Dual colorimetric and luminescent assay for dipicolinate, a biomarker of bacterial spores

Kasey J. Clear, Sarah Stroud and Bradley D. Smith*

A binary mixture of Tb\(^{3+}\) and pyrocatechol violet (PV) forms a 1 : 1 Tb\(^{3+}\)/PV complex that can be used in a dye displacement assay. Addition of dipicolinate (DPA) to the Tb\(^{3+}\)/DPA complex simultaneously produces a PV color change from blue to yellow and luminescence emission from the newly formed Tb\(^{3+}\)/DPA complex.

Many endospore-forming bacteria produce infectious disease in animals and humans.\(^1\) For example, species of Bacillus and Clostridium genera are the causative agents of anthrax and tetanus, respectively. Endospores are fascinating examples of biological evolution, and they are endowed with a remarkable set of structural properties that allow them to survive for very long periods of time even under extreme conditions.\(^2\) The possibility of endospores being exploited as biological warfare agents is an ongoing national security concern, and there is an urgent need for new and improved methods of killing endospores.\(^3\) One way to find novel antibiotics is to screen chemical or photochemical libraries using a high throughput assay that identifies lead candidates for further testing and development.\(^4\)

In the case of endospores, the classical method for determining antibiotic efficacy employs a 24 hours agar plating process to measure the number of viable colony forming units after treatment.\(^5,6\) This method consumes considerable resources and is difficult to develop into a high throughput assay. Alternative assays in the literature focus on the detection of biomarkers that are associated with endospores.\(^6\) One of the best known biomarkers is dipicolinate (DPA), a small molecular weight dianion that makes up approximately 5–15\% of spore dry weight.\(^7\) Previous studies have demonstrated that DPA is released by dead endospores and that the amount of released DPA is a surrogate biomarker for degree of endospore death.\(^8\) Thus, we have initiated a project to develop a high throughput screening assay that reports the amount of DPA released by a treated sample of endospores. We expect that the assay will utilize multiwell plates with each microwell containing a separate population of treated endospores. Since the assay can be manipulated to generate reasonably high levels of DPA, the most important design feature is not DPA sensitivity but rather technical convenience. Here, we report our first advance in the project, namely, a dye displacement assay that produces a dual luminescent and colorimetric response to DPA.

The most common modern method for measuring DPA levels employs a Tb\(^{3+}\) luminescence assay that is based on selective transfer of DPA excitation energy to a strongly bound Tb\(^{3+}\).\(^7,9\) While this assay is effective for detecting low levels of DPA, the need for a suitable excitation/detection system is a potential burden for labs with limited resources.\(^10\) A colorimetric assay that allows DPA detection using naked eye or a cheap digital camera is inherently attractive. The classic literature colorimetric method reacts DPA with Fe\(^{2+}\) ions to form a colored complex with 440 nm absorption.\(^11\) Although straightforward, this method is quite insensitive, so we decided to develop a new, more sensitive assay. We were drawn to literature reports of dye displacement assays that employ binary mixtures of visible\(^12\) or fluorescent\(^13\) dyes and lanthanide cations. Specifically, independent research groups have shown that a complex of Yb\(^{3+}\) and pyrocatechol violet (PV) can be used to optically detect phosphate and polyphosphate anions in buffered aqueous solution.\(^12,14\) The phosphate anions displace the PV dye from the Yb\(^{3+}\) and produce a color change from blue to yellow. This precedence led us to consider the dye displacement assay that is illustrated in Fig. 1 with photoactive Tb\(^{3+}\) as a replacement for the Yb\(^{3+}\). Since DPA is known to have high affinity for Tb\(^{3+}\),\(^15\) we wondered if it could displace a bound PV dye and produce two simultaneous optical responses: (a) a detectable PV color change from blue to yellow, (b) selective luminescence emission from the Tb\(^{3+}\)/DPA. The general concept of a dual colorimetric and luminescent sensing system has been reported previously,\(^16\) but to the best of our knowledge this is the first example utilizing a dye displacement process.

The photograph and associated absorption data in Fig. 2 shows that PV does undergo a substantial color change from...
yellow to blue in the presence of TbCl₃ in buffered aqueous solution. Job’s method of continuous variation was applied to confirm the binding stoichiometry of the ensemble as 1:1 Tb³⁺/PV (Fig. 3). In addition, an absorbance titration curve fitted nicely to a 1:1 binding model (Fig. 3) and provided an association constant of \((4.2 \pm 1.4) \times 10^4\) M⁻¹ in 10 mM HEPES buffer, pH 7.0, 295 K.

The data in Fig. 4 show that DPA is able to displace the PV and return the color from blue back to yellow. The striking color changes take place almost instantly, can be seen easily with the naked eye and are readily detected as ratiometric responses using a simple spectrometer. The DPA detection dynamic range of 0–100 μM corresponds approximately to the amount of DPA in a 1 mL microwell containing \(10^8\) Bacillus spores. It is notable that the sensitivity of this colorimetric Tb³⁺/PV displacement system (5 μM limit of detection) is more than ten times greater than the classic DPA/Fe²⁺ formation assay.

Shown in Fig. 5 is a set of luminescence spectra for the same titration of DPA into a solution of Tb³⁺/PV. The DPA is excited by the 278 nm light and undergoes intersystem crossing to a triplet state from which there is selective energy transfer to the emissive 5D⁴ level of the bound Tb³⁺. Relaxation of the excited lanthanide leads to the characteristic emission profile seen in Fig. 5. As shown by the inserted graph, emission intensity of the strong 545 nm peak corresponding to the 7F₅ \(\rightarrow\) 5D⁴ transition increased linearly with added DPA. It appears that the displaced PV does not affect the photophysics of the newly formed Tb³⁺/DPA complex, an important finding because PV can act as a fluorescence quencher under certain circumstances.

With further optimization, the dual response system reported here may be well suited as a high throughput assay for the DPA that is released from dead spores. In principle, the color change from blue to yellow should allow very rapid
identification of microwells that have higher amounts of dead spores and released DPA (in some cases heat treatment may be needed to accelerate the DPA release). Follow up studies of specific wells that have been identified as positive hits during the initial colorimetric screen should subsequently include a luminescence scan using a spectrometer to confirm that the colorimetric response is due to formation of a Tb\(^{3+}\)/DPA complex and not a false positive caused by other analytes (e.g., phosphates, aromatic dicarboxylates) or the antibiotic candidate displacing the PV from the Tb\(^{3+}\). It may also be possible to immobilize the Tb\(^{3+}\) on a solid phase and develop a robotic version of this dual colorimetric/luminescence assay.\(^{13b,20}\)

From a broader perspective, it seems possible that the dye displacement system in Fig. 1 could be adapted for other chromophore-containing analytes such as tyrosine phosphate derivatives,\(^{21}\) nucleotides,\(^{21b}\) and salicylates.\(^{22}\)

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**Notes and references**