

Dipicolinic acid (DPA) assay revisited and appraised for spore detection

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Delayed gate fluorescence detection of dipicolinic acid (DPA), a universal and specific component of bacterial spores, has been appraised for use in a rapid analytical method for the detection of low concentrations of bacterial spores. DPA was assayed by fluorimetric detection of its chelates with lanthanide metals. The influence of the choice and concentration of lanthanide and buffer ions on the fluorescence assay was studied as well as the effects of pH and temperature. The optimal system quantified the fluorescence of terbium monodipicolinate in a solution of 10 μM terbium chloride buffered with 1 M sodium acetate, pH 5.6 and had a detection limit of 2 nM DPA. This assay allowed the first real-time monitoring of the germination of bacterial spores by continuously quantifying exuded DPA. A detection limit of 10^4 *Bacillus subtilis* spores ml^{-1} was reached, representing a substantial improvement over previous rapid tests.

Introduction

The need for routine detection of microbes is regularly highlighted by reports of outbreaks of contamination causing illness and, in some cases, fatality. In recent years, industrial practice has tried to move towards the development of *rapid* methods of microbial detection which can be easily deployed in routine monitoring in some comparatively diverse sample environments. The main technique which is presently available uses bioluminescence since, with one important exception, the cells of all living things contain adenosine triphosphate (ATP) which can be readily detected in a pathway linked with the enzyme firefly luciferase. The method has proven so successful that bioluminescence is gradually being accepted as the standard for detection of all organisms containing ATP.

The most important organisms that cannot be detected using this technology are bacterial spores. This is because bacterial spores have extremely low metabolic rates and contain only negligible quantities of ATP,^{1,2} so they escape detection and can persist for decades: they are highly resistant to heat, radiation, chemical attack and UV light treatment. However, certain spore-forming bacterial species are pathogenic and are the causative agents of food poisoning or serious disease. The species most toxic to humans are *Clostridium botulinum*, *C. perfringens*, *C. tetani* and *Bacillus anthracis*. The less toxic species *B. cereus* and *B. subtilis*, although rarely fatal, are nonetheless regular causative agents of food poisoning. The related species *B. thuringiensis* is also of interest due to its use on a large scale as an insecticide,¹ however, at the present time, there is no satisfactory rapid method capable of providing complementary information to that available from the bioluminescence assay and detecting these bacterial spores in an industrial or healthcare environment.³

The aim of this work was to demonstrate an analytical method which could be developed for the detection of bacterial spores. The required sensitivity for such an assay is largely a function of its particular application: *B. cereus* (infective dose 10^5 – 10^{11} viable cells or spores^{4,5} depending on the enterotoxins) is commonly found in dairy and rice products, *B. subtilis* is found in pastry products, meat, apple juice, seafood and rice and is the causative agent of 'ropy bread'.⁴ An appropriate analytical system for screening dangerous levels of *Bacillus* sp.

should be able to detect substantially below the level which is considered hazardous and a reasonable goal would be to detect $<10^4$ spores ml^{-1} ; therefore, the work reported herein was designed to assess this target initially. However, many other important spore-forming species are more toxic or the levels more critical. For example, only a *single* spore of *C. botulinum*^{6–9} need survive food processing and subsequently germinate in a food product and be consumed to lead to botulism, so that future analytical targets may consider considerably lower limits!

The required limits seem particularly suitable for immunoassay (100 spores of a particular strain of *B. anthracis* have been detected¹⁰), but currently no *universal* strain-independent surface antigen has been identified which could be targeted in a universal immunoassay type reaction.¹¹ The chosen assay method must be able to detect all bacterial spores (compare with the ATP target analyte of the bioluminescence assay).

A universal constituent of bacterial spores is dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid);^{12,13} it represents 5–15% of the total mass.¹⁴ The link was first established by Powell,¹³ who showed that it was secreted by *B. megaterium*, and it has been isolated from all wild type *Bacillus* and *Clostridium* species analysed and also in *Sporosarcina ureae*. It has also been found to be secreted by some spore-forming moulds such as *Penicillium citreo-viridae* where it acts as a metal scavenger.¹⁵ Some mutants which do not contain DPA have been isolated, but these are rare, so that DPA appears to offer a useful analytical indicator of the presence of bacterial spores; it has never been detected in vegetative cells.

DPA from the spore core is suitable for detection *via* a range of analytical techniques,^{16–32} with HPLC methods perhaps currently reporting the best detection levels on real samples (7×10^5 spores of *M. polyspora* per gram of salmon).^{17,18} However, complexes of DPA with lanthanide metals, such as terbium, europium and dysprosium, are highly fluorescent and this property, although first used as an assay method for terbium²⁹ ($\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 545 \text{ nm}$), gives data showing a clear detection of 300 nM DPA, although this limit was neither quoted nor an aim of the study. The first use of Tb^{3+} for the express purpose of quantifying DPA³⁰ showed a detection limit of 6 μM and in a *B. subtilis* spore suspension³¹ gave a lower

detection limit of 4.4×10^5 cfu ml⁻¹ (1 cfu = 1 colony-forming unit). Although 1 cfu is approximately equal to 1 spore, aggregation and incomplete germination reduce the accuracy of this measure. However, in follow-up work,³² some freshly prepared spore suspensions, including *B. subtilis*, did not give any signal at all, which casts doubt on this result.

Although the determination of DPA *via* the fluorescence of the lanthanide complex has not been adopted as a standard method, it has clearly been explored, and seems the most promising approach for further investigation to establish an effective assay for DPA able to complement the bioluminescence assay for ATP. The study reported herein 'revisits' the fluorescence due to lanthanide–DPA complexes with a particular aim of optimisation of the assay for the purpose of the determination of spore contamination.

Materials and methods

Chemicals

Common salts and buffers were purchased from Sigma (Poole, UK) or Aldrich (Poole, UK). Pyridine-2,6-dicarboxylic acid, 99% (dipicolinic acid, DPA), terbium(III) chloride hexahydrate, 99.9%, europium(III) chloride hexahydrate, 99.99%, and dysprosium(III) chloride hexahydrate, 99.9%, were purchased from Aldrich.

Buffers were prepared in distilled water as follows: sodium acetate buffer was prepared at the appropriate concentration and an equal concentration of acetic acid was added until the required pH was reached; Trizma base and Trizma.HCl were prepared at the desired concentration and mixed to give the required pH; glycine buffer was pH adjusted by addition of HCl or NaOH at the desired concentration of glycine.

Fluorimetry

Fluorimetry was performed using a Perkin-Elmer LS50B fluorimeter (Cambridge, UK). The light output is given in arbitrary units on a scale of 0–1000. Other parameters are stated under each graph. A quartz cuvette or disposable poly(methyl methacrylate) (PMMA) cuvette (FSA, Loughborough, UK) was used. The latter absorbs approximately half the excitation light at 275 nm. Fluorescence lifetimes were calculated from the exponential decay in light output. Having established the exponential lines of best fit, the fluorescence lifetimes of the terbium complexes were determined from the fluorescence intensity after 0.1 ms and 1.1 ms, with a gate of 0.1 ms in each case; 10 µs was used in the case of dysprosium due to the shorter fluorescence lifetime. This is the minimum gate time available with the instrument used. A small blank (<1% of the signal strength) due to scattered light from the end of the excitation flash was measured using buffer and is subtracted in the results shown. Error assessment (including random errors) was performed separately for each measurement parameter and the combined errors are given with each data set.

B. subtilis spore suspension

B. subtilis type NCTC 3610 from a glycerol stock was shaken at 37 °C overnight in 2XSG broth (Difco (West Moesey, UK) nutrient 1.6% w/v, KCl 0.2% w/v, glucose 0.1% w/v, 10⁻³ M Ca(NO₃)₂, 10⁻⁴ M MnCl₂ and 10⁻⁶ MFeSO₄ pH 7) and centrifuged (4 °C, 6000g, 10 min). The spores were resuspended four times in sterile ultrapure water and recentrifuged (4 °C, 1000g, 20 min) that day, once the next day and three times one week later. The loosely packed top layer, representing cell debris, was removed and the suspension was diluted by a factor

of 20 and examined in a haemocytometer (Improved Neubauer Haemocytometer supplied *via* Merck, Poole, UK). Spores were counted under a microscope in 10 randomly selected squares, each 1/400 mm² and 0.1 mm deep, giving 15.7 ± 1.2 spores per square ($n = 4 \times 10$), which represents $(1.26 \pm 0.08) \times 10^9$ spores ml⁻¹ in the suspension.

Results and discussion

Many lanthanide complexes display fluorescence;³³ although the fluorescence of aqueous lanthanide ions and their *inorganic* compounds is weak, the Judd–Ofelt model,^{34,35} which considers the transition probabilities between electronic state wavefunctions, shows that the intensities of these emissions can vary substantially with the chemical environment of the emitting ion.^{36–39} Lanthanide ion fluorescence displays two useful analytical properties: the fluorescence lifetime may be as much as several milliseconds, and the ion fluorescence may be substantially enhanced by energy transfer from a chelating ligand.^{40,41} Moreover, ligands may absorb light *at a frequency characteristic of the ligand* and transfer it to the triplet states of the lanthanide ion (known as indirect fluorescence), and they have thus proved invaluable as labels⁴² and for quantifying the lanthanides themselves.^{29,43}

Direct excitation of lanthanide–DPA complexes has been reported to give emission bands increased in magnitude by different factors [2–20 (Sm³⁺) or 30–330 (Dy³⁺),^{37–39}], so that the choice of lanthanide⁴⁴ must be confirmed for optimum DPA assay. The emission spectra of lanthanide monodipicolinates in acetate buffer (Table 1) showed that terbium and dysprosium gave the strongest signals and europium produced a weak signal, but several other metals investigated (samarium, holmium, gadolinium, praseodymium) gave negligible fluorescence. From the fitted exponential decay due to the lanthanide chlorides, a fluorescence half-life for Ln(DPA)⁺ and Ln³⁺ (Fig. 1) in the presence and absence of DPA showed that, in the case of dysprosium, the lifetime is extremely short, but in general the extended fluorescence lifetime typical of the lanthanide ion is readily seen. Comparison of the fluorescence enhancement ratio (defined as the fluorescence intensity per terbium atom of a terbium chelate divided by the fluorescence intensity of unchelated terbium in the same buffer conditions) due to the ligand environment (Table 2) has not been reported previously, but the 'inverse' assay, where excess DPA is used in the simultaneous detection of europium, dysprosium, gadolinium and terbium,⁴³ suggested that lanthanide dipicolinates have similar fluorescence yields under these conditions. The present work gives substantially different yields, with terbium clearly the best lanthanide for the detection of DPA due to its bright fluorescence, long fluorescence lifetime and high enhancement ratio.

Lanthanide ions in solution can display a range of co-ordination numbers,⁴⁴ and with DPA as a tridentate ligand^{45,46}

Table 1 Excitation maxima for Ln(DPA)⁺ (delay = 0.1 ms, gate = 9.9 ms)^a

Metal ion in Ln(DPA) ⁺	Wavelength/nm	Area	Total area	% of Tb(DPA) ⁺ signal
Tb(DPA) ⁺	490	3300	8500	100
	545	4700		
	585	490		
Dy(DPA) ⁺	481	1570	2200	26
	573	610		
Eu(DPA) ⁺	593	290	570	7
	616	280		

^a Sm, Gd, Ho, Pr monodipicolinates gave insignificant signals.

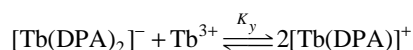
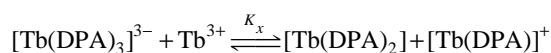
the lanthanide dipicolinates generally reported are:⁴⁷ $[\text{Ln}(\text{DPA})_3]^{3-}$, $[\text{Ln}(\text{DPA})_2(\text{H}_2\text{O})_3]^-$, $[\text{Ln}(\text{DPA})(\text{H}_2\text{O})_6]^+$.

The trisdipicolinates are formed in the presence of excess DPA and have been most extensively characterised.^{48,49} Due to the exclusion of co-ordinating water molecules when DPA binds, they are expected to have brighter fluorescence than the monodipicolinates. Previous reports comparing the fluorescence intensity for the different lanthanides probably considered just the trisdipicolinates, whereas the data above

compare different dipicolinates. This is a critical observation since, unless the fluorescence intensity of $[\text{Tb}(\text{DPA})]^- \leq 1/2[\text{Tb}(\text{DPA})_2]^{2-} \leq 1/3[\text{Tb}(\text{DPA})_3]^{3-}$, the greatest sensitivity in a DPA assay would be achieved at a maximum proportion of $[\text{Tb}(\text{DPA})]^-$ with respect to the other dipicolinates, but consideration of the equilibrium between different lanthanide complexes indicates that, unless excess lanthanide is present, some DPA will be free in solution rather than complexed with lanthanide ions. Tuning the concentration of reagents is thus paramount to achieving the desired limit of detection for DPA.

Effect of terbium concentration

The formation constants K_1 to K_3 for the complexes with terbium are all high (8.68, 7.43 and 5.92 respectively⁵⁰) and $\text{Tb}(\text{DPA})_3^{3-}$ will not exist as the *only* species at the target DPA concentrations for spore detection. This will generate the following equilibria:



$K_x = K_1/K_3 = 10^{2.76}$; $K_y = K_1/K_2 = 10^{1.25}$ which show that the formation of the monodipicolinate will be strongly favoured when the lanthanide is present in excess, but the background fluorescence due to unchelated lanthanide ions at too high a lanthanide concentration could limit the detection level. The effect of different terbium chloride concentrations on DPA determination in 1 M sodium acetate buffer, pH 5.6 is shown in Fig. 2. At low concentrations, the equilibrium moves away from the formation of terbium monodipicolinate reducing the slope of the calibration line. Rosen *et al.*³¹ have detected DPA using 31.4 μM TbCl_3 in 50 mM Trizma, pH 7.7, and state that the limit of detection may be reduced by lowering the concentration of TbCl_3 , but they overlook the several equilibria involved or which terbium dipicolinate is most desirable to form for a DPA assay. In Fig. 2 the critical concentration where the reduced sensitivity begins to be seen occurs at around 1 μM TbCl_3 and is substantial for 100 nM TbCl_3 . The equilibrium constant for the formation of terbium monodipicolinate in water is $10^{8.68}$; therefore, a dilute solution of DPA with terbium should be 25% dissociated at 6.3 nM TbCl_3 , rather than 1 μM as observed. However, several other competitive equilibria must also be considered in this discussion, *e.g.*, acetate from the buffer may compete with DPA to bind terbium, particularly at the high

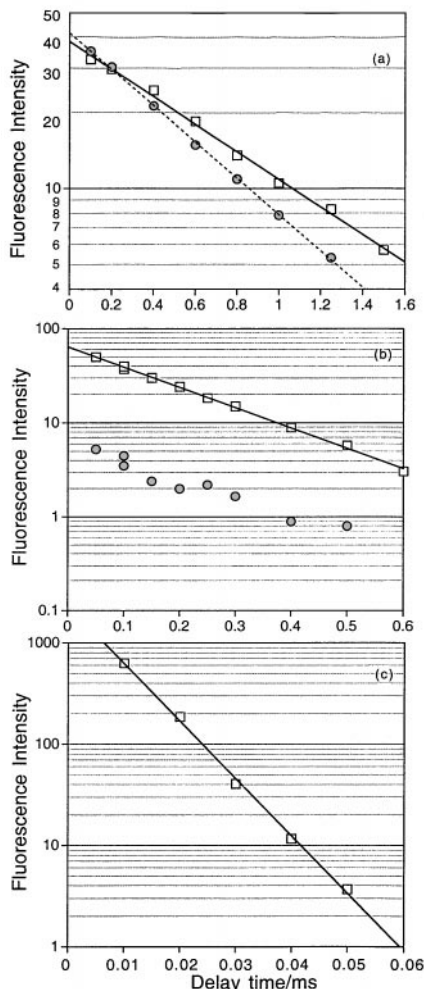


Fig. 1 Decay of fluorescence intensity. Slit width 15 nm; gate time (a) 0.1 ms, (b) 0.5 ms, (c) 10 μs ; delay time varied. (a) 10 mM TbCl_3 (\bullet); 100 μM TbCl_3 + 484 nM $\text{Tb}(\text{DPA})^+$ (\square) 100 μM TbCl_3 + 484 nM DPA gives approximately 484 nM $\text{Tb}(\text{DPA})^+$. $\lambda_{\text{ex}} = 275$ nm, $\lambda_{\text{em}} = 543$ nm. (b) 10 μM $\text{Eu}(\text{DPA})^+$ (\square) and 10 mM EuCl_3 (\bullet). Signal from EuCl_3 was weak and no exponential decay curve could be fitted to the data points with statistical significance. 100 μM EuCl_3 + 10 μM DPA gives approximately 10 μM $\text{Eu}(\text{DPA})^+$. $\lambda_{\text{ex}} = 275$ nm, $\lambda_{\text{em}} = 616$ nm. (c) 10 μM $\text{Dy}(\text{DPA})^+$ (\square). 100 μM DyCl_3 + 10 μM DPA gives approximately 10 μM $\text{Dy}(\text{DPA})^+$. 10 mM DyCl_3 in the absence of DPA produced negligible light output and so is not shown. $\lambda_{\text{ex}} = 270$ nm, $\lambda_{\text{em}} = 481$ nm.

Table 2 Summary of fluorescence data for $\text{Ln}(\text{DPA})^+$ (conditions as for Fig. 1)

Metal	Monodipicolinate fluorescence intensity (% of Tb)	Enhancement ratio	Monodipicolinate fluorescence lifetime/ μs	Aqueous ion fluorescence lifetime/ μs
Tb	100	22 300	790	600
Dy	27	> 1000	7.7	n.d. ^a
Eu	8.1	9000	200	~ 200
Sm	1.9	n.d. ^a	n.d. ^a	n.d. ^a

^a n.d. not determined.

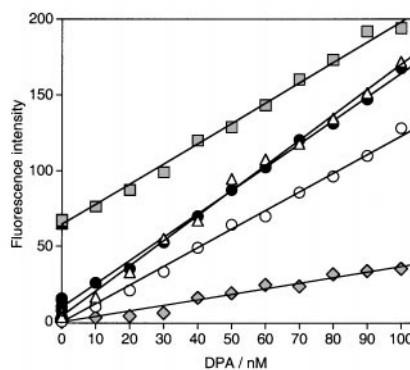


Fig. 2 Preliminary DPA calibration curve for different concentrations of terbium chloride: 1 mM (\square), 100 μM (\triangle), 10 μM (\bullet), 1 μM (\circ), 100 nM (\blacklozenge). $\lambda_{\text{ex}} = 275$ nm, $\lambda_{\text{em}} = 543$ nm. Slit width, 15 nm, delay 0.1 ms, gate 9.9 ms, 1 M sodium acetate pH 5.6. Random intensity measurement error $\pm 1\%$. Combined errors in each data point measurement $\pm 3\%$ (y axis).

concentrations used, and the pH affects the DPA^{2-} population. Nevertheless, the calibration curves presented here clearly offer greater promise than that reported by Sacks³⁰ with a detection limit of 6 μM in 250 μM TbCl_3 in citrate buffer (250 mM).

Influence of buffer

The buffer system found to result in the greatest light output was acetate, pH 6.0 [Fig. 3(a)], and the highest enhancement ratio was given by glycine, pH 4.0. At low pH, the results appear totally intuitive; DPA^{2-} will become protonated to HDPa^- and H_2DPA , which will not chelate terbium in the same manner, but otherwise this shows a substantially different pH response to that which is described in the literature for terbium trisdipicolinate obtained in an excess of DPA. Barela and Sherry²⁹ reported that the fluorescence of 2 μM and 20 μM Tb^{3+} in the presence of excess (224 μM) DPA is constant between pH 5 and pH 10 with no influence from borate, glycine, cacodylate, acetate and Trizma buffers, but that imidazole, biphthalate, bicarbonate, hepes and phosphate buffers all inhibit to some extent, probably by competing with DPA^{2-} for Tb^{3+} binding.

Comparing the changes in fluorescence lifetime [Fig. 3(b)] and intensity (Figure 3a) at high pH allows competition and quenching effects to be distinguished. Glycine at pH 9 and pH 10 shows a marked decrease in fluorescence intensity without a decrease in fluorescence lifetime, so quenching is not indicated, suggesting that the amount of fluorescence decreases with pH, probably as a result of binding hydroxyl or glycinate ions. With Trizma, a similar reduction in terbium dipicolinate should occur

with increased pH, since Trizma can form strong tridentate chelates to displace DPA or form a mixed-ligand Tb:DPA:Trizma chelate, but these chelates also cause quenching so that both a reduced fluorescence intensity and an enhanced rate of quenching are seen. Rosen *et al.*³¹ used 31.4 μM TbCl_3 in 50 mM Trizma, pH 7.7 for assay of DPA and reported that the terbium ion precipitates above 1 mM in Trizma, which supports the idea that Trizma strongly chelates terbium. Since Barela and Sherry²⁹ found that terbium trisdipicolinate was not affected and the fluorescence lifetime of terbium monodipicolinate in Trizma is similar at pH 8 and pH 7 despite a substantial drop in fluorescence intensity, this suggests that Trizma is involved as a chelate. Due to the poor solubility and the poor fluorescence properties found here, Trizma does not appear to be a suitable buffer for lowering the detection limit for DPA, but acetate buffer, pH 5.6 is most promising for further use (a slightly higher fluorescence intensity was found for pH 6.0, but since this is close to the upper pH limit of the buffering capabilities a compromise on pH is desirable). This is also the pH used by Barela and Sherry²⁹ and gives the longest fluorescence lifetime.

Fig. 4 shows that the fluorescence intensity also increases with buffer concentration and the concomitant increase in fluorescence lifetime suggests that the increase may be related to improved shielding from quenching by exclusion of water from the co-ordination shell. Thus, the brightest fluorescence is achieved at the highest concentrations of acetate; this contrasts with the effect of buffer concentration on unchelated aqueous terbium which tended to decrease with buffer concentration.

DPA assay

Fig. 5 shows the detection of both DPA and an equimolar mixture of CaCl_2 and DPA. When spores germinate, they rapidly secrete Ca^{2+} and DPA^{2-} in a 1:1 ratio but the terbium concentration (10 μM TbCl_3) selected here is in sufficient excess (the equilibrium constant for the formation of calcium dipicolinate is $10^{4.4}$ compared with $10^{8.7}$ for terbium monodipicolinate⁵⁰) so that the assay is tolerant to calcium. However, correctly quantifying the assay detection limit is difficult since the SD of the background noise was of the same order or less than the minimum resolution of the particular instrument. Thus, from an inspection of the data, a detection limit of < 2 nM DPA is suggested using PMMA disposable cuvettes (which absorb partially at 275 nm, halving the fluorescence intensity) and of < 1 nM for quartz cuvettes.

Fig. 5(b) shows an upper detection limit for the assay of approximately 30 μM DPA, as the supply of unchelated terbium

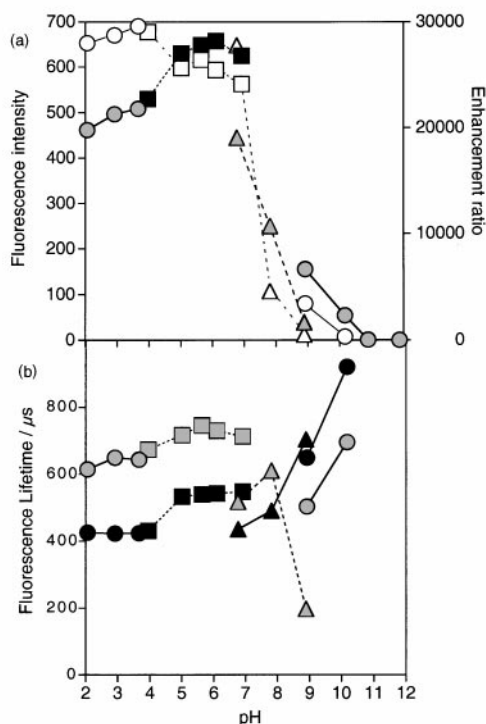


Fig. 3 Variation with pH of (a) fluorescence intensity, enhancement ratio and (b) fluorescence lifetime of 1 μM terbium monodipicolinate and 10 nM TbCl_3 in several buffers. (a) Fluorescence intensity: left axis. Glycine (●), acetate (■), Trizma/Cl (▲) buffers. Enhancement ratio: right axis. Glycine (○), acetate (□), Trizma/Cl (△) buffers. (b) Fluorescence lifetime 1 μM terbium monodipicolinate: glycine (●), acetate (■), Trizma/Cl (▲) buffers. 10 nM TbCl_3 : glycine (●), acetate (■), Trizma/Cl (▲) buffers. Conditions: 1 μM DPA + 100 μM TbCl_3 prepared at 100 \times concentration in deionised water and then diluted into 50 mM buffer. Excitation 275 nm, emission 543 nm, 15 nm slit width, delay 0.1 ms, gate 4.9 ms. pH checked before and after measurement giving less than ± 0.05 variation. Enhancement ratio calculated by comparison with 10 mM TbCl_3 in the same conditions. Combined errors in each data point measurement $\pm 1\%$ (x axis); $\pm 1\%$ intensity (y axis) and $\pm 2\%$ enhancement ratio (y axis).

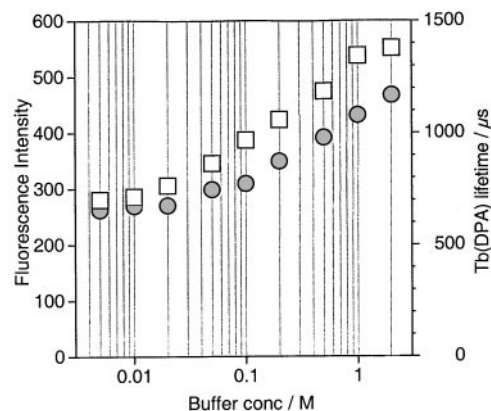


Fig. 4 Variation in fluorescence intensity and lifetime of 500 nM terbium monodipicolinate with sodium acetate buffer concentration. Fluorescence intensity: left axis, □. Fluorescence lifetime: right axis, ●. 40 μl of a stock solution of 10 mM TbCl_3 + 500 nM DPA in distilled water was added to 3960 μl buffer [giving 500 nM Tb(DPA)^-]. $\lambda_{\text{ex}} = 275$ nm, $\lambda_{\text{em}} = 543$ nm, 15 nm slit widths, delay 0.1 ms, gate 9.9 ms.

becomes limiting; this indicates that a detection range of four orders of magnitude is possible for a terbium concentration of 10 μM . The range can be extended to a higher concentration of DPA by the addition of more terbium chloride, but as shown in Fig. 2 the lower detection limit would be compromised.

Detection of bacterial spores

The tuned assay is now in range to appraise its application in the detection of bacterial spores; this was achieved with a fresh suspension of *B. subtilis* (1.3×10^9 spores ml^{-1}) which was diluted with distilled water, heat shocked at 70 $^{\circ}\text{C}$ for 30 min and then further diluted with DPA assay solution (10 μM TbCl_3 in 4 ml of 1 M sodium acetate buffer, pH 5.6). DPA is released on germination, which was triggered by the addition of 1 mM L-alanine at room temperature. Fig. 6(a) shows that it is possible to monitor bacterial spore germination in real time using terbium monodipicolinate fluorescence. Fluorescence from 90 min germination samples gave a quantitative correlation with spore count [Fig. 6(b)] (log-log shows the upper detection limit explored by reducing the sensitivity of the emission detector). To check whether the L-alanine-triggered germination was reliable in releasing all DPA from the spores, a 1000-fold diluted spore sample was autoclaved and a small volume added

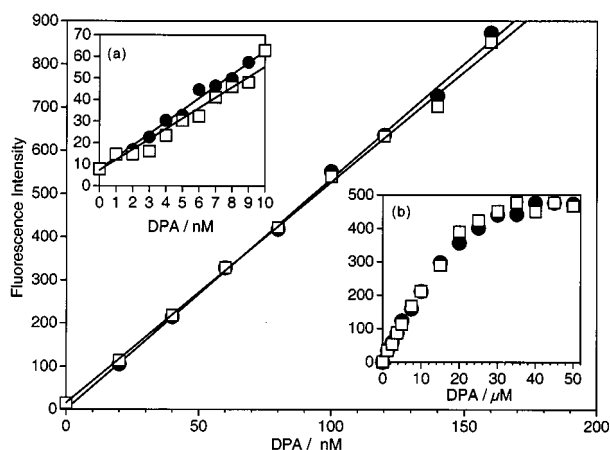


Fig. 5 Fluorescence detection of DPA (\square) and CaDPA (\bullet). $\lambda_{\text{ex}} = 275$ nm (15 nm slit width), total emission mirror (20 nm slit width), delay 0.1 ms, gate 9.9 ms. 1 M sodium acetate, pH 5.6, 10 μM TbCl_3 . Samples with calcium contained equimolar DPA and CaCl_2 . Inset (a) shows the expansion of low concentration range; inset (b) shows the high concentration range extended by using $\lambda_{\text{em}} = 543$ nm. Combined errors in each data point measurement $\pm 2\%$ (x axis); $\pm 1\%$ intensity (y axis).

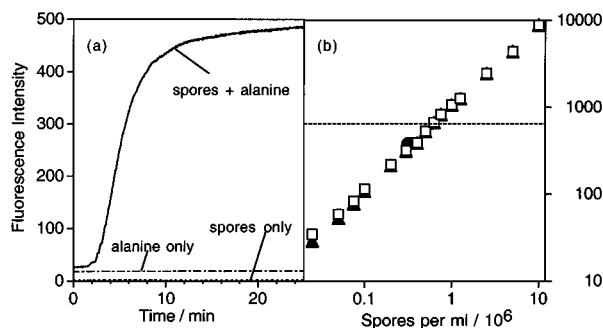


Fig. 6 (a) Germination of 1.6×10^6 spores of *B. subtilis* in 4 ml of DPA assay solution; germination solution contained 1 mM L-alanine. Blanks recorded with just 1 mM L-alanine and spores only. (b) Fluorescence detection of germinated bacterial spores (\square) Total emission mirror (20 nm slit width), delay 0.1 ms, gate 9.9 ms. (\blacktriangle) $\lambda_{\text{em}} = 543$ nm (20 nm slit width), delay 0.1 ms, gate 0.5 ms and scaled to give comparison with total emission data. 3.2×10^5 autoclaved spores (\bullet) and 200 nM DPA (-----) are shown for comparison. Combined errors in each data point measurement $\pm 5\%$ (x axis); $\pm 1\%$ intensity (y axis).

to the same DPA assay solution. The resulting fluorescence intensity coincides with the calibration in Fig. 6(b).

From the autoclaved sample, it is deduced that each spore of *B. subtilis* contained 3.65×10^{-16} moles of DPA (therefore 1 spore $\text{ml}^{-1} = 365$ fM DPA) (35% of the amount in a spore of the larger species, *B. megaterium*, 1.05×10^{-15} moles⁵¹). The detection limit is 10 000 spores ml^{-1} (based on the PMMA cuvettes), which represents an improvement of at least an order of magnitude compared to the lowest reported detection level for a DPA-based spore assay (121 000 spores ml^{-1} .³²)

Conclusions

Measurement of the fluorescence of the terbium chelate of DPA was shown to be capable of quantifying < 2 nM DPA, but it was clear that lower limits might be realisable through dedicated instrumentation and taking advantage of the alternative methodologies in processing the fluorescence signal; the large difference between excitation and emission frequency (Stokes shift) is favourable for achieving low sample blanks and thus maximising the detection limits.

The assay was optimised to be tolerant of calcium and to be able to determine DPA concentrations across four orders of magnitude; it had a lower limit of detection of 10^4 spores ml^{-1} (*B. subtilis*) and it was shown that ungerminated *B. subtilis* spores gave only a low fluorescence with terbium, but their presence could be unambiguously determined by addition of germinant, whereupon the spore DPA was exuded. This sheds further light on the often irreproducible data obtained from DPA spore assays of stored spore suspensions without germination,³² which determine just DPA which has either leached from bacterial spores during storage or is present in spore coats and is immediately available to complex TbCl_3 .

Previous to this work, the lowest limits of detection reported for bacterial spores by a DPA assay were 7×10^5 spores ml^{-1} (*B. cereus*),¹⁷ 4.4×10^5 spores ml^{-1} (*B. subtilis*) and 1.2×10^5 spores ml^{-1} (*B. Globigii*).^{31,32} The success of this assay and its ease of use are encouraging. Nevertheless, greater sensitivity would be desired for wider application; a dedicated low cost detection system can improve the instrumental limit seen here, but more exciting for the future development of this assay into a format which can complement the ATP bioluminescence assay is the prospect that the chelating properties of DPA allow a solid phase assay to be imagined, whereby the exuded DPA is first 'captured' from the sample to the solid phase. The DPA assay proposed here is well suited to the determination of DPA captured by the solid phase and the technique allows considerable 'concentration' of the sample and thus a lower limit of detection is expected. This development will be reported shortly.

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