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Biocatalytic Synthesis of Fluorescent Conjugated Polyserotonin

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Abstract: Polyserotonin was enzymatically synthesized using Horseradish peroxidase (HRP) as the catalyst. This novel conjugated polymer exhibited good fluorescent properties with significantly higher Stokes shift than its monomer. The enzymatic polymerization eliminated the need for extensive purification of the product (typically necessary for the removal of residual metal catalyst) allowing the product to be naturally fluorescent as synthesized. The reaction was monitored using UV-Vis spectrophotometry and the polymers were characterized using Fluorescence, Fourier Transform Infrared Spectroscopy (FTIR) and Thermogravimetry. The possibility of using polyserotonin for the detection of nitro-aromatic compounds, including 2,4-dinitrotoluene (DNT) and trinitrotoluene (TNT), through fluorescence quenching at parts per million levels in solution has also been demonstrated.

Keywords: Polyserotonin; enzymatic polymerization; fluorescence; sensing

1 Introduction

Conjugated polymers have attracted considerable attention due to their tunable optical and electronic properties, rendering them suitable in numerous applications including organic electronics and sensing [1]. Conjugated polymers have been used commercially for the fluorescence sensing based detection of nitroaromatic compounds. The reported mechanism is that of electron transfer through π-π stacking of conjugated fluorescent polymers and nitroaromatic compounds [2]. One well-known class of fluorescent polymers utilized in the detection of nitroaromatic compounds is poly(*p*-phenylene ethynylene) derivatives. The device known as FIDO (Fluorescence Impersonating Dog Olfaction) has been commercially utilized for several years [3]. However, most fluorescent conjugated polymers are often synthesized using metal-salt catalysts and multi-step synthesis [3]. In many cases, the fluorescence of those polymers is quenched due to the strong binding with residual metal-salt catalysts. Extensive purification steps are usually required to obtain the fluorescent conjugated polymers. Moreover, most starting materials and solvents involved in the preparation of synthetic fluorescent conjugated polymers are very toxic. Enzyme catalysis has been utilized for numerous oxidative polymerization reactions including for the synthesis of fluorescent conjugated polymers [4]. Enzymes have the unique ability of carrying out transformations under milder reaction conditions (pH, aqueous media), simplifying very complex reactions, lowering the number of steps involved in purification of the products. Greener solvents (e.g., water) and/or mixed solvent based aqueous system are often utilized as the reaction media [5,6]. The enzyme used for carrying out oxidative polymer synthesis are oxidoreductases. The most common examples are Horseradish peroxidase (HRP) and Soybean peroxidase (SBP) [7]. Both catalysts have been used for the synthesis of a variety of conjugated polymers such as polyanilines, polypyrroles

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and polyphenols (conducting polymers and high thermal-resistant polymers) [7,8]. However, there has been very few reports of fluorescent conjugated polymers synthesized using enzyme catalysis [9,10,11]. Recently, we have demonstrated the possibility of synthesizing fluorescent oligomers of indole using soybean peroxidase as catalyst under mild conditions [4].

Serotonin (5-hydroxytryptamine) is a well-known neurotransmitter in mammals and plays important roles in the regulation of emotion, sleep, and appetite [12]. As shown in Fig. 1, serotonin is an indole derivative comprising a bicyclic structure with primary amine-attached pyrrole fused to a phenol molecule. It is primarily found in the gastrointestinal tract, blood serum, and the central nervous system. It has also been reported to be found in many plants such as legumes, walnuts, hickory nuts, plantains, kiwifruits, and tomatoes [13]. Based on its ability of forming complex with various types of proteins and metal ions, serotonin was used to modify glassy carbon electrode to detect norepinephrine and uric acid [14]. In serotonin, both -OH and -NH_2 groups have good binding ability therefore it can potentially be more effective in sensing applications compared to parent indole. From having bicyclic structure, serotonin exhibits good fluorescence [15]. The highly conjugated structure of the polymer may enhance the fluorescence properties render them suitable for sensing applications. In this work, polyserotonin was polymerized using HRP as the catalyst. UV-Vis and fluorescence spectroscopy were used to monitor the chemical reaction progress and characterize the absorption and fluorescence properties. To study the effectiveness of this conjugated polymer for sensing, nitroaromatic compounds (DNT and TNT) were exposed to polyserotonin in solution while continuously monitoring the fluorescence.

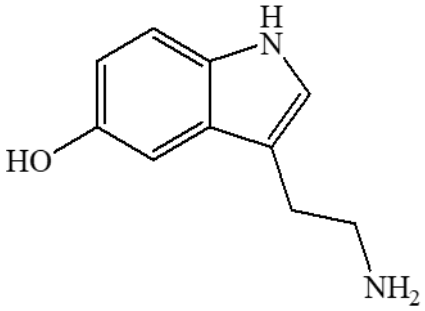

Figure 1: Chemical structure of serotonin monomer

2 Experimental

2.1 Materials

Serotonin hydrochloride (>97%) was purchased from Alfa Aesar and used without further purification. Peroxidase from Horseradish (HRP-Type II, RZ = 1.9), 2,4-Dinitrotoluene (DNT, 97%) and 2,4,6-Trinitrotoluene solution (TNT, 1000 μg/ml in acetonitrile) were purchased from Sigma Aldrich. Quinine sulfate, used as reference standard in quantum yield determination, was also purchased from Sigma Aldrich. The 29-32% Hydrogen peroxide (H_2O_2) from Alfa Aesar was diluted with deionized water to prepare 3% (v/v) stock solution for polymerization. The deionized water was obtained from a Millipore Elix® 3 system equipped with Proguard® 2 filters. All other chemicals (such as Ethanol, Acetonitrile and Sodium hydroxide) were reagent or higher grade of purity and used as received. Cellulose ester dialysis membrane (100-500 Dalton molecular weight cut-off) was obtained from Spectra/Por® Biotech.

2.2 Enzymatic Polymerization of Serotonin

5 mM of serotonin monomer was prepared in 10 ml of mixing solvents between deionized water and ethanol (9:1 v/v). The pH of the monomer solution was adjusted to 7.5 using 1 M sodium hydroxide solution. Then, 4 mg of HRP was dissolved into monomer solution. After homogenization, 1 ml of 3%

H_2O_2 was added into the solution, the solution immediately turned from colorless to reddish-brown color. The reaction was carried out under magnetic stirring for 24 hours at room temperature. The product was purified by dialysis against deionized water to facilitate the removal of unreacted monomers.

2.3 Characterizations of Polyserotonin

The enzymatic polymerization reaction was monitored using UV-Vis spectrometer (Agilent 8453). 10 µl of sample solution (before and after adding H_2O_2) was diluted with 290 µl of water to a total volume of 300 µl in one-millimeter path length quartz cuvette. The UV spectrum was recorded at 1 nm interval in the range of 190-1100 nm wavelength with integration time of 0.5 seconds. The molecular weight was determined using Gel Performance Chromatography (GPC, Agilent 1100 series) with a RI detector calibrated using polystyrene standards. GPC analysis was performed using a PLgel 5 µm Mixed-D column (Agilent Technologies) with N,N-Dimethylformamide (DMF) mixed with 1% Lithium chloride as mobile phase at flow rate of 0.3 ml/min. About 10 mg of dried polymer (from solvent evaporation and dried under vacuum oven) was dissolved in 1 ml of DMF. The polymer solution was filtered through 0.2 µm polytetrafluoroethylene (PTFE) filter membrane (VWR International) before being injected into the GPC. FTIR Spectra of monomer and polymers were obtained on Nicolet 4700 FTIR spectrometer with a Smart Orbit Attenuated Total Reflectance (ATR) accessory. The wavenumber range was 4000-400 cm^{-1} with the resolution of 4 cm^{-1} and 32 scans was used. The thermal stability of serotonin monomer and polymer was analyzed using Thermogravimetric Analyzer (TGA, TA Instruments Q50). Approximately 10 mg of samples was weighed in a ceramic pan and heated up to 750ºC at heating rate of 20 ºC/min. All samples were run under nitrogen atmosphere with constant flow at 60 ml/min.

2.4 Fluorescence Analysis and Quenching Studies of Polyserotonin in DNT and TNT Detection

The fluorescence intensity of the monomer and polyserotonin was measured by using Fluorescence Spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer). 100 µl of polymer or monomer solution was diluted with 2900 µl of deionized water and used for the measurements. The fluorescence quantum yield (QY), the ratio of the number of photons emitted to the number of photons absorbed, was calculated using the absorbance value from UV-Vis spectrophotometer and fluorescence intensity from fluorescence spectrophotometer. QY was calculated using the comparative method [16] by using the Eq. (1),

$$QY = QY_R \left[\frac{A}{A_R}\right]\left[\frac{n^2}{n_R^2}\right] \quad (1)$$

where A is the slope of the line obtained from the plotting integrated fluorescence intensity area against the absorbance for difference concentrations of the fluorophore. 'n' is the refractive index of solvent and the subscript 'R' refers to the reference fluorophore of known QY. Quinine sulfate was used as reference standard fluorophore. The QY_R value of quinine sulfate dissolved in 0.5 M sulfuric acid was reported to be 0.546 [17]. The refractive index of solvent was measured by using Abbe Refractometer (Bausch&Lomb).

For sensing studies, fluorescence quenching of polyserotonin in the presence of 2,4-dinitrotoluene (DNT) and 2,4,6-trinitrotoluene (TNT) was measured using fluorescence spectrophotometer. In this work, 100 µl of polymer solution was diluted with acetonitrile (2.9 ml) in presence of pre-selected concentrations of DNT or TNT. The fluorescence quenching (the reduction in the fluorescence intensity) is attributed to the presence of the analytes. The fluorescence quenching sensitivity was calculated based on the Stern-Volmer constant (K_{SV}), as stated in the Eq. (2) [2,17]. The K_{SV} value is the formation constant of the complex formed between the fluorophore and the analyte molecule. From this equation, it is obvious that a higher value of K_{SV} represents high sensitivity of the fluorophore towards the analyte [18,19],

$$\frac{I_0}{I} = 1 + K_{SV}\,(M_c) \quad (2)$$

where I_0 and I are the initial fluorescence intensity and fluorescence intensity upon addition of the analyte, respectively, and M_c is the molar analyte concentration. K_{SV} can be obtained as a slope of the graph of analyte concentration versus I_0/I. All samples were tested for at least three times for fluorescence quenching studies.

3 Results and discussion

3.1 Enzymatic Polymerization of Serotonin

Recently we have reported the effect of buffer salts on fluorescence intensity in the case of enzymatically synthesized polymers [4]. It was found that buffer salts and polyelectrolytes can interact electrostatically with conjugated polymers leading to quenching of fluorescence emission. To avoid this issue, the enzymatic reaction was carried out in water/ethanol mixtures. The enzymatic reaction was monitored by UV-Vis spectrophotometer. The absorbance spectrum of serotonin monomer indicates clear $\pi \rightarrow \pi^*$ transition from the bicyclic structure as in the case of indole monomer [20]. Most indole derivatives usually show three main absorbance peaks from 215-330 nm. In case of serotonin, there are two peaks near 250-325 nm region with maximum absorbance peak (λ_{max}) at 277 nm and a small shoulder at about 300 nm as shown in Fig. 2. The absorption of indole derivatives above 260 nm is a superposition of bands due to two-electron transition. The region in the range of 215-230 nm is also overlapping with the absorption of polar solvent (water and ethanol), therefore the remaining absorption peak from serotonin has not been observed in this range. After the 24 hours polymerization reaction, the peak intensity in the range of 250-325 nm decreased because of monomer is consumed in the reaction. A longer tail absorption up to 600 nm was formed due to the absorption of polyserotonin species with extended conjugation. Polyserotonin synthesized from this enzymatic polymerization has molecular weight in oligomer range with M_n ~ 1200 Dalton, M_w ~ 1500 Dalton and molecular weight distribution at about 1.25 as determined by GPC.

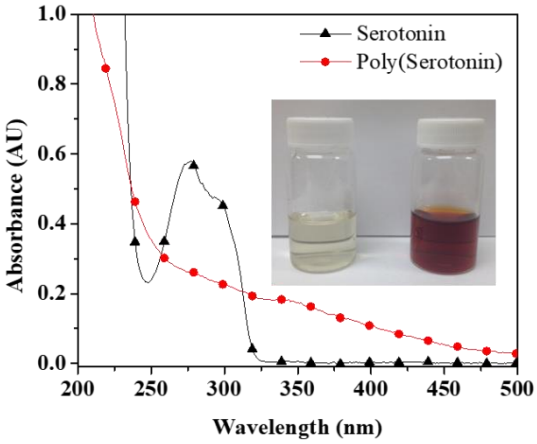

Figure 2: UV-Visible absorption and photograph of solutions of serotonin monomer (left) and polyserotonin (right)

3.2 Spectroscopic Characterization

The FTIR spectrum of serotonin monomer (Fig. 3) shows strong absorption peak at about 3385 cm^{-1} which was attributed to the N-H stretching from pyrrole ring and $-NH_2$ (as a single sharp peak). The broad peak in the range of 3300-3100 cm^{-1} was assigned to -OH vibrational mode while the C-N (amine) from having indole based-structure was observed at 1370 cm^{-1} [21,22]. The vibrational peaks in the range of 3100-2900 cm^{-1} were assigned to C-H stretching of aromatic and aliphatic structure. The peaks in the range of 1600-1400 cm^{-1} and 850-700 cm^{-1} are the vibrational modes of the C-H and C-C bonds in

aromatic structure. Moreover, the peak at 1522 cm^{-1} can be ascribed to the stretching and deformation vibration of the N-H bond [23].

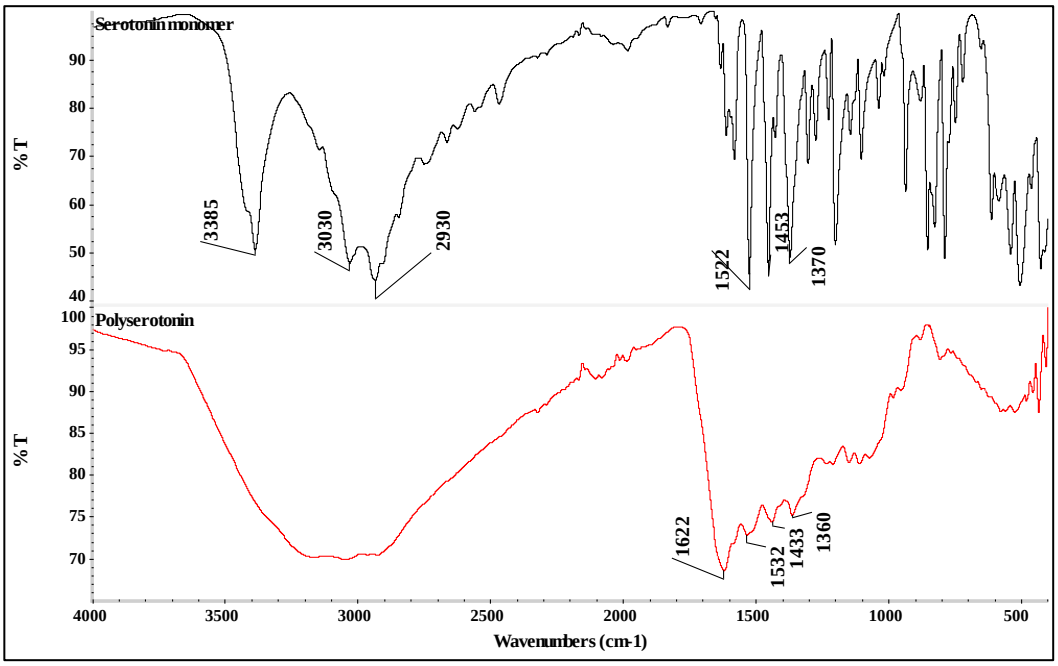

Figure 3: FTIR spectra of serotonin monomer and polymer

The oxidative oligomerization of serotonin has been reported earlier [24,25,26,27,28]. The dimer was claimed as the main product from these reactions. However, the trimer and tetramer were also observed in the case of electrochemical reactions [26]. The main linkages have been reported to be through the benzene ring at C(4)-C(4´) and C(4)-O-C(5´) [24,25,26]. The linkage between benzene and pyrrole ring was proposed through N(1)-C(4´) position [25]. The FTIR of polyserotonin as shown in Fig. 3 showed broad peak in the range 3650-2000 cm^{-1} predominantly from moisture absorption [10]. However, in this region, it is hard to conclude a N(1)-C(4´) linkage purely from the missing N-H absorption. In addition, the FTIR of polymer showed the disappearance of 740 cm^{-1} peak (assigned to out-of-plane deformation of the C-H bond). Therefore, the other possible linkage for the formation of the dimer is through C(2)-C(2') as reported in polymerization of indole [4]. The ^{1}H-NMR of serotonin monomer and polymer were recorded. The ^{1}H-NMR results are shown in Supporting information and the possible linkages are also summarized.

3.3 Photoluminescence Characteristics

As shown in Fig. 4, polyserotonin exhibits significantly higher photoluminescence compared to the monomer due to higher conjugated backbone. This highly conjugated structure with the $\pi \rightarrow \pi^*$ transition helps improve electronic delocalization. The conformation difference between monomer and polymer in the solution also affects the emission [29]. The difference between the maximum wavelength of excitation and emission for a particular fluorophore ($\lambda_{emission}$–$\lambda_{excitation}$) is known as “Stokes shift” [30,31]. Stokes shift is a very important property for utilization of these fluorophores in sensing applications. It allows photons emitted by the fluorophore to be detected against the background, without interference from the photons used for excitation. This shift can range from few nanometers to over several hundred nanometers depending on the molecular structure of fluorophore. From a practical perspective, higher Stokes shift is preferred since the measurement of fluorescence can be made more accurately.

Polyserotonin showed Stokes shift (over 100 nm) two times higher than that of the monomer. Tab. 1 summarizes fluorescence properties of serotonin monomer and polymer. In addition, the influence of monomer and oxidant concentration and solvent on the fluorescent emission properties of the polymer are shown in the Supporting information.

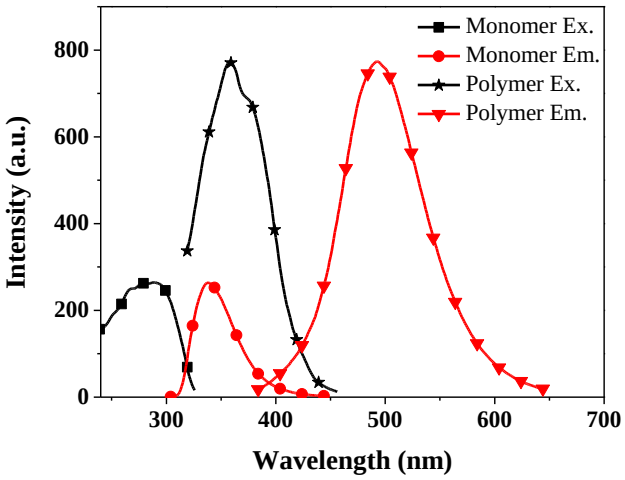

Figure 4: Fluorescence spectra of excitation (Ex.) and emission (Em.) of serotonin monomer and polyserotonin

In this work, polyserotonin showed low quantum yield of 0.017 in water and 0.024 in dimethyl sulfoxide (DMSO). The quantum yield of serotonin monomer was about 0.28 similar to the reported value in literature [15]. Polyserotonin exhibits low fluorescence quantum yield values, compared to the monomer and reference material, most likely because of the highly articulated structure that results in self-quenching. The relationship between the molecular weight and fluorescence quantum yield value had been reported for natural phenolic compounds (such as catechin and epicatechin) and synthetic polyphenols (such as poly(4-hydroxyphenylacetic acid)) [10,32,33].

Table 1: Excitation/emission wavelength and Stokes shift of serotonin monomer and polymer.

Material	Excitation Wavelength ($\lambda_{excitation}$, nm)	Emission Wavelength ($\lambda_{emission}$, nm)	Stokes Shift ($\lambda_{emission} - \lambda_{excitation}$, nm)
Serotonin Monomer	280	339	59
Polyserotonin	360	490	130

3.4 Detection of Nitroaromatics by Polyserotonin through Fluorescence Quenching

Fig. 5 shows fluorescence quenching spectra of polyserotonin in presence of increasing concentration of DNT and TNT. With increasing analyte concentration, the fluorescence intensity of polymer solution decreases. This quenching process is mainly based on electron donor-acceptor mechanism since nitroaromatic compounds (DNT and TNT) are usually electron deficient while polyserotonin is a good electron donor [2]. The fluorescence quenching sensitivity was calculated based on Stern-Volmer constant (K_{SV}). A higher value of K_{SV} represents higher sensitivity of the fluorophore towards the analyte [17,31]. In this research, polyserotonin has K_{SV} value about 2700 M^{-1} for both DNT and TNT. In comparison to other polyphenols synthesized in the same procedure, polyserotonin exhibited moderate sensitivity in nitroaromatic compounds detection [10]. The limited sensitivity of this polymer is

due to extensive of the polymer in solvent [17]. In addition, polyserotonin showed high K_{sv} value upon dilution (data not shown).

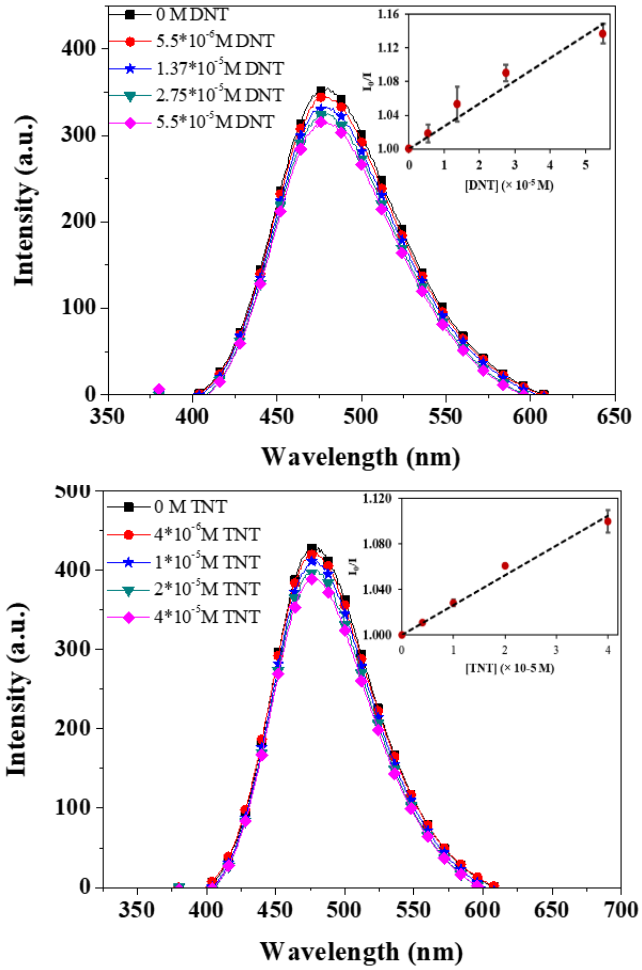

Figure 5: Fluorescence spectra and Stern-Volmer plot of polyserotonin at various concentrations of analytes (A) DNT and (B) TNT

3.5 Study of Thermal Stability of Polyserotonin

The results of thermogravimetric analysis of serotonin monomer and polyserotonin is presented in Fig. 6. The serotonin monomer is stable till 250ºC due to the inter/intramolecular hydrogen bonding. Polyserotonin exhibited higher char remaining at 750ºC compared to the monomers. Both the serotonin monomer and polyserotonin exhibited an initial weight loss between 2-6% at 80-120ºC, attributed to the loss of moisture absorbed by the samples. Apart from the dehydration step, serotonin monomer and polymer exhibit three distinct decomposition steps. The decomposition of side chain attached to bicyclic structure occurs in the range of 220-300ºC, decomposition of aromatic structure (-OH groups and aromatic moieties) occurs at 300-400ºC [34]. Finally, the further decomposition of the remaining product and formation of crosslinked char occurs [35,36]. Serotonin monomer exhibits around 31% char remaining while polyserotonin ends up with over 50% carbonaceous char residue remaining at the end of the test. This provides additional corroborative evidence for the formation of the polymer.

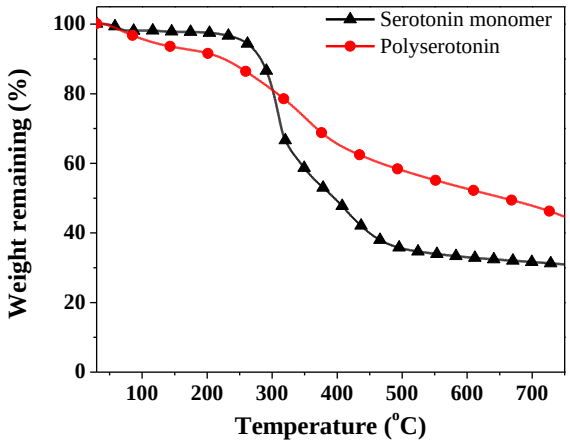

Figure 6: TGA thermogram of serotonin monomer and polymer under nitrogen atmosphere

4 Conclusions

Polyserotonin has been successfully synthesized using HRP as the catalyst under mild conditions in a benign solvent system (water and ethanol) at room temperature. The polymeric product was inherently fluorescent and exhibited Stokes shift greater than 100 nm. Polyserotonin exhibits good sensitivity for detection of nitroaromatic compounds such as DNT and TNT in solution (down to hundreds of parts per billion) through fluorescence quenching. This approach to inherently fluorescent conjugated polyserotonin opens new possibilities in the use of 'greener' methodologies for the creation of fluorophores that can be utilized in numerous applications.

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