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## COMMUNICATION

## Engineering lead-sensing GFP through rational designing

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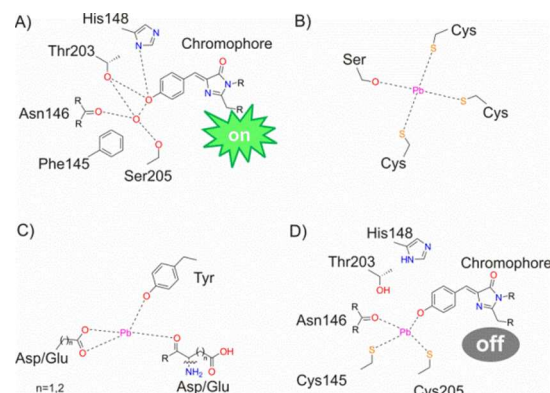
**Lead is one of the most hazardous metals ubiquitous in the environment, causing serious health hazards to organisms. Recently, fluorescent proteins such as GFP and Dsred were utilized for the development of reagent-less rapid metal sensors. Here, we demonstrate the development of a lead-sensing GFP that is highly sensitive to lead in micro molar concentrations.**

Metals are the most important constituent, playing a wide role in various industrial applications in the economic-driven world.<sup>[1, 2]</sup> Due to the importance of metals in industrial applications, industrial effluents have become the principal source of metal contamination in the environment.<sup>[1]</sup> Among the contaminants, heavy metals such as lead and mercury are the most toxic metals in the environment, causing health hazards to humans and other organisms. Therefore, the development of metal-binding peptides for sensing and sequestering purposes is necessary. Moreover, the quantitative detection of lead and mercury in the environment and biological systems is an important and challenging task. Traditionally, metal responsive repressors/activators<sup>[3]</sup> and chemo sensors<sup>[4]</sup> were used for the measurement of metal. Each method has disadvantages, such as induced background expression, impermeability to cells and poor metal selectivity.<sup>[3]</sup> In addition, a highly sensitive and selective lead-sensing DNzyme was recently reported.<sup>[5]</sup> Despite its application, it also has disadvantages such as a complex procedure for DNzyme production, fluorophore labelling, intermolecular quenching, temperature sensitivity and reactivity towards nucleases.<sup>[5, 6]</sup>

Fluorescent proteins have a wide range of applications in various fields, and have been extensively studied and well characterized.<sup>[7]</sup> Due to their inherent and engineered metal binding property, these proteins were efficiently utilized for studying *in vivo* metal dynamics and for the detection of heavy metals.<sup>[7]</sup> In the recent decades, green fluorescent protein (GFP) and its spectral variants have been utilized as FRET partners for the ratiometric detection of metals such as Ca<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Cd<sup>2+</sup>, and Pb<sup>2+</sup> by fusing with metal binding peptides/domains or engineering metal binding sites.<sup>[8, 9]</sup> Another approach was also developed by engineering the metal binding site to be proximal to the chromophore of fluorescent protein, working under the fluorescent turn-off and turn-on mechanism. Using this

approach, binding sites for Zn<sup>2+</sup>, Cu<sup>2+</sup>, and Hg<sup>2+</sup> have been engineered to date using natural and unnatural amino acids.<sup>[4, 10, 11]</sup> Although a FRET-based fluorescent indicator for Pb<sup>2+</sup> was reported<sup>[9]</sup>, it faces drawbacks such as poor selectivity, weak signal sensitivity in terms of fluorescence ratio and lack of characterization of the FRET probe under intracellular conditions.<sup>[12]</sup> To overcome these problems, a highly selective and sensitive lead sensor can be attained efficiently by utilizing the later approach.

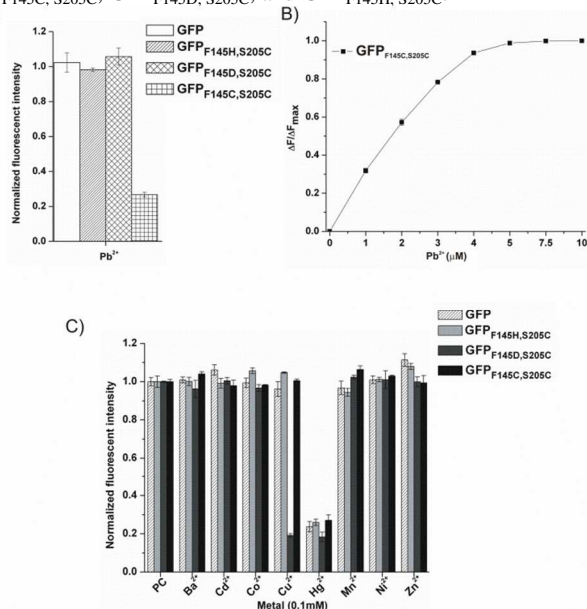
The primary objective of this study was to develop a highly selective and sensitive lead sensor through the introduction of a lead binding site near the chromophore. First, we analysed the GFP chromophore coordinating with a water molecule to form a hydrogen bond network with the side chain atoms of Ser205 and Thr203 and the main chain atom of Asn146 (Fig. 1A). Engineering a metal binding site in this region may help in the development of efficient lead binding and enable a fluorescent turn-off mechanism. Deriving common lead-binding patterns from proteins whose structures co-crystallized with lead may help in the design of a lead binding site.<sup>[13]</sup>



**Fig. 1** Engineering of a lead binding site proximal to the GFP chromophore. (A) Molecular interactions of fluorescent protein with a water molecule and its nearby residues. (B-C) Lead-binding patterns derived from crystal structures of a protein that coordinates lead. (D) Modified GFP chromophore and its proposed lead-binding and fluorescence turn-off mechanism.

We first analysed the protein data bank for lead-coordinating proteins and obtained 36 protein structures (Fig S1).<sup>[14]</sup> From these structures, six different kinds of lead-coordinating sites were observed, containing amino acids such as Cys, His, Asp and Glu which favour the lead binding. Out of the six lead coordinating patterns, pattern I and II were selected for engineering the lead binding site (Fig. 1B,1C).

In general, highly stable proteins must be chosen to undergo further mutation without loss of their functional properties.<sup>[10, 15]</sup> Despite stability, it is better to carry out molecular modelling studies to prevent deleterious effects of mutations and predict the precise orientation of amino acids which favour metal binding. Since the side chains of Ser205, Thr203 and Chromophore Tyr are involved in the interaction, model were developed using *in silico* mutation at Ser205, Thr203 and chromophore Tyr with amino acids such as Cys, His, Asp and Glu, respectively. Ser205 could accommodate only Cys, as replacement with His, Asp, and Glu creates steric clashes with nearby amino acids. Similarly, Thr203 could accommodate the above mentioned amino acids, but the orientation may not favour metal binding. Therefore, it would not be an appropriate site for the engineering of a metal binding site. Further, a bulky residue Phe145 proximal to the chromophore that could accommodate His, Cys and Asp mutation was observed. In general, the chromophore tyrosine of GFP can be replaced only with aromatic amino acids to maintain its fluorescence. It can therefore be replaced with His. So, Generating double mutants with a combination of His, Cys and Asp at Ser205, Phe145 and Tyr66 might enable the production of an efficient lead sensor. Finally, four different variants were generated: GFP<sub>Y66H, S205C</sub>, GFP<sub>F145C, S205C</sub>, GFP<sub>F145D, S205C</sub>, and GFP<sub>F145H, S205C</sub>.

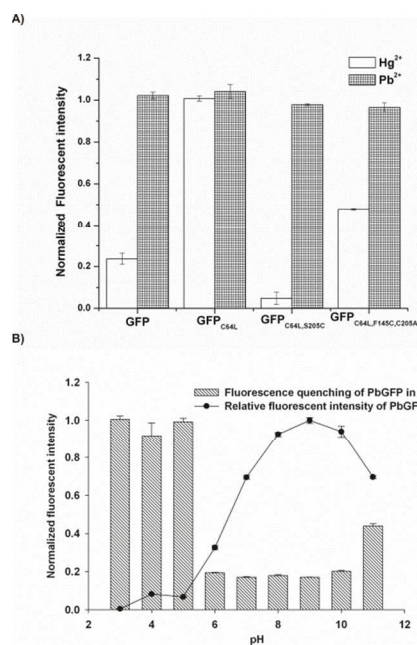


**Fig. 2** (A) Fluorescence intensities of 1  $\mu\text{M}$  GFP variants treated with 100  $\mu\text{M}$  lead. (B) Fluorescence quenching of 1  $\mu\text{M}$  GFP<sub>F145C,S205C</sub> treated with different concentration of lead (0-10  $\mu\text{M}$ ). (C) Metal selectivity assay of GFP variants to different transition metals.

Among them, GFP<sub>Y66H, S205C</sub> failed to show fluorescence, which demonstrated that our GFP variant cannot accommodate His at the 66<sup>th</sup> position. Interestingly, GFP<sub>F145C,S205C</sub> showed fluorescence quenching with lead (Fig. 2A). Further, the sensitivity of GFP<sub>F145C, S205C</sub> was measured by treatment with various concentration of lead (1-10  $\mu\text{M}$ ). This GFP<sub>F145C,S205C</sub> had high lead binding affinity, with a lead binding constant of  $654 \pm 0.16$  nM (Fig. 2B). The selectivity of

the generated variants was then measured by treating them with 100  $\mu\text{M}$  concentration of 14 different metals. Likewise, a similar fluorescence quenching was for GFP<sub>F145D,S205C</sub> when copper was added instead of lead. As this research was focusing on the development of a lead sensor, GFP<sub>F145C,S205C</sub> was further characterized. No comparable response was observed for GFP<sub>F145C,S205C</sub> with other metals, except mercury (Fig. 2C).

In contrast, all of the generated variants, including wild type GFP, demonstrated fluorescence quenching with respect to 100  $\mu\text{M}$  of mercury (Fig. 2C). Further, the binding constant of GFP<sub>F145C, S205C</sub> for mercury was found to be  $20.95 \pm 5.71$   $\mu\text{M}$ , which is 30 times less than for the lead atom. This showed GFP<sub>F145C,S205C</sub> to be sensitive enough towards lead when compared to mercury. It is well known that the sulphhydryl group plays a role in mercury binding.<sup>[14]</sup> To get rid of the mercury binding and improve the selectivity towards lead, the protein structure was analysed for cysteine residues. Since the wild type GFP itself contains three cysteine residues at 48, 64 and 70, mutation of the residues was tested. Interestingly, mutation of the cysteine at the 64<sup>th</sup> position, proximally close to the chromophore with leucine, resulted in a mercury insensitive variant. However, the C64L mutation in GFP<sub>F145C,S205C</sub> did not improve its selectivity due to the presence of Cys205 and 145 (Fig. 3A). To deduce the roles of Cys at 145 and 205 in lead sensing, mutations were conducted with Phe and Ala, respectively. These variants lost their lead-sensing nature, exhibiting only mercury-sensing activity. From the above results, it is clear that these two residues were highly responsible for the lead sensing. Therefore, it was not possible to make the variant selective towards lead against mercury. The variant was named PbGFP, and further characterization was carried out.

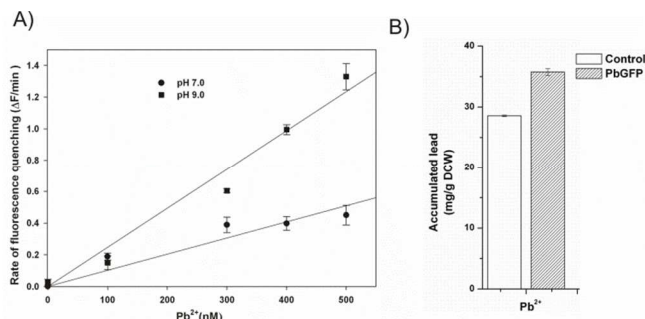


**Fig. 3** (A) Effect of Hg<sup>2+</sup> and Pb<sup>2+</sup> on the GFP variants. (B) Effect of pH on GFP in the presence and absence of Pb<sup>2+</sup>. Line representation shows fluorescent intensity of PbGFP in the absence of Pb<sup>2+</sup>. Bar chart represents the fluorescent quenching of PbGFP in the presence of 100  $\mu\text{M}$  Pb<sup>2+</sup>.

The binding affinity of PbGFP with lead and mercury was then measured. Similar to GFP<sub>F145C,S205C</sub>, PbGFP also showed similar binding activity with mercury and lead. The binding activity was  $700 \pm 0.2$  nM for lead and  $20 \pm 3.8$   $\mu\text{M}$  for mercury. To further investigate whether the fluorescent quenching was due to the binding

of lead with PbGFP, the metal chelating agent EDTA was used to chelate the lead to prevent binding with PbGFP, which may prove that lead binding induces fluorescent quenching. Simultaneous addition of 500  $\mu\text{M}$  EDTA showed 90% fluorescence, which demonstrated  $\text{Pb}^{2+}$  binding induced fluorescence quenching (Fig. S8). In general, metal binding induces conformational changes in the secondary structure of protein. The far-UV CD spectral analysis of PbGFP in 20 mM MOPS showed similar results to those previously reported, and a similar peak in the presence of  $\text{Pb}^{2+}$  (Fig. S9). This result indicated that the fluorescence quenching is not based on a structural change, but may be due to the formation of a non-fluorescent lead-GFP complex. Further, to examine the effect of pH on the fluorescent intensity of PbGFP and its metal binding property, the protein was treated at different pH ranges from 4.0 -11.0 in the presence and absence of lead. Interestingly, PbGFP showed high fluorescent emission intensity at pH 9.0 compared to pH 7.0. Although the fluorescent emission intensity was high at pH 9.0, the quenching efficiency in the presence of lead was similar from pH 6.0 to 10.0 (Fig.3B). In addition, the binding constant of PbGFP for lead was measured at pH 9.0 ( $815 \pm 0.2$  nM), and was found to be almost similar to that at pH 7.0. This shows there was not much binding difference between pH 7.0 and pH 9.0.

Previously, metal ion quantitation of the reported metal sensors was obtained through static parameters such as the amount of fluorescence quenching and the ratiometric fluorescence change. In this study, we attempted to utilize the dynamic parameter quenching rate for the determination of metal concentration. Quantitation of the metal concentration based on the quenching parameter is more sensitive than that of the parameters utilized earlier. The experiment was carried out by measuring the quenching rate against various concentrations of the lead. At pH 9.0, the rate of fluorescence of PbGFP was higher than at pH 7.0, while the rate of quenching was linear against various concentrations of lead (Fig. 4A).



**Fig. 4** (A) Fluorescence quenching rate of PbGFP in the presence of  $\text{Pb}^{2+}$  at pH 7.0 and pH 9.0. (B) Bioaccumulation of lead using *E.coli* expressing PbGFP.

In addition to metal sensing, the bioaccumulation of metal is also an important task to remove toxic elements from the environment. Mostly, metal-binding peptides and metallo proteins have been widely used for the bioaccumulation of heavy metals from the environment. In the case of PbGFP, it could be utilized as a metal sensor as well as a metal accumulating protein due to its high affinity towards lead. Therefore, the bioaccumulation property of PbGFP was measured and compared with an *E.coli* control. *E.coli* showed the accumulation of 28 mg of lead per gram dry cell weight, which validated the earlier report.<sup>[16]</sup> The PbGFP-expressing *E.coli* produced herein accumulated 9mg/g dry cell weight in excess of the *E.coli* without PbGFP (Fig. 4B). This demonstrated that PbGFP can be efficiently utilized for both metal sensing and sequestering purposes.

## Conclusions

In summary, a novel lead-sensitive fluorescent protein-based sensor was successfully developed by engineering a lead binding site near the chromophore. This variant is highly sensitive to lead and selective towards heavy metals such as lead and mercury. The lead-sensing GFP developed shows linear fluorescence quenching with respect to increasing concentrations of lead. In addition, PbGFP can also be utilized for lead bioaccumulation purposes. Further, GFP<sub>S205C,F145C</sub> and GFP<sub>C64L,C205A,F145C</sub> also showed copper sensing activity, which depict that the fluorescent protein is an excellent alternative tool for the development of metal sensors. In this study, we wish to highlight the structural insights of the GFP chromophore that could be used further for the design and evolution of metal sensors.

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