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# Steady-State and Frequency-Domain Lifetime Measurements of an Activated Molecular Imprinted Polymer Imprinted to Dipicolinic Acid

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We recently demonstrated the synthesis and fluorescence activity associated with an optical detector incorporating a molecular imprinted polymer (MIP). Steady-state and time-resolved (lifetime) fluorescence measurements were used to characterize the binding activity associated with MIP microparticles imprinted to dipicolinic acid (DPA). DPA is a unique biomarker associated with the sporulation phase of endospore-forming bacteria. Vinylic monomers were polymerized in a dimethylformamide solution containing DPA as a template. The resulting MIP was then pulverized and sorted into small microscale particles. Tests were conducted on replicate samples of biologically active cultures representing both vegetative stationary phase and sporulation phase of *Bacillus subtilis* in standard media. Samplers were adapted incorporating the MIP particles within a dialyzer cartridge (500 MW). The permeability of the dialyzer membrane permitted diffusion of lighter molecular weight constituents from microbial media effluents to enter the dialyzer chamber and come in contact with the MIP. Results showed dramatic (10-fold over background) steady-state fluorescence changes (as a function of excitation, emission and intensity) for samples associated with high endospore biomass (DPA), and a frequency-domain lifetime of 5.3 ns for the MIP–DPA complex.

**KEY WORDS:** Molecular imprinted polymer; dipicolinic acid; frequency-domain; time-resolved fluorescence; steady-state.

## INTRODUCTION

Molecular imprinted polymers (MIPs) represent an innovative approach to the nanoscale-level selectivity, detection, and control necessary to recognize a variety of biological and chemical agents. In addition, the nanoscale modification of molecular design of polymers by imprinting provides a powerful approach to controlling electronic and optical properties of materials as well as processability [1]. The attractive quality of MIPs is the target-template used to create a highly specific binding cavity yielded from the ligands utilized during copolymerization. This cavity feature of the MIP provides a "memory" of the template molecule and a resulting bonding preference to that target. To demonstrate the detection of a common biological constituent by MIP, we concentrated on dipicolinic acid.

Dipicolinic acid (DPA) is a unique, environmentally stable compound produced during sporulation by *Bacillus* mother cells and can be used as a signature biomarker. This compound can make up greater than 10% of the cell dry weight, and significant fractions can be lost during the sporulation event [2]. DPA possesses a 450 nm fluorescence emission (380 nm $\lambda_{ex}$) and is a fair candidate for fluorescence detection methods. Unfortunately many

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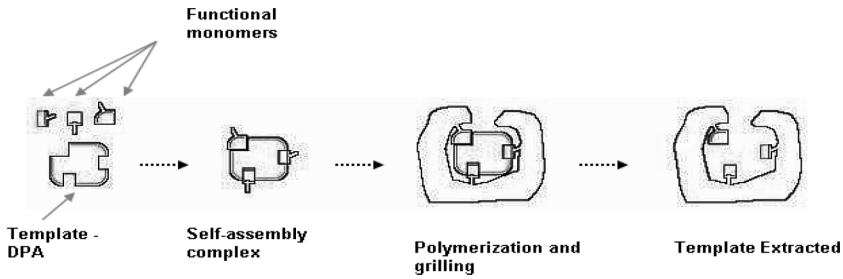


**Fig. 1.** MIP preparation.

other UV absorbing compounds such as fluorophores containing aromatic amines, proteins and nucleic acids occur in the same region and therefore intrinsic fluorescence alone is not sufficiently selective for positive identification of DPA within complex mixtures[3]. Selectivity enhancement can potentially be achieved by selective and reversible sorption of DPA by MIPs. Since MIPs can be developed to exhibit high preferential affinity to DPA, they can selectively adsorb and concentrate DPA from a mixed background. Theoretically, the fluorescence for the MIP-DPA complex could be increased, shifted or quenched. In this paper we report on an analysis of the fluorescence of a DPA-imprinted MIP in biological media. The results indicate that the fluorescence is increased upon formation of the MIP-DPA complexes, which is very promising for selective detection of this important biomarker.

## METHODS

### Bacterial Cells and Endospores

For this study, ten replicate samples of the species *Bacillus subtilis* were prepared. These bacteria represent a select group of organisms that are *Bacillus* shaped, Gram +, and endospore-forming. Cultures were isolated from stock-cultures maintained on nutrient agar slants at 4°C. Active *B. subtilis* cultures were prepared by growth in nutrient broth at 35°C as a shaker culture. Suspensions of bacteria were then centrifuged to form cell pellets. Sporulation was induced upon transfer of the *B. subtilis* vegetative cells to sporulation broth media. MIP detection of DPA was conducted on ten replicate samples of biologically active cultures representing various microbial growth phases including vegetative stationary phase and sporulation.

### Microbial Characterization and Enumeration—PLFA Analysis

We characterized ten samples in terms of total cell and spore counts, in various stages of growth, by means of membrane-linked phospholipid fatty acid (PLFA) content. PLFA analysis was used to profile all microbial phases and potential cross-contamination in the culture samples. This was done to ensure sample purity, generate statistics based upon microbial community profiles (including endospores), and provide information regarding growth phase constituents. Multivariate principal components based factor analysis was used to determine which samples exhibited communality. This technique permitted samples of similar consortia to be paired or clustered based upon vegetative, spore, or other common biomass constituents.

### Molecular Imprinted Polymers

Molecular imprinted polymers (MIPs) were synthesized by the polymerization of functional monomers and a crosslinker in the presence of DPA as a template. The template, functional monomer, and crosslinker form a self-assembled complex in solution prior to polymerization. This complex is formed due to noncovalent hydrogen and ionic bonding. Removing the template yields a polymer exhibiting receptor sites that are complementary to the template molecule (Fig. 1) [4,5].

For optimum performance, the polymer porosity, particle dimensions and overall composition must be precisely controlled. For fluorescence detection it is also required to reduce the level of aromatic impurities in the polymer. This was achieved through the appropriate selection of monomers and by removing traces of aromatic impurities from the compounds used in the imprinting process. The functional monomers used were acrylamide and 4-vinylpyridine. Bisacrylamide was a crosslinker and 2,2′-azobis(2-methylpropionitrile) was used as a radical initiator. All compounds were purchased from Sigma-Aldrich. The mixture of monomers was dissolved in $N, N$-dimethylformamide by ultrasonic assistance and the reaction proceeded at 60°C for 24 hr. Both imprinted and nonimprinted polymers were synthesized according to the same procedure and composition, except

the nonimprinted material was not exposed to the template. After the polymerization, the bulk polymer was ground by mortar and pestle and the template was removed by extraction in a Soxhlet apparatus for 4 hr with 200 mL of methanol. By double sedimentation in ethanol, small particles with diameters in the range of 10–20 $\mu$m were collected. Additional extraction of the DPA template was found to be required because fluorescent methods are very sensitive to small amounts of residual DPA. The collected particles were repeatedly washed on a 0.47 $\mu$m polyester membrane mounted in a Nalgene filter holder with a mixture of methanol and HCl (9:1) until the UV absorbance of the filtrate at 270 nm measured below 0.005 AU. Eventually, the particles were collected and dried in vacuum and fractioned for testing of the polymer sensitivity (Pestov, 2003, Personal communication).

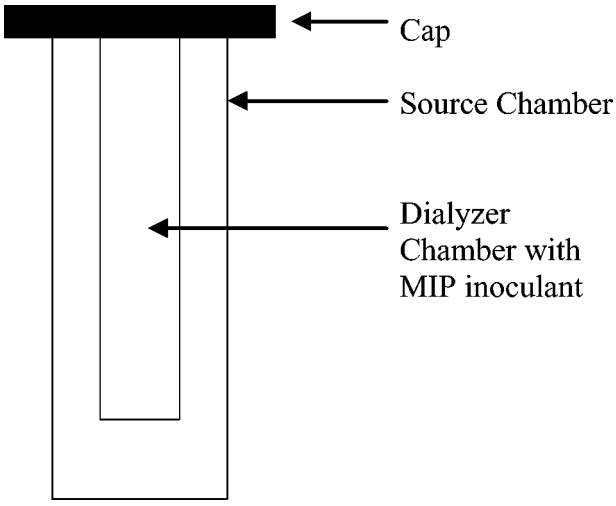


**Fig. 2.** Dialyzer apparatus schematic showing internal chamber (bag) configuration.

### Sampling Apparatus

A thorough literature review demonstrating the stable physical properties of sensors incorporating molecular imprinted polymers against bio-destruction makes them ideal artificial receptors, i.e., biosensor sensing elements, for detection systems against chemical and biological toxins, drugs, and environmental contaminants [5]. Active (real-time) sampling of the biological culture media was accomplished using membrane dialyzers containing a suspension of MIP (2 $\mu$g) in 5 mL of distilled water injected into the inner sample chamber. Each dialyzer cartridge (500 MW design) was openly permeable to external biological constituents (i.e. DPA), allowing diffusion across the membrane into the inner chamber, while the larger MIP (at $>$200,000 MW) was unable to escape (Fig. 2).

### Fluorescence Measurements: Steady-State and Frequency-Domain Lifetime

Samples were extracted from the inner chambers of the dialyzer cartridges and subjected to fluorescence spectral analysis using the JY Horiba Fluoromax-3 and Fluorolog Tau-3 spectrometers (xenon arc lamp). Also, each biological sample was measured for its steady-state, intrinsic fluorescence response to develop a background signature prior to exposure to the MIP. Spectral measurements included both absorption (excitation) and emission scans. Each sample was aqueous, held at 22–25°C (room temperature), and at a near neutral pH prior to measurement. All samples were measured in 5 mL quartz cuvets using right angle (90°) viewing geometry with the emission beam recorded orthogonal to the excitation beam.

Lifetime or ($\tau$) is a measure of the decay time of a fluorophore or fluorophores in a mixture. The lifetime ($\tau$) represents an added fourth dimension to detecting and recording target constituents based upon unique decay times (typically in nanoseconds and/or picoseconds) presented by fluorescent materials. Frequency-domain lifetime measurements were used to characterize the fluorescence lifetime of the MIP under optimal and nonoptimal conditions. These conditions involved the MIP being isolated or placed within a mixture. Time-resolved emission spectra (TRES) (based on steady-state and individual lifetime data) were measured using an excitation wavelength of 300 nm, over an emission wavelength range of 310–510 nm (sample measured every 20 nm). This wavelength represented nonoptimized detection conditions at a common absorption for both the MIP and ancillary UV fluorophores. A frequency range of 10–100 MHz was used (with 8 frequencies points), while ludox was used as a scattering standard. TRES measurements were performed to fully understand the MIP detection limitations under nonoptimized circumstances. To accomplish this, we targeted the common aromatic amino acid tryptophan (300 nm $\lambda_{ex}$, 350 nm $\lambda_{em}$) because of its known presence in the sample and its known lifetime ($\tau$). Due to the MIP's broad absorption, targeting the shorter wavelength UV region did not prove to be problematic. The experimental multifrequency data were analyzed using software that attempts to fit an appropriately chosen model that minimizes the deviations of the model from the data. The reduced chi-squared values for multiple-lifetime analysis reflect the goodness of fit of the model [6].

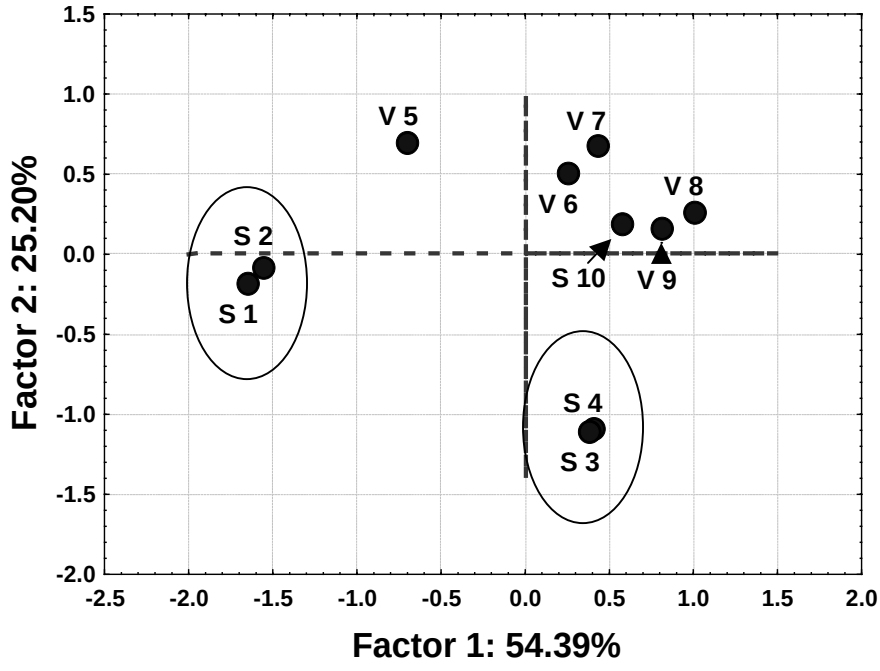


**Fig. 3.** Principle components analysis showing community similarities based upon PLFA composition. Samples indicating DPA detection by MIP are shown as "S." Samples of vegetative cells are indicated by "V." S10 appears to be an outlier caused by a dominance of vegetative cells entering the sporulation phase.

## RESULTS

### Microbial Characterization and Enumeration–PLFA Analysis

Phospholipid fatty acid (PLFA) analysis profiled the microbial biomass associated with the samples and showed communality of samples as indicated by the principal components plot (see Fig. 3). The plot relates those samples indicating DPA detection by MIP activity (S1, S2, S3, S4, and S10) with samples containing mostly vegetative cells (V5, V6, V7, V8, and V9) based on phospholipid fatty acid content. Sample S10 is an outlier possibly suggesting very early sporulation with a dominance of active vegetative cells. The samples paired in communality, which is to say they are similar in community structure (e.g., they have the same endospore or vegetative cell components of *B. subtilis* in phases consistent with DPA production). This is not to say that DPA was *not* present in other samples, but that these samples stood out among those tested as having both reactive DPA and similarity of microbial communality.

### Steady-State

Figure 4 presents intrinsic, steady-state spectra for biological media containing spore-forming *B. subtilis* samples. These samples represent supernatant fractions from media effluents where PLFA analysis showed very little vegetative biomass, but high spore counts ($>1.9 \times 10^5$). These data were measured at excitation 355 nm. The relatively low photon counts ($<2.5 \times 10^6$) of the background effluent samples offered a stark contrast to the high emissions recorded with the MIP polymer probe.

Both absorption and emission spectra for the MIP-DPA samples are presented in Figs. 5 A and B. Absorption and emission scans show molecular activity, or the "turning on" of the MIP in the presence of the target compound

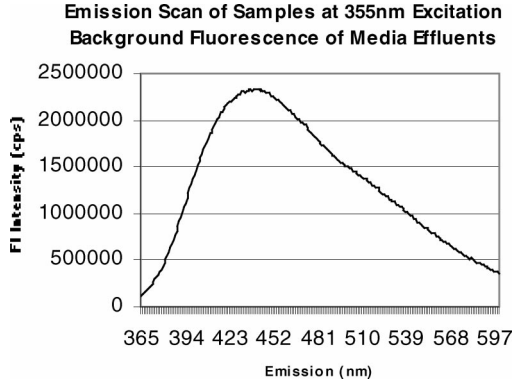


**Fig. 4.** Emission spectra for biological samples.

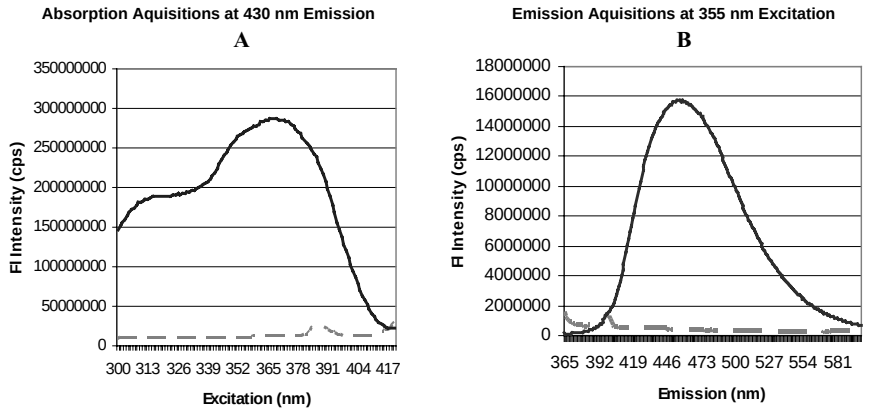


**Fig. 5.** (A and B) Absorption (LEFT) and emission (RIGHT) scans for sporulation samples detected by MIP. Dashed lines indicate fluorescent controls: de-ionized water and un-inoculated media.

(DPA). The data also show (for comparison) uninoculated media controls and distilled water lacking any biological activity (dashed bottom traces). The MIP absorption has a broad UV structure with a steep fall off at about 380 nm. The absorption/excitation maxima are actually closer to 365 nm for the samples presented. The emission shows an intense fluorescence ($16 \times 10^6$ cps) at 450 nm when excited at 355 nm. This emission can be characterized as a broad 75 nm Stoke's Shift given the position of the excitation wavelength (355 nm). The emission of the activated MIP over the intrinsic background fluorescence is near 10-fold and demonstrates the utility of this probe analysis.

### Frequency-Domain Lifetime

Frequency-domain lifetime measurements were used to characterize the MIP. Figure 6 (top) shows the single exponential decay lifetime plot for an MIP sample ($\tau =$ 5.3 ns, 355 nm $\lambda_{ex}$, 450 nm $\lambda_{em}$, $\chi^2 = 0.775$). Also, time-resolved emission spectra (TRES), generated from measured steady-state and individual lifetime data (Fig. 6, bottom), show the resolved frequency-domain lifetime ($\tau$) of the active polymer in a mixture of biological media using an overlapping UV wavelength of absorption. These data show the nonoptimized optical resolution of the polymer against a predominant aromatic amino acid (tryptophan) ($\tau = 2.3$ ns for a single exponential decay model, 300 nm $\lambda_{ex}$, 350 nm $\lambda_{em}$, $\chi^2 = 0.962$).

## DISCUSSION

Our results indicate that molecular imprinted polymers can be used to selectively enhance the fluorescence signal from DPA. Calibrated spectrofluorometry measured and characterized the steady-state emission intensity maxima of the bound polymer as 10-times greater than ancillary background fluorophores such as aromatic amines, proteins and nucleic acids. Fluorescence lifetime measurements of this MIP also revealed the successful detection of DPA under both optimal (selective wavelength) and less-than-optimal (overlapping wavelengths) conditions against a strong UV fluorescing background. This was due to the longer decay time of the MIP compared to background fluorophores. The fluorescence detection of the MIP-DPA complex was similar to assays involving lanthanides such as terbium, but more sensitive to lower levels of DPA present in (sporulation) media. Additionally, where terbium exhibits a long-lifetime approaching micro-seconds, the MIP exhibits a shorter decay (5.3 ns). This decay, however, is still longer than associated biological fluorophores and with increased sensitivity [7].

Our laboratory tests on MIP sensitivity showed decimal dilutions of DPA in methanol yielded fluorescence signal recovery at concentrations as low as 0.15 mM using 0.3 g of MIP. A time-course analysis showed immediate response from the polymer to DPA with internal quenching taking place after 2 hr. However, DPA was concentrated in methanol, and solvent interaction and relaxation are factors to be considered, possibly affecting the resulting fluorescence yield (Pestov, 2003, Personal communication).

The molecular sensing attributes of the MIP are best characterized using Janssen *et al.* whom estimated that there is approximately 41.6 $\mu$g of DPA per milligram of dried *Bacillus* spores [8]. As mentioned previously, DPA is known to make up approximately 10% of the spores' dry weight [2]. Furthermore, Hathout *et al.* estimated that 5 mg of *Bacillus* spores (dry weight) corresponded to approximately $2.4 \times 10^8$ spores [9]. Using this information, we estimated approximately 800–2000 fg of DPA per spore.

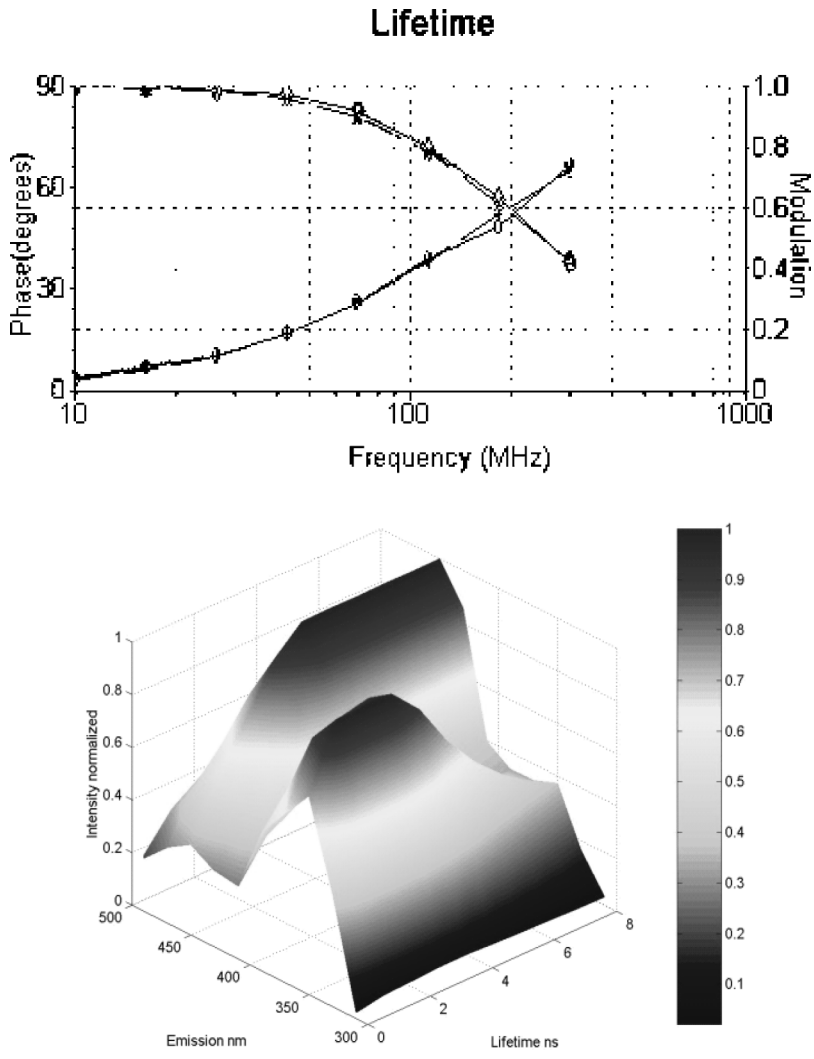


**Fig. 6.** (Top) Modeled frequency-domain lifetime plot for MIP ($\tau = 5.3$ ns, 355 nm $\lambda_{ex}$, 450 nm $\lambda_{em}$, $\chi^2 = 0.775$). (Bottom) Comparative lifetime plots for MIP ($\tau = 5.3$ ns, 450 nm $\lambda_{em}$, in background) and Tryptophan ($\tau = 2.3$ ns, 350 nm $\lambda_{em}$, in foreground), using the overlapping excitation wavelength of 300 nm.

Our calculations indicated that (based on the 5 mL sample volume) the MIP was able to detect comparable low levels of DPA.

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