Enzymatically and Combinatorially Generated Array-Based Polyphenol Metal Ion Sensor

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Phenolic polymers were synthesized via soybean hull peroxidase catalysis and used as metal-based sensor components in a polymer array. A sensor array for Fe⁶⁺, Cu²⁺, Co²⁺, and Ni²⁺ has been developed consisting of 15 phenolic homopolymers and copolymers generated from five phenolic monomers by peroxidase-catalyzed oxidative polymerization. Sensing was based on the change of intrinsic polyphenol fluorescence upon addition of a metal ion or a metal ion mixture to an aqueous suspension of a polyphenol. Importantly, the fluorescence response of copolymers differed, in some cases dramatically, from the constituent homopolymers and was dependent upon the relative ratio of monomers that comprise the polymer. This finding suggests that an extremely broad range of sensor arrays can be generated from a limited number of phenolic monomers. Using a statistical analysis, histograms constructed for the four different metal ions yielded unique fingerprints of the array response and can be used to identify specific metal ions.

Introduction

Traditional sensors operate with high sensitivity and selectivity by tailoring a specific sensor element to the analyte of interest (1, 2). When multiple analytes are present, this requires the deployment of an equal number of specific sensors. An alternative approach is to employ an array of rather nonselective sensors and then utilize a recognition technique to identify patterns of sensor elements that respond to specific analytes (3, 4). Such a design mimics the mammalian olfactory system, which consists of a large array of receptors (5, 6). Odorants interact with a combination of receptors, and the pattern of receptor—odorant interactions is processed in the brain (7).

Similarly, the relatively new field of “chemical nose” technology employs a series of sensors laid out in an array (8–10). Interactions of an analyte or analyte mixture with specific sensors in the array yield a pattern of sensor responses. The result is a specific “fingerprint” for each analyte present (11). A major advantage of this sensing technique is that each sensor element need not be extremely sensitive or selective for a given analyte. Rather, a series of somewhat less sensitive sensor elements can be used to yield a fingerprint response for a specific analyte. This has paved the way for simpler and less costly materials to serve as components of newer sensors. Such chemical noses have been used in several applications including the detection of volatile organic compounds (VOCs) (11, 12), fish freshness (13), and beer flavor (14).

Metal ions are ubiquitous in nature and also represent a significant man-made pollutant (15, 16). Unfortunately, the development of sensors with high sensitivity, exquisite selectivity, and broad sensing capability (e.g., capacity to sense many different metals in isolation or in mixtures) remains a hurdle. This is not due to the transducing mechanisms needed for optimal sensor design but rather the lack of effective sensor elements that interact sensitively and selectively with a given metal analyte. Thus, metal sensing would appear to be ideally suited toward array-based sensor design as long as a large and diverse sensor array could be constructed. For example, simple polymers may serve as sensor elements as long as a large enough matrix of polymers is employed that have different levels of interactions with different metal ions.

Polyphenols are known to interact with metal ions, and the resulting polymer—metal complexes show altered fluorescence properties as compared with the uncomplexed polyphenols (17, 18). Biocatalysis, in general, and peroxidase catalysis specifically, has proven to be a highly effective method to synthesize polyphenols (19–21). The broad selectivity of peroxidases toward phenolic monomers enables a single enzyme system to be used to generate a diverse array of polyphenols from a number of simple phenolic monomers in a combinatorial fashion. In our previous work, we demonstrated that soybean peroxidase could be used to generate polyphenols that could bind metals with different selectivities and sensitivities and that these polyphenols could be used as the basis for a metal-sensing system (22). In the present study, we extend the use of peroxidase into the realm of combinatorial biocatalytic synthesis of polyphenols as metal-ion sensors by preparing a simple 15-member array based on both homo- and copolymers from five simple phenols.

Materials and Methods

Soybean hull peroxidase (SBP) was purchased from Sigma (St. Louis, MO). Phenols were purchased from Aldrich (Milwaukee, WI). All other chemicals and solvents were of the highest grade commercially available.

Polyphenols were synthesized from five simple phenols: p-cresol, p-methoxyphenol, p-phenylphenol, p-hydroxyphenylacetic acid, and p-hydroxybenzoic acid, either
as homopolymers or copolymers. Polymer synthesis was performed in aqueous buffer (50 mM Bis-Tris propane, pH 7.0) containing 20–33% (v/v) dimethylformamide to aid in phenol dissolution. The reaction mixture contained a phenol at a concentration of 10 mM and 0.1 mg/mL SBP in a volume of 100 mL. The reaction was initiated by addition of 10 mM H₂O₂, which was slowly added over a period of 4 h. For reactions with charged phenols (e.g., p-hydroxyphenylacetic acid and p-hydroxybenzoic acid), the reaction volume was decreased to 10 mL and the phenolic concentration was increased to 100 mM to ensure precipitate formation during polyphenol synthesis. Copolymer synthesis was performed in an identical manner, except that two phenols at a concentration of 5 mM each were used.

The polymers precipitated out of solution and were collected and washed thoroughly with deionized water in 0.45 μm pore size centrifuge filters from Alltech (Deerfield, IL). The fluorescence excitation and emission spectra of the polyphenols were measured in suspension in 50 mM pH 7.0 Bis-Tris propane buffer. The effect of a metal on the fluorescence of a polyphenol was monitored in 0.45 M3) responds much differently. This is not due to the sole influence of (eq 1), where I₀ and I represent fluorescence intensity of the polyphenols in the absence and presence of metal ions, respectively, at the maximum emission wavelength.

\[ \Delta F = \frac{I_0 - I}{I_0} \]

of the polyphenols in the absence and presence of metal ions, respectively, at the maximum emission wavelength.

In the present study, external fluorophores were not used. Instead, intrinsic fluorescence of polyphenols was used to sense metal ion binding. A 15-member array of homo- and copolymers is depicted in Table 1 along with specific MEWs obtained. The yield of polymer formation (based on conversion of the individual monomers) was uniformly >85%. A wide range of MEWs was obtained (from 360.8 to 444.8 nm). ΔF values of the fluorescence response of polymers upon metal binding are shown in Table 2 for both 1 mM Cu²⁺ and 0.2 mM Fe³⁺. The ΔF values for 1 mM Cu²⁺ ranged from 0.12 to 0.42, showing significant fluorescence quenching of the fluorescence of the polyphenolics. Moreover, the response of the individual polymers to Cu²⁺ was different depending on the nature of the polymer and copolymer. In some cases, this difference was striking. For example, the p-methoxyphenol homopolymer (M₃-M₃) and the copolymer of p-methoxyphenol with p-phenylphenol (M₂-M₃) respond to 1 mM Cu²⁺ nearly identically, whereas the copolymer of p-methoxyphenol with p-cresol (M₁-M₃) responds much differently. This is not due to the sole influence of 

### Results and Discussion

In our previous work (22), we demonstrated that polyphenols, prepared by peroxidase-catalyzed oxidative polymerization of simple phenols, could be used as sensor elements for specific metal ions either alone or in small mixtures. Specifically, polyphenols prepared from para-substituted phenols such as cresol, phenylphenol, and methoxyphenol copolymerized with ca. 10% (mol/mol) fluorescein or calcein were capable of detecting Fe³⁺, Fe²⁺, Cu²⁺, Zn²⁺, and Mn²⁺, at concentrations as low as 10 μM. The fluorescence intensity of the polymers were altered in the presence of a metal. The influence of the metal ion binding on the fluorescence was described by a fluorescence intensity change ratio, ΔF (eq 1), where I₀ and I represent fluorescence intensity of the polyphenols in the absence and presence of metal ions, respectively, at the maximum emission wavelength.

\[ \Delta F = \frac{I_0 - I}{I_0} \]

of the polyphenols in the absence and presence of metal ions, respectively, at the maximum emission wavelength.

### Table 1. Maximum Emission Wavelengths (MEWs, nm) for the Polyphenol Array

<table>
<thead>
<tr>
<th>monomer</th>
<th>M₁</th>
<th>M₂</th>
<th>M₃</th>
<th>M₄</th>
<th>M₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td>P₁₁</td>
<td>P₁₂</td>
<td>P₁₃</td>
<td>P₁₄</td>
<td>P₁₅</td>
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<td>P₂₃</td>
<td>P₂₄</td>
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<tr>
<td>M₃</td>
<td>X</td>
<td>X</td>
<td>P₃₃</td>
<td>P₃₄</td>
<td>P₃₅</td>
</tr>
<tr>
<td>M₄</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>P₄₄</td>
<td>P₄₅</td>
</tr>
<tr>
<td>M₅</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>P₅₅</td>
</tr>
</tbody>
</table>

### Table 2. Fluorescence Intensity Change Ratio, ΔF, of Each Polyphenol Sensor Element in a 15-Member Sensor Array

<table>
<thead>
<tr>
<th>monomer</th>
<th>M₁</th>
<th>M₂</th>
<th>M₃</th>
<th>M₄</th>
<th>M₅</th>
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<tr>
<td>M₁</td>
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<td>0.21</td>
<td>0.19</td>
</tr>
<tr>
<td>M₄</td>
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<td>X</td>
<td>X</td>
<td>0.36</td>
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<td>M₅</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*p-M1, p-cresol; M2, p-phenylphenol; M3, p-methoxyphenol; M4, p-hydroxyphenylacetic acid; M5, p-hydroxybenzoic acid. All copolymers were prepared at a 1:1 phenolic monomer substrate ratio (see text for details). ΔF values different from those of their constituent homopolymers. Indeed, a more diverse fluorescence response was obtained. A more sensitive response to Fe³⁺ was obtained. A more diverse fluorescence response was obtained based on the nature of the polyphenol. For example, the p-hydroxybenzoic acid homopolymer (M₅-M₅) yielded a ΔF value of −0.78, indicating substantial fluorescence enhancement in the presence of 0.2 mM Fe³⁺, while all other homo- and copolymers yielded fluorescence quenching. As with Cu²⁺, different copolymers yielded different fluorescent responses. This is vividly shown by comparing the p-hydroxybenzoic acid homopolymer (M₅-M₅) to any of its copolymers.

Given that the copolymers had ΔF values different from those of their constituent homopolymers, it occurred to us that the fluorescence response of metal ions of a given copolymer can be tuned solely by adjusting the ratio of one phenolic monomer to another in the copolymer. This can be done simply by using different concentrations of phenolic monomers in the peroxidase-catalyzed polymerization reaction. The ratio of phenols in a copolymer did influence the fluorescence response to the presence of a metal ion, as shown in Figure 1. When M₁—M₅ copolymer pair (p-cresol-co-p-hydroxybenzoic acid) responded differently to a given metal (either 1 mM Cu²⁺...
or 0.2 mM Fe$^{3+}$ at different concentrations of M$_5$ in M$_1$.

In the case of Cu$^{2+}$, the ΔF doubled in going from pure p-cresol to 75% p-hydroxybenzoic acid/25% p-cresol. Thus, the fluorescence response of a given metal can be influenced both by the nature of the copolymer and by the composition of the copolymer. This is similar to the finding by Dickinson et al. (23) in the sensing of VOCs using an array of copolymers from two commercially available monomers. Theoretically, a nearly unlimited number of copolymer elements in an array can be generated combinatorially by manipulating a finite number of commercially available monomers. The larger the array of sensing elements that shows different responses the more similar the array is to the mammalian olfactory system.

We have expanded the array-based format to provide “fingerprints” of sensor fluorescence responses. A histogram of the metal ion was obtained as a pattern of the metal ion, its concentration, and the polyphenol array by using a statistical average, ΔZ, shown in eq 2, where

$$\Delta Z = \frac{\Delta F - x}{y} \quad (2)$$

x and y represent the average and standard deviation of each ΔF value. Figure 2 shows the overall response of the 15-member array on four metals: 0.2 mM Fe$^{3+}$, 1.0 mM Cu$^{2+}$, 1.0 mM Co$^{2+}$, and 1.0 mM Ni$^{2+}$. Each metal’s “fingerprint” is sufficiently distinct to enable rapid identification of the analyte.

In conclusion, a sensor array for metal ions composed of 15 phenolic homopolymers and copolymers has been generated from five phenolic monomers by peroxidase-catalyzed oxidative polymerization. Using a statistical analysis, histograms for four different metal ions yield unique fingerprints of the array response and can be used to identify specific metal ions. Although not shown, this approach could distinguish individual metal ions at concentrations as low as 10–50 μM, a value that is relevant to many metal-containing environments. This work shows that it is possible to generate polyphenols combinatorially and in array-type format, which can then

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**Figure 1.** Fluorescence intensity change ratio, ΔF, under the influence of metal ions (1.0 mM Cu$^{2+}$ (■) and 0.2 mM Fe$^{3+}$ (●)) of the copolymers depends on the ratios of the two monomers, p-cresol (M$_1$) and p-hydroxybenzoic acid (M$_5$), in the initial substrates.

**Figure 2.** Histograms of four metal ions generated from the 15-member homo- and copolymer array from five phenolic monomers represent “fingerprints” of the metal ions, their concentration, and the sensor array.
be screened against specific metal ions for identification of metal ion sensor elements. Because of the different metal ion binding capability of the heteropolymers, a nearly infinite variety of sensor elements can be generated by a manageable number of commercially available phenols. Pattern recognition techniques can then be employed (4) to distinguish between different metal ions or metal ion mixtures.

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References and Notes


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