Biomarker Detection

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Ratiometric Fluorescent Detection of an Anthrax Biomarker at Molecular Printboards**

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Anthrax is an acute disease, concurrently a potential biological warfare agent caused by *Bacillus anthracis*. The accurate, rapid, sensitive, and selective detection of *Bacillus* spores plays a vital role to prevent a biological attack or outbreak of disease.[1] Bacterial spores contain a main core cell which is enclosed by protective layers. As a major component of these protective layers, bacterial spores contain up to 1M dipicolinic acid (DPA), accounting for 5–15% of the dry mass of the bacterial spore.[2] Hence, DPA is a convenient biomarker for these spores.

In recent years a number of biological and chemical detection methods for *Bacillus anthracis* spores have been investigated. Biological methods are based on polymerase chain reactions (PCR)[3] and immunoassays.[4] Important chemical methods employ vibrational spectroscopy (FT-IR, Raman, and surface enhanced Raman (SERS))[5] and photoluminescence.[6] Among them, lanthanide ion (Ln^{3+}) based luminescent detection of DPA has been most promising owing to the unique photophysical properties of Ln^{3+}–DPA chelates including their bright luminescence upon sensitization by DPA, the long luminescence lifetime compared to free Ln^{3+}, and the concomitantly high luminescence-enhancement ratio upon coordination of DPA to the Ln^{3+} center.[7] Besides the use of DPA itself as a sensitizer, ratiometric fluorescent detection of anthrax spores can be achieved through the displacement of a different sensitizer by DPA.

Molecular recognition processes at monolayers on surfaces offer advantages over solution-based sensing, such as a fast response time, minimization of analyte sorption time to the receptor, and real-time and real-space measurements.[8] Glass is an appropriate substrate for fluorescence detection of chemical species owing to its transparency, inertness to light, and easy modification with a monolayer of organic adsorbates.[9] Microarrays on glass allow for rapid, simultaneous, and multiple analyte sensing on glass slides. Previous studies have indicated that fluorescent monolayers on glass can be employed in the fabrication of microarrays by soft lithography techniques, such as microcontact printing (μCP) which is an efficient and low-cost method to create patterned surfaces. Using μCP on a glass substrate enables the use of fluorescence microscopy for direct visualization of the fluorescent patterns created.[10]

Ratiometric detection of chemical species, that is, the recording of the relative fluorescence intensities at two different wavelengths, has attracted interest owing to an increased accuracy and reproducibility of analyte detection compared to measurements performed at a single wavelength.[11] Strong ratiometric fluorescence responses have been achieved in solid films and fluorescent monolayers as well as in solution.[12]

Herein we present a novel platform for the ratiometric detection of the *Bacillus anthracis* biomarker DPA with high sensitivity and selectivity on a supramolecular monolayer surface. We employ so-called molecular printboards,[13] which are monolayers of β-cyclodextrin (β-CD) on a surface to which building blocks are attached in a noncovalent fashion that allow ratiometric DPA sensing. To our knowledge, this is the first lanthanide-based surface-receptor system for the detection of DPA, as well as the first example of ratiometric DPA detection at a surface.

In a previous study we have demonstrated the surface-assisted sensitized luminescence of Eu^{3+} ions on a molecular printboard.[14] In the current study we fabricate these luminescent patterns for the ratiometric detection of DPA on a receptor surface, as outlined in Scheme 1. Two building blocks have been used in this study: an ethylenediamine tetraacetic acid (EDTA) based ligand (**1**) for binding Eu^{3+}, and a naphthalene-based antenna (**2**) for coordination to Eu^{3+} through the carboxylate moiety. Both building blocks have adamantyl groups for immobilization onto the β-CD monolayer. To fabricate patterned sensing surfaces, a stepwise procedure was followed. Briefly, in the first step, an equimolar mixture of **1** and **2** was printed onto the β-CD monolayer by μCP to generate surface patterns of the ligand pairs. After thorough rinsing with water and drying, the patterned surface was imaged by fluorescence microscopy using filter B ($300 < \lambda_{exc} < 400$ nm, $410 < \lambda_{em} < 510$) and filter R ($300 < \lambda_{exc} < 400$ nm, $\lambda_{em} = 615$ nm). As such, filter B collects the naphthalene emission of **2**, while filter R only collects the Eu^{3+} emission of **1**-Eu^{3+}. Both filters have excellent selectivity. Subsequently, the glass slides were immersed in a $EuCl_3$ solution for 30 min to convert **1** into **1**-Eu^{3+}, monitored by fluorescence imaging. Subsequently, substrates were incubated into aqueous solutions of DPA with different concentrations (pH 6.5) for 10 min with continuous stirring. After

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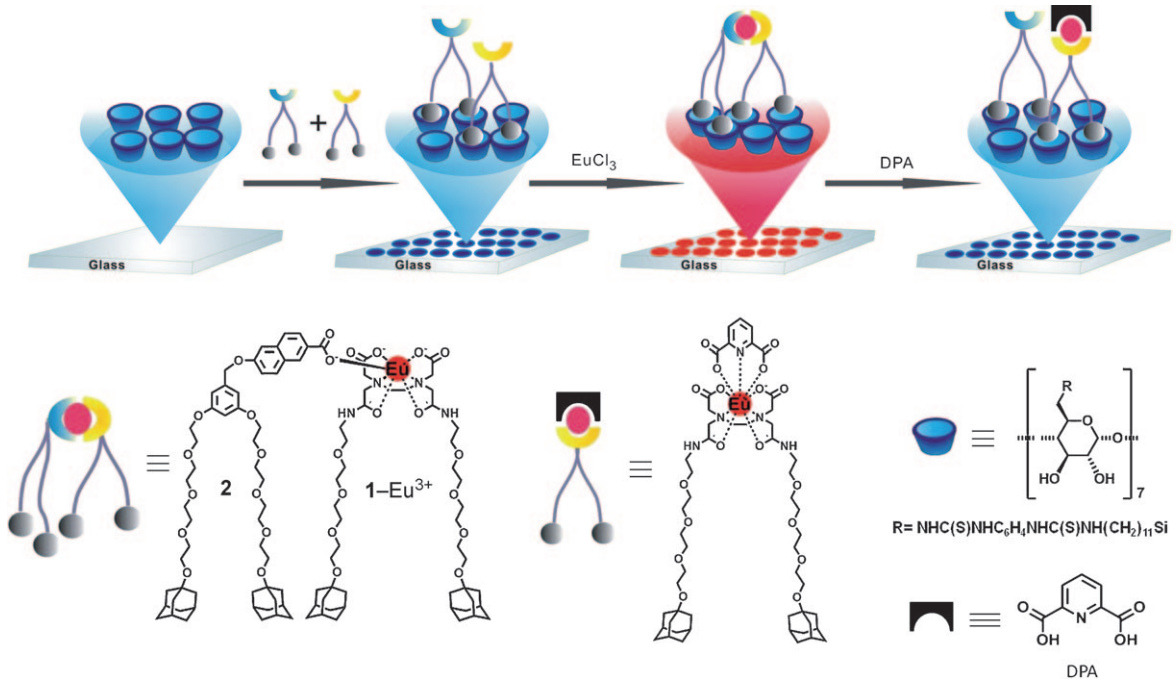

Scheme 1. The construction of the supramolecular sensing-surface system and the detection of the anthrax biomarker DPA (top) and compounds used in this study (bottom).

rinsing and drying, the substrates were again imaged. It is important to note that direct DPA excitation at 270 nm is inhibited by the narrow band excitation filter at 350 nm.

Fluorescence images for all steps are shown in Figure 1 a. In the first step, only the blue naphthalene emission was observed using filter B and no Eu^{3+} emission was seen by filter R. After incubation in the $EuCl_3$ solution, the red emission of Eu^{3+} appeared (filter R) and the blue emission of naphthalene decreased (filter B) because of energy transfer from the europium-coordinated naphthalene moiety of **2** to the Eu^{3+} center of **1**-Eu^{3+}, as studied before.[14] In the last step, the blue emission was recovered and the red emission disappeared because of the displacement of the naphthalene moiety by DPA and the concomitant loss of energy transfer between the antenna and the Eu^{3+} center. The fluorescence images clearly indicate that the recovery of the naphthalene emission is practically complete in the presence of only 200 nM DPA. This concentration, corresponding to 7×10^5 spores per mL, is about three orders of magnitude lower than the infectious dose of the spores.[7f,15] To determine the ratiometric sensing behavior of the DPA binding, values of blue over red emission intensities (I_B/I_R) were plotted for the different samples (Figure 1 b).

As a demonstration of DPA coordination to Eu^{3+} ions on the surface, a UV/Vis spectrum of DPA–**1**-Eu^{3+} on quartz was compared to a solution spectrum of 0.5 mM aqueous DPA–**1**-Eu^{3+}. To avoid the overlap of the naphthalene and DPA absorbances, only **1**-Eu^{3+} was printed on a β-CD monolayer on quartz, then incubated into 1 mM DPA (pH 6.5). The UV/Vis spectrum (Supporting Information; Figure S1) showed the characteristic broad absorption bands between 260–280 nm as also observed in solution. This result confirms that DPA forms a complex with **1**-Eu^{3+} on the surface, but also that the large excess of DPA does not remove the Eu^{3+} ion from ligand **1**, even at high (mM) concentrations. This effect is attributed to the fact that the stability constant of the EDTA-Eu^{3+} complex is nearly eight orders of magnitude larger than that of the DPA-Eu^{3+} complex in water.[7a,16]

The fluorescence intensity ratio of the Eu^{3+}-based sensing platform as a function of time upon addition of 200 nm DPA is depicted in Figure 2 a (see also Supporting Information; Figure S2). It clearly indicates that the sensing response is complete within 10 min.

To determine the detection limit of DPA, the intensity ratios for the detection substrates were plotted against the concentration of DPA (Figure 2 b). Treatment of the surface with relatively high concentrations of DPA resulted in the expected maximal increase of the blue to red ratio, and reached saturation around 200 nM of DPA. However, at lower concentrations of DPA, the ratio was lower, and a detection limit of approximately 25 nM of DPA was obtained.

To show the selectivity of our system as a sensing platform for DPA, ratiometric fluorescence changes of patterns upon addition of different aromatic ligands, such as the *o*/*m*/*p*-phthalic acids, nicotinic acid and its two isomers (picolinic and isonicotinic acid), and nicotinamide adenine dinucleotide (NAD) were investigated in 200 nM aqueous solutions at pH 6.5 (Figure 3 and Supporting Information; Figure S3). The small or negligible fluorescence changes for these ligands, combined with the lower occurrence of these species in the spores,[7e] demonstrates the excellent selectivity of the system for DPA.

In conclusion, we have demonstrated that the supramolecular europium-based luminescent sensing platform developed on glass substrates can be employed for the ratiometric fluorescent detection of the anthrax biomarker

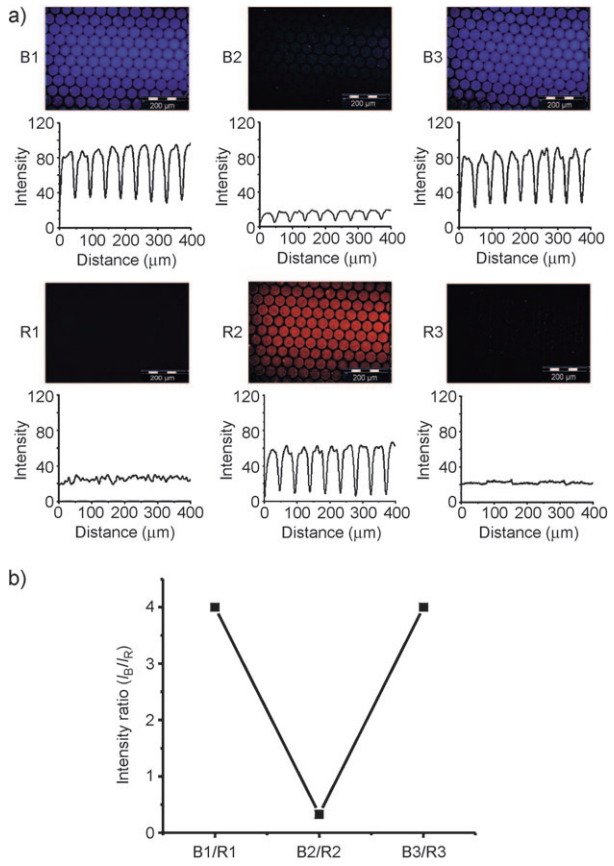

Figure 1. a) Fluorescence microscopy images and corresponding intensity profiles of 50 μm dots on β-CD monolayers of an equimolar ratio of **1** and **2** (B^1, R^1), subsequently incubated in a solution of $EuCl_3$ for 30 min (B^2, R^2), then rinsed with MilliQ water and immersed into a 200 nM solution of DPA (B^3, R^3). b) Plot of the ratios of the maximum intensities of blue (B) and red (R) images upon Eu^{3+} and DPA binding.

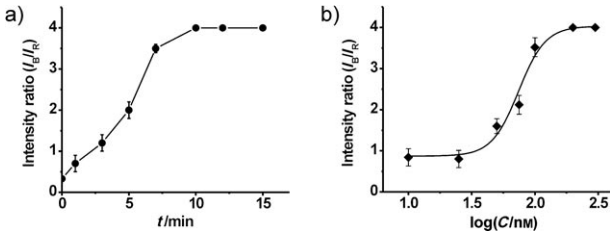

Figure 2. a) Plot of fluorescence intensity ratios versus incubation time upon addition of 200 nM DPA at pH 6.5. b) Ratiometric fluorescence change of supramolecular sensing surface as a function of DPA concentration.

DPA. The results show the excellent nanomolar sensitivity of the system towards DPA. Moreover, the system has a remarkable selectivity over other potentially competitive aromatic ligands in water. This surface-assisted sensing system opens new avenues to build solid-state devices for the detection of biologically relevant ions and bacterial spores. From this point of view, real-time monitoring and imaging of analytes can be extended with supramolecular systems to yield new classes of sensing platforms on surfaces or in microfluidic devices. For creating a practical sensor

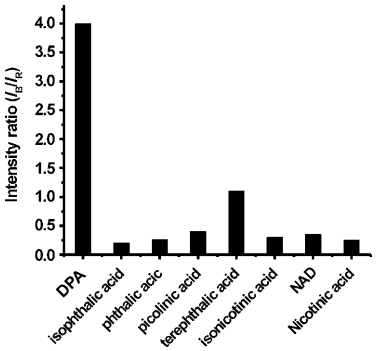

Figure 3. Evaluation of the response of the supramolecular sensing surface to different competitive aromatic ligands (200 nM for each ligand).

device, more attention needs to be paid to investigating other antenna systems to eliminate the need for UV excitation, shorter fabrication routes, and other possible cross-sensitivities. Overall, the system exemplifies the power of non-covalent strategies to create sensing platforms because different small building blocks can be easily designed and synthesized and subsequently assembled onto such a platform to create a functional system.

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