}$ uptake in the cells is a tightly regulated process, which normally allows for very little free intracellular Mn$^{2+}$ without active uptake by DMT1.

Supramolecular displacement assays have found widespread scope of application.16 but there have been few applications demonstrated in a cellular context. There are many challenges to the application of such methods in biological systems, including solubility, cell permeability, potentially variable subcellular localization of components, biocompatibility, orthogonality of the chemistry to intracellular receptors and metals, among others. The present assay requires a metal sensitive reporter ion could permit a wider scope of application.

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Modification of CB with chelating moieties that would provide more selective association of Mn$^{2+}$ would improve quenching efficiency of the blue dye and would increase sensitivity. Also, linking the two dyes together covalently could guarantee delivery of equivalent concentrations to the cells and simplify the calibration required for quantitative studies.28 Use of a more widely biocompatible reporter ion could permit a wider scope of application.

Notes and references

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