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Effects of terbium chelate structure on dipicolinate ligation and the detection of *Bacillus* spores

L.S. Barnes [a], K.R. Kaneshige [a], J.S. Strong [a], K. Tan [a], H.F. von Bremen [b], R. Mogul [a,*]

[a] Chemistry Department, California State Polytechnic University, Pomona, 3801 W. Temple Ave., Pomona, CA 91768, USA
[b] Department of Mathematics and Statistics, California State Polytechnic University, Pomona, 3801 W. Temple Ave., Pomona, CA 91768, USA

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ABSTRACT

Terbium-sensitized luminescence and its applicability towards the detection of *Bacillus* spores such as anthrax are of significant interest to research in biodefense and medical diagnostics. Accordingly, we have measured the effects of terbium chelation upon the parameters associated with dipicolinate ligation and spore detection. Namely, the dissociation constants, intrinsic brightness, luminescent lifetimes, and biological stabilities for several Tb(chelate)(dipicolinate)$_x$ complexes were determined using linear, cyclic, and aromatic chelators of differing structure and coordination number. This included the chelator array of NTA, BisTris, EGTA, EDTA, BAPTA, DO2A, DTPA, DO3A, and DOTA (respectively, 2,2′,2″-nitrilotriacetic acid; 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ethylenediamine-N,N,N′,N′-tetraacetic acid; 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; 1,4,7,10-tetraazacyclododecane-1,7-diacetic acid; diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid; 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid; and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid). Our study has revealed that the thermodynamic and temporal emission stabilities of the Tb(chelate)(dipicolinate)$_x$ complexes are directly related to chelate rigidity and a ligand stoichiometry of $x = 1$, and that chelators possessing either aromaticity or low coordination numbers are destabilizing to the complexes when in extracts of an extremotolerant *Bacillus* spore. Together, our results demonstrate that both Tb(EDTA) and Tb(DO2A) are chemically and biochemically stable and thus applicable as respectively low and high-cost luminescent reporters for spore detection, and thereby of significance to institutions with developing biodefense programs.

1. Introduction

The use of terbium-sensitized luminescence for the detection of *Bacillus* spores, such as anthrax, has garnered much interest in recent years due its applications in biodefense [1,2] and microbial diagnostics [3,4]. The underlying strategy to the terbium-based detection is the formation of luminescent coordinate complexes between terbium (the reporter reagent) and dipicolinic acid, which is a secondary metabolite found in high abundance in *Bacillus* spores (5–15% by dry weight) [5]. In chemical terms, dipicolinic acid (DPA), pyridine-2,6-dicarboxylic acid, is a tridentate fluorophore that complexes terbium to form Tb(DPA)$_x$(H$_2$O)$_{9-3x}$, where $x = 1$, 2, and 3 [6,7]. These particular complexes are of spectroscopic significance as the triplet state of DPA transfers energy to bound terbium, ultimately resulting in long-lived luminescent emissions with large Stokes shifts in the visible spectrum [8,9]. For these reasons, therefore, DPA serves as an excellent biochemical marker and through use of sensitized lanthanide luminescence is fully applicable towards the development of rapid and cost-effective detection protocols for homeland security.

Essential to the reliable detection of DPA in the field or laboratory settings is the formation of chemically and biochemically stable terbium complexes of known stoichiometry, as biological extracts contain a multitude of metal-binding (and therefore metal-competitive) biomolecules and anions such as carbohydrates, peptides/proteins, gluconic acids, phosphate, oxalate, and more [10,11]. To date, however, $TbCl_3$ remains to be the most commonly used reagent for spore detection [1,12–14] despite its potential for rapid ligand exchange (or biochemical competition) due to the labile coordination sphere when dissolved in solution (Tb(H$_2$O)$_9$). As a consequence, the luminescence-based quantitative analysis of spore counts or DPA concentration are unnecessarily complicated, as complexes of mixed DPA stoichiometry, emission brightness, and emission lifetimes are formed under biological conditions.

To overcome these issues, therefore, the role of chelated terbium was recently introduced as an effective means in which to restrict the number of bound DPA and subsequently inhibit ligand competition [3,15]. While these reports clearly demonstrate the advantages of chelator-inclusion, there remains to be few, if any, studies in the literature that systematically address the effects of chelate structure and chelate

* Corresponding author.
E-mail address: rmogul@csupomona.edu (R. Mogul).

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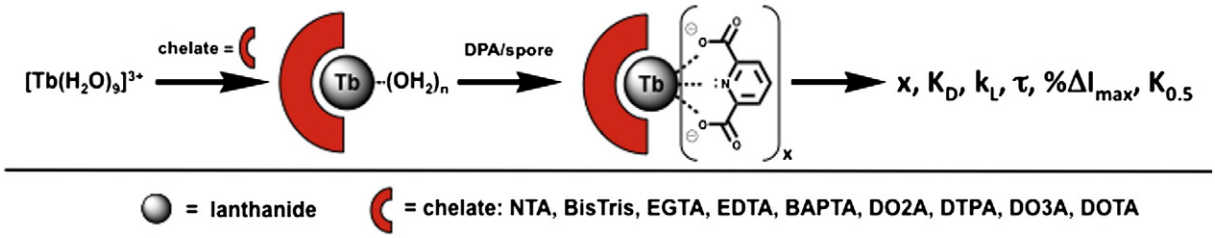


Scheme 1. Strategy designed to elucidate the effects of chelate structure upon the stoichiometry, formation, luminescence brightness, luminescence lifetime, and biological stability of terbium–dipicolinate complexes.

coordination number upon the formation and luminescence of terbium–dipicolinate complexes. Accordingly, we have studied an array of differing terbium chelates using the experimental strategy outlined in Scheme 1 and have elucidated the parameters of stoichiometry (x), formation (K_D), brightness (k_L), lifetime (τ), and biological stability ($\%\Delta I_{max}$, $K_{0.5}$) for several different Tb(chelate)$(DPA)_x$ complexes. Specifically, we have varied the chelate structure by utilizing the linear, cyclic, and aromatic chelators of NTA, BisTris, EDTA, EGTA, BAPTA, DO2A, DTPA, DO3A, and DOTA (Scheme 2), which together encompass the coordination number range of 4–8.

To our knowledge, this work represents the most holistic study to date regarding the effects of chelate structure and chelate coordination number upon terbium–dipicolinate complexes and the detection of extremotolerant *Bacillus* spores. Our analyses have revealed that chelate rigidity, coordination number, and ligand stoichiometry each independently impact the luminescence and biological stabilities of the differing Tb(chelate)$(DPA)_x$ complexes. Moreover, our biological luminescence stability studies were performed in extracts of the extremotolerant and sporulated form of *Bacillus pumilus* SAFR-032, which is a desiccation and radiation resistant microorganism originally isolated from the spacecraft assembly facilities at the Jet Propulsion Laboratory during construction of the Mars Phoenix lander [16–18]. Hence, our fundamental structural analysis of terbium chelation and its impact on spore detection has revealed both chemical and biochemical trends that have immediate application towards the development of reagents for NASA-related planetary protection endeavors, routine environmental monitoring, and first responder assays for homeland security.

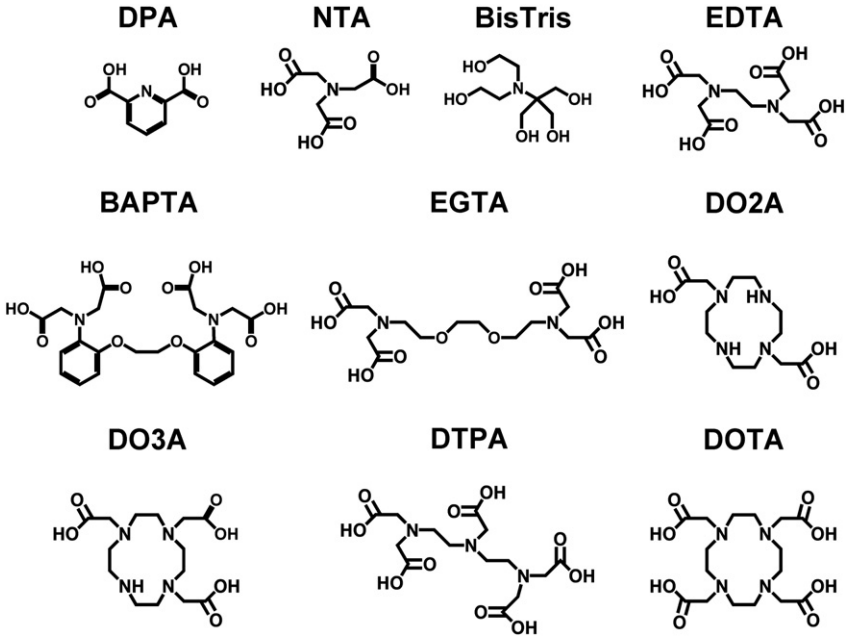
2. Materials and methods

2.1. Materials

All chelates were purchased as the acid or free salt with the exception of DO2A and DO3A, which were obtained as the *t*-butyl protected esters: DPA (Alfa Aesar, 98%), NTA (EMD Chemicals, >98.0%), Bis–Tris (Acros Organics, 99+%), Na_4EDTA (Aesar, 98%), EGTA (Omnipur, >97%), BAPTA (Fluka Analytical, >95.0%), DO2A-*t*-Bu-ester (Macrocyclics), DTPA (Alfa Aesar 97%), DO3A-*t*-Bu-ester (Macrocyclics), and DOTA (Macrocyclics). Terbium (III) chloride hexahydrate (99.90%) and trifluoroacetic acid (99%) were purchased from Acros Organics. Sodium acetate trihydrate and 10× phosphate buffered saline (0.10 M total phosphate, 1.4 M NaCl, 27 mM KCl, pH 7.4) were purchased from VWR. All synthetic glassware were acid washed in a 1:1 mixture of HNO_3:H_2SO_4 for 1 hour at room temperature, thoroughly rinsed with ultrapure water and dried in an oven before use. All terbium stock solutions were prepared in 0.01 M HCl, whereas all ligation reactions were performed in filtered (0.22 μm) 0.10 M sodium acetate buffer (pH 5.5). Ultrapure water was used throughout (~18 MΩ/cm) for all aqueous solutions.

2.2. Synthesis of DO2A and DO3A

Deprotection of DO2A-*t*-Bu-ester (400.6 g/mol) and DO3A-*t*-Bu-ester (514.7 g/mol) (Macrocylics) were carried out in trifluoroacetic acid (TFA) as described. For each protected chelate, 80 mg were



Scheme 2. Chelators and ligands listed in order of coordination number.