Preparing Fluorescent Conjugated Polymers through One Pot Enzymatic Polymerization for Sensing Applications

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Abstract Fluorescence based polymeric sensors are simple, extremely sensitive, versatile and adaptable for detection of different types of analytes. However, the multi-step synthesis using toxic chemicals followed by purification is often required for obtaining these fluorophores. Here, the possibility of utilizing enzymes as the catalyst, for the one step polymerization of naturally occurring monomers to yield fluorescent conjugated polymers is presented. Four monomers, 4-Hydroxyphenylacetic acid (HPA), Hydroxytyrosol (HDT), Chlorogenic acid (CGA) and Serotonin (Sero) were polymerized using Horseradish peroxidase as the biocatalyst. The broad peaks of IR spectrum were obtained from all polymers comparing with the sharp peaks of monomers. The decrease of UV-Vis intensity at significant wavelength of each monomer was found when the polymer was formed. These polymers exhibit fluorescence with significant stokes shift around 100 nm rendering them useful in fluorescence quenching-based sensors. In the detection of lead ion, DNT and TNT in solution through fluorescence-quenching are studied. Both nitro aromatic compounds were detected from electron interaction between phenol and nitro-aromatic compound structures that poly(HPA) and poly(Sero) detected at 1 ppm level. From having carboxylic group, poly(HPA) detected lead at 50 ppb at neutral pH.

1. Introduction
Fluorescence-based sensors have been used to detect various specific analytes such as explosive materials, metal ions and biological materials.[1,2] These sensors can be interacted with the analytes (receptors) depending on their chemical structure and mechanism. The fluorescent characteristic changing is relied on the interaction and also the contribution of the analytes concentration. From many reported sensor mechanisms, the turn off or quenching based sensors, reduction in fluorescent intensity or shifting the particular wavelength range, are simplest fluorescent techniques. However, many fluorescent polymers used in sensors, for example polythiophenes and poly(p-phenylene ethynylene), require multi-step synthesis and purification by using more toxic chemicals.[3,4] To solve these problems, enzyme catalysis has been introduced. This catalyst has been introduced in many chemical reactions including polymerization from the non-toxic nature of enzymes. Enzymes have a unique ability to simplify transformations with less step like one pot method. The enzymatic chemical reactions can be...
carried out under mild reaction conditions including pressure, temperature and pH and also in greener solvents (aqueous based or mixed solvent systems)[5].

In this research, four naturally occurring phenol monomers were chosen as starting materials to polymerize fluorescent materials. These are including 4-hydroxyphenylacetic acid (HPA), hydroxytyrosol (HDT), chlorogenic acid (CGA), and serotonin (Sero)[6,9]. These monomers were selected from having different functional groups that have a potential to detect different analytes through different mechanisms. The oxidoreductase enzyme namely Horseradish peroxidase (HRP) had been selected as catalyst to polymerize these four monomers. The biocatalytic synthesis in this work is one-step reaction. Enzymatically synthesized polyphenols were then characterized by using spectroscopic techniques including UV-Vis absorption, FTIR, NMR and fluorescence spectroscopy. To determine the sensing abilities of these four polyphenols, the quenching studies were carried in the presence of nitroaromatic compounds including dinitrotoluene (DNT) and trinitrotoluene (TNT) in an organic solvent of acetonitrile and Lead (II) nitrate in water.

2. Materials
Hydroxytyrosol (TCI America), serotonin hydrochloride (Alfa Aesar), 4-Hydroxyphenylacetic acid, Chlorogenic acid and Horseradish Peroxidase (HRP-Type II, RZ ~ 1.9 from Sigma Aldrich) were purchased and used without further purification. 3[v/v] of Hydrogen peroxide (H2O2 from Alfa Aesar) stock solution was prepared by diluted with de-ionized water. All analytes, 2,4-dinitrotoluene (DNT), 2,4,6-trinitrotoluene (TNT) and lead (II) nitrate, were purchased from Sigma Aldrich and used as received. Other remaining organic solvents (such as Acetonitrile and Ethanol, etc.) were reagent grade.

3. Methods
3.1. Enzymatic Synthesis
10 milligrams of each monomer was firstly dissolved in the mixtures of deionized water and ethanol [9:1 v/v] to prepare the starting monomer solution. The pH of the solution was then adjusted to 7.5 by using acidic or alkaline solution depending on starting pH. Then, 4 mg of HRP catalyst was added into the adjusted monomer solution following with slow dropping of H2O2 (1 ml). The solution was continuing stirred at room temperature for 24 hours and removed of unreacted monomer by dialyzing against deionized water at least 48 hours.

3.2. Characterization
3.2.1. UV-Vis Absorption Spectroscopy. To monitor the enzymatic polymerization, the reactions were investigated by using a UV-Vis spectrometer (Model - Agilent 8453). The sample solution (about 0.1 ml) was added into 2.9 ml of a mixture solution (water and ethanol at 9:1 v/v) in a quartz cuvette. The UV spectrum (in the wavelength range of 190-1100 nm) was then recorded.

3.2.2. Spectroscopy Studied. The spectroscopic spectra of monomers and polymers were characterized with two techniques. Thermo Scientific Nicolet 4700 FTIR instrument with a Smart Orbit Attenuated Total Reflectance (ATR) accessory in the frequency range of 4000 - 400 cm-1 with a resolution of 4 cm-1 and 32 scans was used to obtain FTIR spectrum. The 1H-NMR spectra were recorded using a Bruker & Spectrospin Advance 500 spectroscopy (500 MHz) with using d6-DMSO as solvent at room temperature (~20 °C).
3.2.3 Fluorescence Analysis Studies of Polyphenols. To compare the fluorescent characteristic, the absorption of each monomer and polymer was measured by using a fluorescence spectrophotometer (Cary Eclipse). The sample solution was diluted with deionized water to optimize the fluorescent intensity. The excitation maxima for each of the solutions was firstly determined in the wavelength of 200 to 700 nm. The fluorescence emission of each sample solution was then measured by using that maximum excitation wavelength.

3.2.4 Fluorescence Quenching Studied in Analysts Detection. Fluorescent quenching behaviour or reduction in the fluorescence intensity of each polyphenol in the presence of DNT, TNT, and lead (II) nitrate, was measured by using the same spectrophotometer in 3.2.3. To eliminate the solubility limit of nitroaromatic compounds (DNT and TNT), the polymer solution was diluted with acetonitrile instead of water while the water was used as solvent for lead (II) nitrate. Moreover, to optimize lead detection condition, the pH of polymer solution was varied in the range of 4 to 9. Fluorescence quenching sensitivity was calculated based on Stern-Volmer constant ($K_{SV}$), provided in the following equation \[ \frac{I_0}{I} = 1 + K_{SV} \cdot M_c \]

Where $I_0$ and $I$ are the fluorescent intensity before and after adding the analyte, respectively. The $M_c$ represents the molar analyte concentration.

4. Results and Discussion

At the starting condition before the enzymatic reaction, all monomer solutions were clear and colorless (except in a yellow tinge for CGA). However, the color of all monomer mixture solution changed rapidly to orange-yellow or dark brown (depending on monomer type) after addition of the HPA and $H_2O_2$ to start polymerization reaction.

4.1 UV-Vis Analysis of Polyphenols

As mention earlier, the UV-Vis spectrophotometer was used to monitor the enzymatic polymerization reaction. The phenol monomers have well-known exhibit high UV absorbance at less than 350 nm \[12\] The examples of UV-Vis spectrum of phenol monomer and polymer are shown in figure 1 and 2 while the spectra peaks of all monomers and polymers are summarized in table 1. Normally, all phenol monomers have a dominant peak represented the $\pi \rightarrow \pi^*$ transition of the phenyl ring (at 224 nm for HPA as shown in figure 1). The remaining small peaks are from the substituted groups such as carbonyl group in HPA monomer structure (from $n \rightarrow \pi^*$ at 276 nm) or the double bond between phenyl ring and ester linkage (figure 2) in CGA chemical structure at 324 nm \[13,14\] After polymerization, most of polymer peaks exhibited a redshift (or disappeared) with longer tail absorption to 400-600 nm depended on monomer type. This redshift, shift to negative, is from electron delocalization of polymer formation, having more conjugated structure, and with the lower energy required for the $\pi \rightarrow \pi^*$ transition.
Figure 1. UV-Visible absorption of HPA monomer and polymer.

Figure 2. UV-Visible absorption of CGA monomer and polymer.
Table 1. UV-Visible Wavelength Peaks of Phenol Monomers and Polymers.

| Materials       | Wavelength peak (nm)                  |
|-----------------|---------------------------------------|
| HPA monomer     | 224 and 276                           |
| Poly(HPA)       | 228 and 285 with long tail till 450 nm|
| HDT monomer     | 225 and 283                           |
| Poly(HDT)       | 250 and 350 with long tail till 465 nm|
| CGA monomer     | 218, 240 and 324                      |
| Poly(CGA)       | 275 with long tail till 500 nm        |
| Sero monomer    | 260, 270 and 300                      |
| Poly(Sero)      | Long tail till 600 nm                 |

4.2. Chemical Structure Characterization by FTIR and $^1$H-NMR

Normally, polyphenols from enzymatic polymerization have highly articulated structures with a combination of monomer units coupled through C-C and C-O-C linkages making it more complicated for clear structure elucidation using NMR spectroscopy (as shown in figure 3 for $^1$H-NMR spectra of HPA and poly(HPA)). Fourier transform infrared spectroscopic technique is usually for suitable for confirming the polymer structure for most of polyphenols synthesized from the enzymatic polymerization reaction.[15] Figure 4 show the example of FTIR spectra of HPA monomer and poly(HPA) polymerized through enzymatic reaction. After purification by removing the unreacted monomer and HRP catalyst from the polymer solution by dialysis, the characteristic peaks especially amide group of HRP are not recognized in the FTIR spectra of all polyphenols. Most polymers have broader peaks as compared with monomers from having more conjugated structure from a combining of phenyl rings with C-C and/or C-O-C linkages.
Figure 3. $^1$H-NMR spectra of HPA monomer and polymer.

Figure 4. FTIR spectra of HPA monomer and polymer.
4.3. Fluorescence Characteristic Properties of Polyphenol

All enzymatic polymerization reactions were carried out in the water-ethanol mixtures instead of buffer solution as reported from the literature. This is from avoiding of buffer salts effect, the interaction between polyelectrolytes (salts) and conjugated polymers leading to quench the fluorescence emission.[16]

Figure 5 and 6 show the example of fluorescence characteristic strum of monomer and polymer of HPA. The fluorescent results; excitation, emission and Stokes shift, of all materials are summarized in table 2. The excitation wavelength in each sample solution was determined at the maximum absorbance. The Stokes shift, the difference between the excitation and emission maxima of a particular fluorophore, is an important property for utilization of these fluorescent materials in sensing applications. It allows photons emitted by the fluorophore to be detected against the background, without interference from the photons used for excitation. Therefore, the shift varies with the molecular structure of fluorescent samples (or fluorophores). From having higher conjugated structure, all polymers (except poly(CGA)) emit at a higher wavelength as compared to their monomers. These results are the evidence to confirm that the chemical conformation differences between monomer and polymer in the solution have an influence on the shift of excitation and emission.[17]

![Fluorescence spectra](image)

**Figure 5.** Fluorescence spectra of excitation (EX) and emission (Em) of HPA monomer and polymer.
4.4. Fluorescence Quenching Studies of Polyphenols in a Presence of DNT and TNT

The quenching mechanism and detective sensitivity of fluorescence sensor are relied on the interaction between analytes and fluorophore resulting in fluorescence intensity. Since nitroaromatic compounds as DNT and TNT are electron deficient, they will quench the fluorescence of electron-rich as conjugated polyphenols after the interaction between them. All polyphenols have good ability to detect TNT at slightly better than DNT. However, the analytical detection ability limit for all conjugated polyphenols are in the range of parts per hundreds of analyte concentration. The fluorescence quenching sensitivity can be calculated based on Stern-Volmer constant (K_{SV}). The K_{SV} is the slope of the graph of the ratio of fluorescence intensity in the absence of the analyte to intensity in the presence of the analyte (I_0 / I) versus the analyte concentration (as shown in figure 7). A higher K_{SV} value of represents the high sensitivity of the fluorophore towards the analyte. All enzymatic synthesized polyphenols showed the K_{SV} values
in the thousands as shown the results in table 3. The limited sensitivity is attributed to extensive aggregation of the polymers in the solutions.

![Fluorescence spectra with Stern-Volmer plot of poly(CGA) at various DNT concentrations.](image)

**Figure 7.** Fluorescence spectra with Stern-Volmer plot of poly(CGA) at various DNT concentrations.

| Table 3 | Summary of Sensitivity of DNT and TNT Detection by Polyphenols through Fluorescence Quenching |
|---------|--------------------------------------------------------------------------------------------------|
|         | **Minimum DNT concentration detection** | **Minimum TNT concentration detection** |
| **Polyphenol** | $\times 10^6$ M | ppm | $K_{SV}$ (M$^{-1}$) | $\times 10^6$ M | ppm | $K_{SV}$ (M$^{-1}$) |
| Poly(HPA)  | 5.5 | 1.0 | 3460 | 4 | 0.9 | 3410 |
| Poly(HDT)  | 55 | 10 | 1120 | 20 | 4.6 | 2170 |
| Poly(CGA)  | 55 | 10 | 1150 | 20 | 4.6 | 2280 |
| Poly(Sero) | 5.5 | 1.0 | 2690 | 4 | 0.9 | 2620 |

4.5. *Fluorescence Quenching Studies of Polyphenols in a Presence of Lead*

The preliminary results showed that only poly(HPA) and poly(CGA) had a potential to detect lead ion in water while the fluorescence quenching ability to detect lead ion of poly(HDT) and poly(Sero) required at the high amount of lead ions (more than 50 ppm). This is from having a carboxylic group (-COOH) in their chemical structure. The carboxylic group has a potential to absorb and interact (form a complex) with lead ion (and also other metal ions as described in the literatures$^{18,19}$). The effect of pH on the detection ability of poly(HPA) and poly(CGA) was investigated as shown the results in table 4. It
was found that both polyphenols have a high ability to detect lead ions at lower concentration during pH 6 to 8. The pKa reported values of carboxyl position of HPA and CGA monomer are 4.25 and 3.5, respectively\(^{20,21}\). At lower pH closed to pKa or lower than, the carboxylic groups are protonated leading to the reduction of the adsorptive activity\(^{18}\). The deprotonation of carboxyl groups to carboxylate ions (\(-\text{COO}^-\)) starts at high pH resulting in highly complex forming between polyphenols and lead ions. The detection ability decreased at high pH from lower solubility of lead ions in water (precipitation)\(^{19}\).

### Table 4. Summary of Sensitivity of Lead (II) Nitrate Detection by Polyphenols through Fluorescence Quenching.

| pH | Minimum Pb$^{2+}$ concentration detection by Poly(HPA) ($\times 10^{-6}$ M) | Minimum Pb$^{2+}$ concentration detection by Poly(CGA) ($\times 10^{-6}$ M) | K$_{SV}$ (M$^{-1}$) | K$_{SV}$ (M$^{-1}$) |
|----|---------------------------------------------------------------------|---------------------------------------------------------------------|------------------|------------------|
| 4  | 55                                                                  | 620                                                                  | 5.5              | 5400              |
| 5  | 28                                                                  | 900                                                                  | 5.5              | 4010              |
| 6  | 0.55                                                                | 5300                                                                 | 2.8              | 6720              |
| 7  | 0.28                                                                | 8900                                                                 | 2.8              | 4870              |
| 8  | 0.55                                                                | 12400                                                                | 2.8              | 5400              |
| 9  | 2.8                                                                  | 3670                                                                 | 2.8              | 3920              |

### Conclusion
The synthesis of four naturally occurring phenols; HPA, PDT, CGA and Sero, have been successfully prepared by using HRP as catalyst under mind condition in this research. The enzymatic reaction is one pot reaction method with less purification step. All phenol monomers and polymers were characterized using UV-Vis, FTIR and Fluorescence spectroscopy to determine and confirm the formation of conjugated polyphenolic structure. By using fluorescence spectroscopic studies, all fluorescent polyphenols exhibit significant Stokes shift up to 100 nm making them suitable for being utilized in quenching based sensors. The nitroaromatic compounds and metal ion detection ability of these polyphenols showed in fifty to hundred parts per billion with a reasonable Stern-Volmer constant number.

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