Chapter 2

Generation of Environmentally Compatible Polymer Libraries via Combinatorial Biocatalysis

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A combinatorial strategy for biocatalytic polymer synthesis has been demonstrated. Two polymer libraries were synthesized. In one, the polycondensation of esters (C_3-C_{10}) with polyhydroxylated compounds (e.g., diols, sugars, nucleic acids, and a natural steroid diol) was performed. The lipase from *Candida antarctica* in acetonitrile was capable of catalyzing the polycondensation of these monomers to give polymers with Mw's as high as 20,000 Da, including the preparation of novel sugar-containing polyesters. In the second library, soybean peroxidase was used to catalyze the oxidative polymerization of phenols to yield materials that bound and sensed metal ions. Histograms were developed from the fluorescent response of the library constituents to yield fingerprints for specific metals. These two libraries, along with their methods of preparation, provide a new paradigm for functional polymer discovery that may have applications in environmentally-benign materials and sensor arrays.

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Enzymatic polymerizations (*1*) have become an effective method for the synthesis of polyphenols (*2-5*), polyesters (*6-9*), and polysaccharides (*10*). Coupled with chemoenzymatic methods (*11*), the scope of biocatalytic polymer synthesis expands even further to include poly(acrylates, acetylenes, ols) among others. In these transformations, enzymes are capable of displaying a high degree of selectivity (e.g. stereo-, regio-, and chemoselectivity), which is a critical advantage over chemical routes to polymer synthesis. Moreover, enzymes operate under conditions that favor environmentally benign and energy efficient synthesis.

To capture the full universe of biocatalytic transformations, we have chosen a path that has been used by nature to synthesize and select for unique materials. Scientists and engineers have applied combinatorial strategies for pharmaceutical and agrichemical discovery, and recently, materials, which include catalysts, dielectric materials, and polymers (*12*). We have now extended this methodology to include enzymatic polymerizations. With the application of combinatorial biocatalysis in polymer synthesis, we are able to synthesize diverse libraries of polymers, and shorten time scales for polymeric materials discovery.

MATERIALS

Materials. All materials and enzymes were purchased from Sigma or Aldrich (St. Louis, MO), unless otherwise stated. Solvents were dried over 3 Å molecular sieves for at least 24 h prior to use for the removal of residual water. The 96-deep well plates were purchased from Alltech associates, Inc (Deerfield, IL). Divinyladipate and divinylsebacate were purchased from TCI America (Portland, OR) and Polysciences, Inc. (Warrington, PA), respectively. Bis(2,2,2-trifluoroethyl)malonate (15.1 g, 39 % yield) and bis(2,2,2-trifluoroethyl)glutarate (19.2 g, 45% yield) were synthesized from a previously published method (*13*). Novozym-435 (lipase B from *Candida antarctica* immobilized on an acrylic resin) and the alkaline protease, Protex-6L, was obtained as gifts from Novo Nordisk Bioindustries (Bagsvaerd, Denmark) and Genencor International (Rochester, NY), respectively.

Enzyme-catalyzed polycondensation reactions. Enzymes (20 mg/ml except for freeze-dried Protex-6L, which was employed at a concentration of 5 mg/ml) were screened in different solvents for their ability to catalyze the transesterification and polymerization of aliphatic and aromatic diols (0.2 M) dissolved in 1.5 ml of a suitable solvent containing 0.2 M divinyladipate (DVA). A 96-deep well plate was shaken at a 45° angle at 250 rpm and 45°C for 24 h in an orbital shaker. The polymers were isolated by evaporating the reaction

solvent in a vacuum oven and subsequently washed in methanol. The pellets were dried, redissolved in DMF, and filtered to remove the enzyme. The filtrate was then placed in an identical 96-deep well plate. For reactions performed in the full array, Novozym-435 (25 mg/ml) was added to a mixture of equimolar concentrations (0.2 M) of an activated diester and a hydroxyl-containing compound dissolved in 1.5 ml of acetonitrile. After 88h the plate was worked up similarly as above. The large-scale synthesis of poly(sorbitol adipate) was performed as follows: 0.2 M sorbitol and 0.2 M DVA were dissolved in 0.2 L of CH_3CN and the reaction was initiated upon addition of 5 g of *C. antarctica* lipase. After 93 h, the polymer was isolated and worked up as described above.

Enzyme-catalyzed polyphenol reactions. Soybean peroxidase (SBP) catalyzed synthesis of a polyphenol array was performed in aqueous buffer (50 mM Bis-Tris propane, pH 7.0, containing 20-33% (v/v) DMF) with five simple phenols as listed in Table 1. The reaction mixture contained 10 mM of a phenol (for homopolymers), or 5 mM each of two phenols (for copolymer), and 0.1 mg/ml SBP in a volume of 100 mL. The reaction was initiated by pumping 1 ml H_2O_2 into a phenol and SBP mixture to obtain a final concentration of 10 mM H_2O_2 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. The precipitated polymers were collected and washed thoroughly with deionized water and filtered through 0.45 μm pore size centrifuge filters from Alltech.

ANALYTICAL MEASUREMENTS

Quantitative analysis of acyl donors, sugars, and diols was performed by gas chromatography (Shimadzu GC-17A) with an AT-1 (Alltech) capillary column (30 x 0.25, 0.10 μm) and helium as the carrier gas. Determinations of polymer molecular weight (M_n and M_w) were made by gel permeation chromatography using a Shimadzu LC-10A LC system (Columbia, MD) differential refractometer (Waters) with the column calibrated using poly(ethylene glycol) standards in DMF. ^{13}C and ^{1}H -NMR spectral data were collected using a Varian Unity 500 NMR spectrometer (Palo Alto, CA).

Measurement of fluorescence response of the polymer array to metal ions. Phenolic polymers were distributed uniformly in 50 mM Bis-Tris propane buffer (pH 7.0). The effect of a metal on the fluorescence of a polyphenol was monitored by fluorescence intensities at specific maximum emission wavelengths

(MEW). The specific response of fluorescence intensity upon the perturbation of metal binding was defined as ΔF in the following equation (Eq. 1):

$$\Delta F = \frac{I_o - I}{I_o} \quad (1)$$

where I and I_0 represent emission intensities at MEWs in the presence and absence of metal ions, respectively. Each ΔF was calculated from a minimum of three independent measurements of I and I_0.

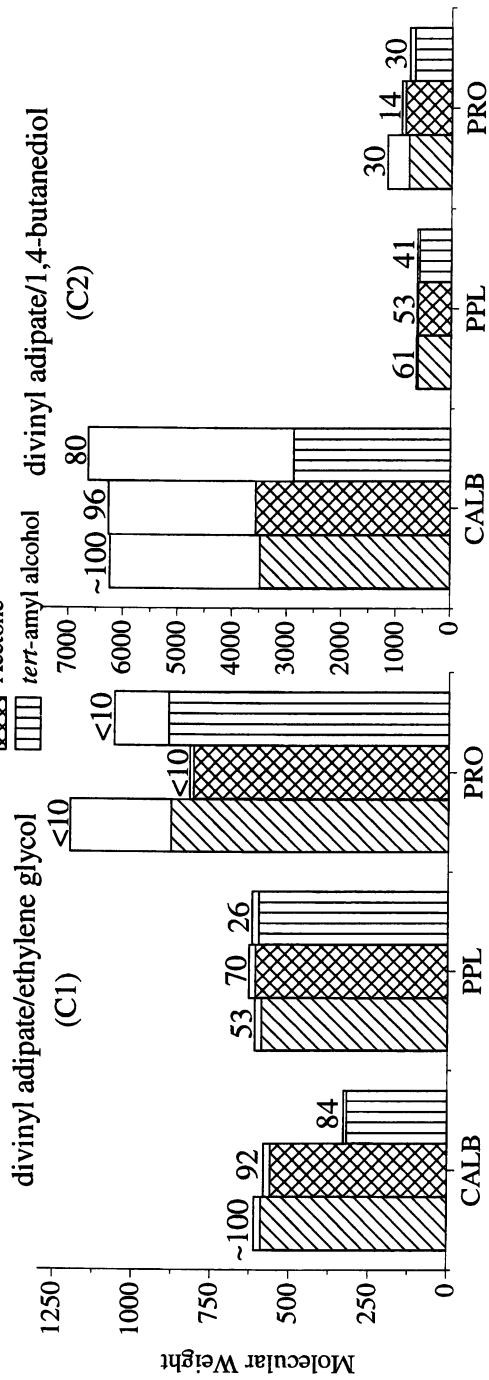
RESULTS AND DISCUSSION

Enyzme-catalyzed polycondensation reactions

Library 1: Divinyl adipate reacted with four diols (3 enzymes x 3 solvents x 4 diols)

An initial screen for combinatorial enzymatic polymerizations used several biocatalysts in different reaction media. With the advent of 96-well plates and automated instruments, screening and the entire polymer discovery process can be conducted efficiently using parallel synthesis. We first constructed a simple polymer array from one acyl donor, divinyl adipate (DVA), and four diols (ethylene glycol, 1,4-butanediol, 1,8-octanediol, and 1,4-benzenedimethanol). Upon review of the literature for enzymatic activity toward polycondensation reactions (*14-16*), three enzymes (Novozym-435, Porcine pancreatic lipase, and Protex-6L) were chosen for their abilities to catalyze the transesterification of DVA with these four diols in three solvents (acetone, acetonitrile, and *tert*-amyl alcohol).

In all cases, oligo-condensation was observed. In addition, as a control, a replicate array was constructed and in the absence of enzyme, no reaction was observed (Figure 1). Reactions catalyzed by *C. antarctica* lipase consistently displayed the highest activity in all three solvents. Porcine pancreatic lipase (PPL) and Protex-6L exhibited smaller molecular weights, which is consistent with lower conversions in step-wise polycondensation reactions. *C. antarctica* lipase catalyzed polymerizations showed M_w in the range of 330 to as high as 6,640, corresponding to a degree of polymerization (DP) of 17. The polydispersity index (PDI) ranged from 1.03 for the smallest oligomers to 1.8 for the larger polymers. Complete conversion of monomer was obtained for *C. antarctica* lipase in acetonitrile, and therefore used in subsequent studies for the construction of the next library.



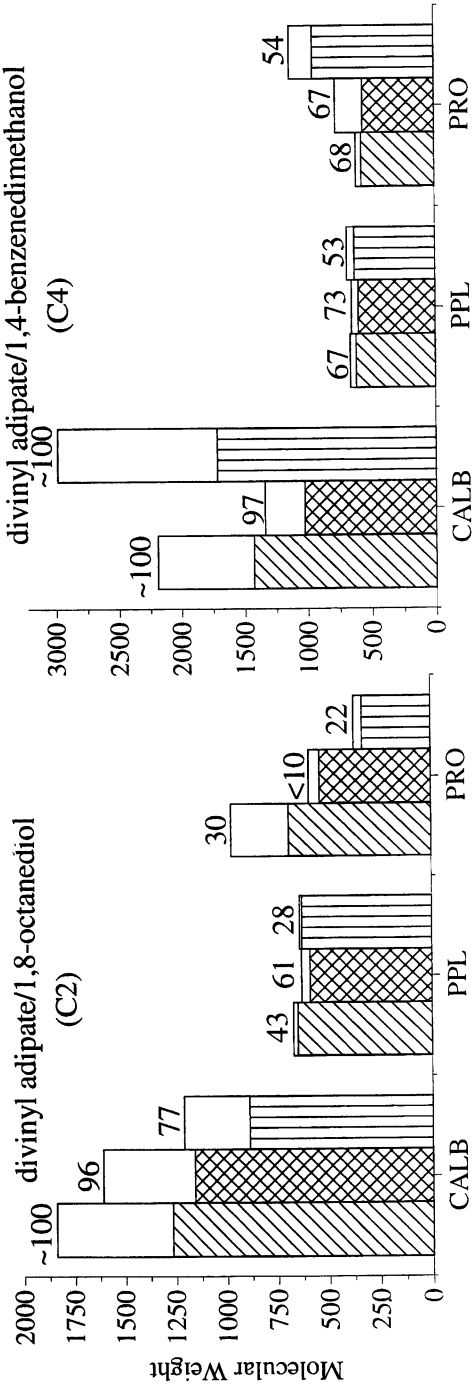


Figure 1. Library 1: Synthesized using catalysts from different sources [C. antarctica lipase (CALB), Porcine pancreatic lipase (PPL), and Protex-6L (PRO)] and by varying reaction mediums (acetonitrile, acetone, and tert-amyl alcohol). M_n=shaded portion; M_w=shaded+clear portion; Numbers refer to conversion of monomer.

Library 2: Activated esters reacted with various acyl acceptors (4 acyl donors x 12 acyl acceptors)

Biocatalyst reaction conditions were the main focus of the first library but it did not highlight the diverse number of polymers that is able to be enzymatically synthesized. To generate polymers with different architectures, we chose an additional eight diols or polyols, whose structures are shown in Figure 2. Therefore, a total of twelve acyl acceptors, which consisted of the same aliphatic and aromatic diols as in Library 1, along with carbohydrates (mono and disaccharides), nucleic acids, and a natural steroid diol, were used. We also included four straight-chain diesters as acyl donors with sizes ranging from C_3-C_{10}. This array was then duplicated with identical reaction mixtures, but without enzyme, and this half of the plate served as a necessary series of controls. Reactions were initiated with the addition of 20 mg/ml *C. antarctica* lipase. After 88h, the reactions were terminated and worked up.

A range of polymer sizes was obtained from this array with aliphatic (**1-3**) and aromatic (**4**) acyl acceptors yielding appreciable molecular weights (Figure 3). The M_w ranged from 1,540 to as high as 6,460 for the polymer synthesized from bis(2,2,2-trifluoroethyl) malonate and ethylene glycol. *C. antarctica* lipase displayed diversified polymerization activity towards the other eight acyl acceptors (sugars/nucleic acids/steroid diol), for example, rather small esters were formed with the disaccharides and all four acyl donors. Notably, polymers formed from sorbitol (**8**) with the four-acyl donors (**A-D**) displayed the highest molecular weights, which ranged from 7,000 to greater than 20,000 Da. On a weight average basis, up to 50 sorbitol molecules were incorporated into the polyester. Sugar containing polyesters are important for its interesting properties, which include a high number of hydroxyl moieties resulting in increased hydrophilicity, and may have importance in hydrogel applications (*17,18*).

The hallmark selectivity of enzymes, specifically *C. antarctica* lipase, is highlighted in this library. Polymers formed from sorbitol and mannitol showed different molecular weights, with the former exhibiting larger polymer formation These two sugar alcohols only differ in the orientation of the hydroxyl moiety at the C-2 position. Hence, *C. antarctica* lipase is able to differentiate slight variations in structure, and highlights the discriminating catalytic power of biocatalytic polymer formation over acid or base-catalysis. The enzyme-catalyzed polyester array was able to uncover the high reactivity and unusually high molecular weight for sorbitol-containing polymers indicating that this approach appears to have a role in new polymer discovery.

Detailed studies were conducted into four specific wells of the lipase-catalyzed array. Two acyl donors, DVA and divinyl sebacate (DVS), were each reacted with 1,8-octanediol and sorbitol with conversion recorded as a function of time. The reactions proceeded quickly with complete conversion of monomer

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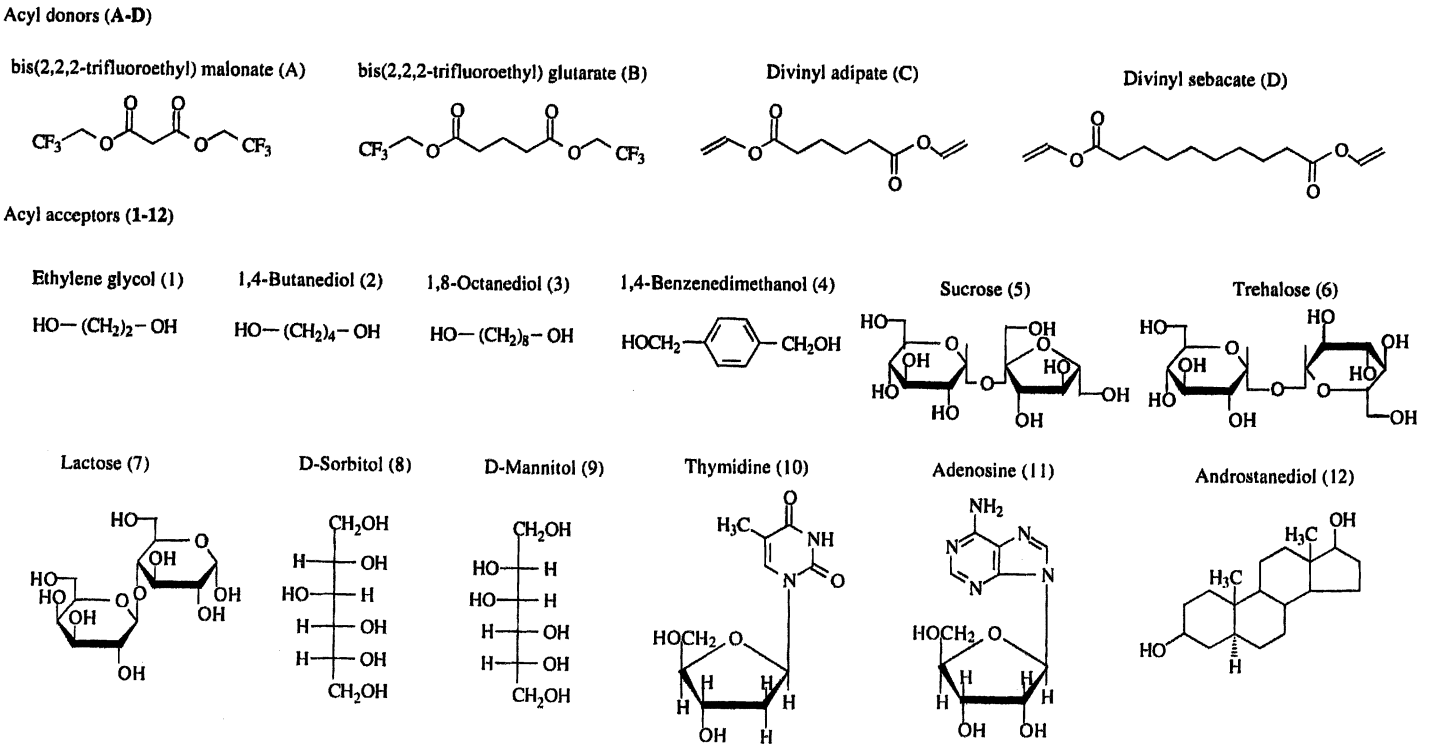

Figure 2. Array of acyl donors and acyl acceptors polycondensed for the synthesis of polymer libraries.

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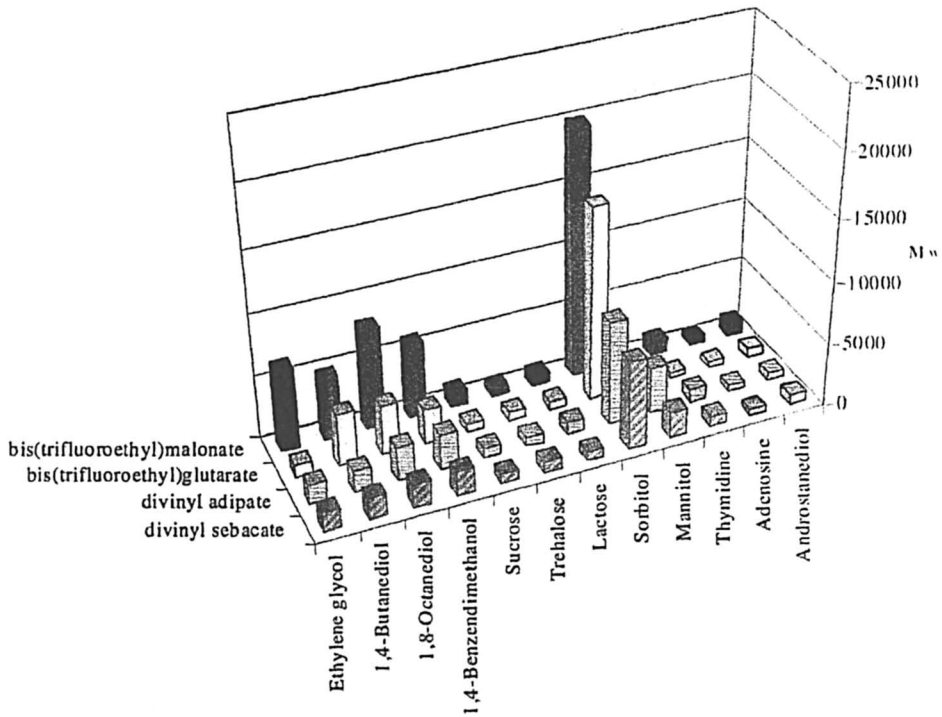

Figure 3. Library 2: Array synthesized from a diverse number of monomers (4 acyl donors x 12 acyl acceptors) to highlight the wide range application of enzymatic polymerizations.

within 25 min and 1 h for DVA/octanediol and DVS/octanediol, respectively. Monomer depletion was dependent on both acyl donor and polyol size with longer reaction times required for sorbitol. The extended monomer reaction times were 2 h and 10 h, for DVA/sorbitol and DVS/sorbitol, respectively.

Large-scale poly(sorbitol adipate) synthesis was conducted in order to investigate the regioselectivity of *C. antarctica* lipase. The polymer was isolated, as described in the materials and methods, and 5.7 grams of polymer was obtained (isolated yield of 49%). Structural analysis (^{13}C NMR) revealed the terminal carbons on the sugar polyol shifting downfield after polymerization from 62.6 to 65.8 ppm and 63.5 to 66.6 ppm for C-1 and C-6, respectively. Carbons adjacent to the primary hydroxyls indicated an upfield shift from 72.4 to 70.7 ppm and 73.8 to 71.1 ppm for C-2 and C-5, respectively. Furthermore, the chemical shift corresponding to the vinyl group on the acyl donor disappeared from the polymer spectrum completely after reaction. Therefore, as result of acylated carbons shifting downfield and adjacent carbons shifting upfield (*19*) a linear polymer was formed from the selective acylation of the terminal hydroxyls on sorbitol. In addition, Uyama and coworkers (*20*) report the synthesis of a similar polymer with sorbitol, poly (sorbitol sebacate), with an analogous conclusion of *C. antarctica* lipase regioselectively acylating the sugar alcohol at the terminal 1- and 6- positions.

Polymer molecular weights were also investigated as a function of time. The M_w for the reaction between 1,8-octanediol and DVA reached a maximum of 1,600 Da and decreased to 1,200 Da after 94 h (Figure 4). The M_w for the reaction between sorbitol and DVA reached a maximum of 21,700 Da and decreased to 13,600 Da after 94 h. These results were unusual since AA-BB polycondensation reactions proceed in a step-wise fashion, whereby the depletion of monomer occurs to produce dimers, trimers, and oligomers, which then condense to generate polymers. However, after 25 min and 2 h, for octanediol and sorbitol, repectively, the molecular weight decreased perhaps due to lower reactivity of the lipase towards higher molecular DVA/octanediol and DVA/sorbitol oligomers, relative to the monomers. Moreover, the polymer size decreases over time, well after the depletion of monomers. We speculate that a small amount of water, introduced either by the solvent and/or the biocatalyst, occurs and this results in partial depolymerization. Therefore, the lipase may catalyze the hydrolysis of polymer, resulting in ester cleavage and decreased polymer sizes.

Russell and coworkers (*21*) extensively investigated the hydrolysis and depolymerization phenomena of biocatalytic AA-BB polycondensed materials. Their discussions present insight into the hydrolysis of DVA when reacted with 1,4-butanediol. The hydrolysis of DVA during small oligomer formation is not significant, but as the polymerization progresses and as the monomer concentration decreases, the enzyme bound water is involved in hydrolysis of the

vinyl end group, thereby limiting the extent of polymerization. Our results are consistent with Chaudhary et al's (*21*) that water has an important role in preventing high molecular weight formation.

Enzyme-catalyzed polyphenol reactions

Peroxidases may be thought of as the ideal combinatorial biocatalyst due to their extraordinarily broad substrate specificity. Peroxidase catalyzes the oxidative polymerization of phenols to generate phenolic polymers in the absence of formaldehyde, and peroxidase catalysis has proven to be a highly effective method to synthesize polyphenols (*5, 22, 23*). The availability of a wide variety of phenolic monomers and the broad substrate specificity of peroxidases enables a single enzyme to be used to generate a combinatorially diverse array of polyphenols (*24, 25*). Polyphenols are known to interact with metal ions, and the resulting polymer-metal complexes show altered fluorescence properties as compared with the uncomplexed polyphenols (*26, 27*). Therefore, enzymatically and combinatorially generated phenolic polymer libraries have application as a sensor array for metal ions.

Sensing for metal ions has been a rather demanding research area. Metal ions are ubiquitous in nature and also represent a significant man-made pollutant (*28, 29*). Metal ions often exist in mixtures and different metal ions may respond similarly to a given sensing material. The emerging technology of 'electronic noses' employs an array of rather non-selective sensors that utilizes a recognition technique to identify patterns of sensor elements that respond to specific analytes (*30, 31*). Such a design mimics the mammalian olfactory system, which consists of a large array of receptors (*32, 33*). Odorants interact with a combination of receptors and the pattern of receptor-odorant interactions is processed in the brain (*34*).

In library 3, we utilize sensor arrays composed of phenolic polymers generated by combinatorial catalysis using SBP and pattern recognition techniques to sense metal ions. The polyphenols prepared are more environmentally friendly than their chemical counterparts, phenol formaldehyde resins, which typically require formaldehyde during the syntheses.

Library 3: Phenolic polymer array used for metal-ion sensing

A phenolic polymer sensing array (15 members) was synthesized combinatorially from 5 phenolic monomers under the catalysis of SBP, as listed in Table 1. ΔF values of the fluorescence response of polymers upon metal binding, along with each specific MEWs when excited at 322 nm, are shown in Table 2 for 1.0 mM of Cu^{+2} and 0.2 mM of Fe^{+3}.

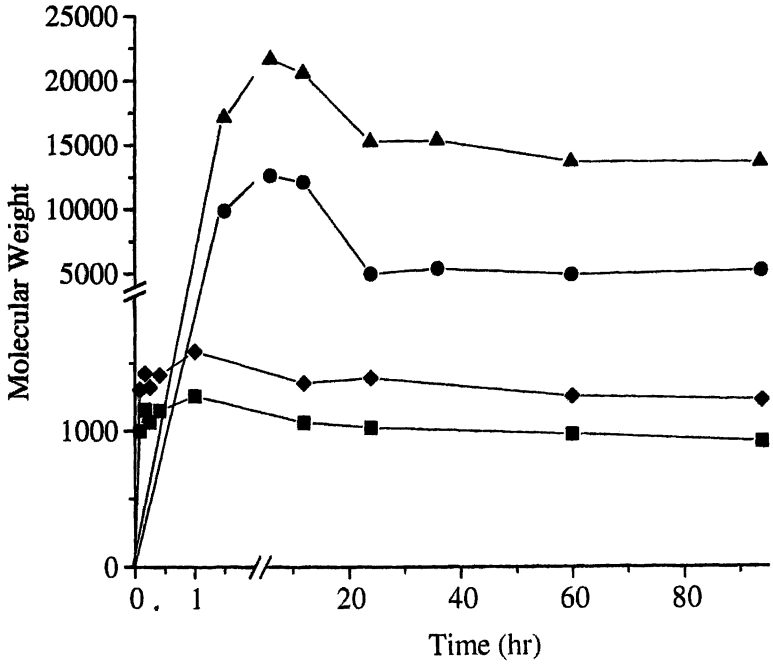

Figure 4. Molecular weight of polymers synthesized from DVA/sorbitol [M_w(▲) and M_n(●)] and DVA/octanediol [M_w(♦) and M_n(■)] as time progresses.

Table 1. Polyphenol array 2 generated of homo- and copolymers from 5 phenolic monomers.

	monomer				
monomer	M_1	M_2	M_3	M_4	M_5
M_1	P_{11}	P_{12}	P_{13}	P_{14}	P_{15}
M_2		P_{22}	P_{23}	P_{24}	P_{25}
M_3			P_{33}	P_{34}	P_{35}
M_4				P_{44}	P_{45}
M_5					P_{55}

M_1: *p*-cresol; M_2: *p*-phenylphenol; M_3: *p*-methoxyphenol; M_4: *p*-hydroxyphenyl acetic acid; M_5: *p*-hydroxybenzoic acid. All copolymers were prepared at 1:1 phenolic monomer substrate ratio (see text for details).

It is clear from Table 1 and 2 that copolymers not only have different MEW from the corresponding homopolymers, they also have different ΔF values of fluorescence response to metal ions. Every element of the 15-member array is measured for fluorescence response upon exposure to 4 different metal ions, Fe^{+3}, Cu^{+2}, Ni^{+2}, and Co^{+2}.

A statistical analysis is used to evaluate the difference of each fluorescence response. ΔZ, which represents the normalized deviation of fluorescence response from the average for each sensing element in the sensing array, is defined as follows in Eq. 2:

$$\Delta Z = \frac{\Delta F - x}{y} \tag{2}$$

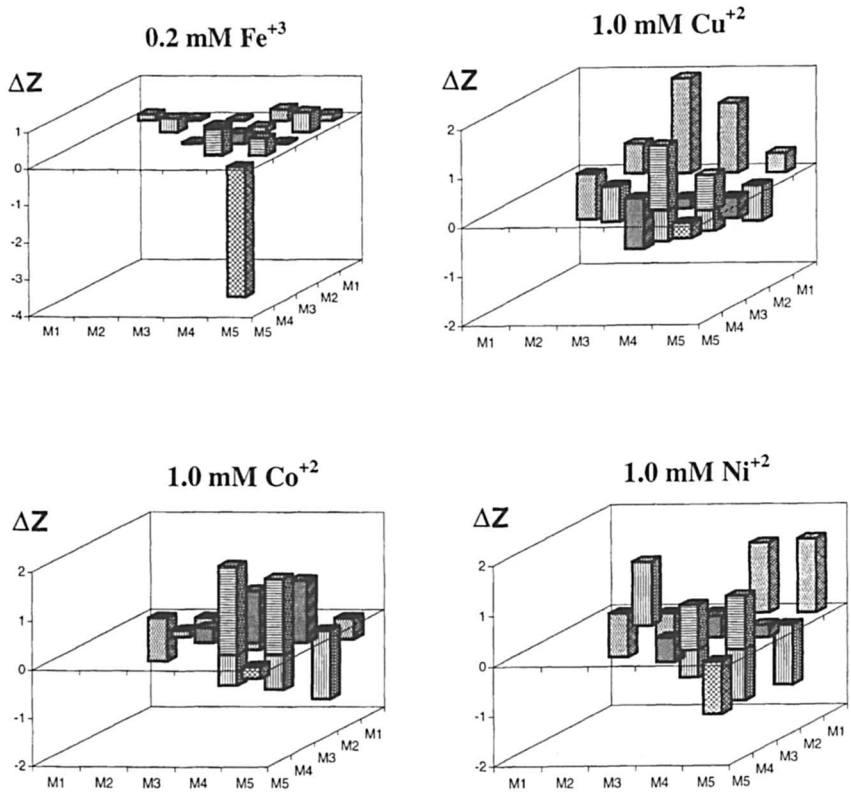

Figure 5. Histograms of 4 metal ions generated from the 15-member homo- and copolymer array, representing "fingerprints" of the metal ions and their concentrations.

Table 2. Maximum emission wavelengths (MEWs) of each element in the 15-member polyphenol sensor array II, and fluorescence intensity change ratio, ΔF, of each element to four different metal ions

		Metal Ions			
	MEW (nm)	*Fe^{+3} (0.2 mM)*	*Cu^{+2} (1.0 mM)*	*Ni^{+2} (1.0 mM)*	*Co^{+2} (1.0 mM)*
P_{11}	360.8	0.52	0.14	0.03	0.03
P_{22}	360.8	0.59	0.16	0.10	0.06
P_{33}	360.8	0.47	0.13	0.04	0.09
P_{44}	408.0	0.70	0.36	0.09	0.16
P_{55}	408.0	-0.78	0.20	0.02	0.06
P_{12}	398.4	0.48	0.29	0.04	0.06
P_{13}	360.8	0.47	0.42	0.05	0.04
P_{14}	360.8	0.57	0.37	0.10	0.09
P_{55}	398.4	0.52	0.27	0.11	0.05
P_{23}	360.8	0.52	0.12	0.02	0.02
P_{24}	440.0	0.51	0.14	0.01	0.01
P_{25}	444.8	0.65	0.16	0.02	0.00
P_{34}	404.0	0.56	0.21	0.07	0.12
P_{35}	404.0	0.47	0.19	0.07	0.13
P_{45}	364.8	0.62	0.30	0.09	0.15

MEW represents maximum emission wavelength for each polymer when excited at 322 nm.

where the x and y represent the average and standard deviation. Based on ΔZ obtained for each sensing element, histograms were generated for the 15-member array using 0.2 mM or 1 mM metal ions as characteristic 'fingerprints' (Figure 5).

In conclusion, the diverse catalytic power of enzymes has been demonstrated with the generation of three unique polymer arrays. Environmentally compatible polymer libraries are synthesized by exploiting biocatalysts in a variety of reaction media, different monomers, and reaction types. This combinatorial array-based biocatalytic approach can be extended and utilized as a tool for polymer materials discovery.

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