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Conducting Matrices of the Poly(-O-Toluidine, PTOL) Film for Immobilizing Uricase

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ABSTRACT: Polyaniline, PANI as conducting polymer has been used as enzyme immobilization matrix in biosensors due to its excellent stability and electrochromic effects, conductivity, and redox reversibility. However, at pH above 4, the electron transfer ability and redox activity decrease, hence it is necessary to modify the PANI structure to increase the PANI pH range. In this study, the PANI derivative, the poly(-o-toluidine, PTOL) film was produced for the uricase immobilization matrix. This study aimed to develop a supporting material that maintains catalytic activity uricase with a facile and inexpensive procedure. The PTOL was prepared by using the chemical oxidative polymerization technique with ammonium persulfate (dissolved in 1.0 M HCl) as an oxidizing agent. The FTIR spectra confirmed the success of PTOL formation by the appearance of its characteristic peaks. The UV-Vis spectra indicated that PTOL was in an emeraldine state with a semi-crystalline structure that was shown by its XRD pattern. While, SEM analysis described that the PTOL film was uniform with some granular aggregation in its surface morphology, but there is no holes or porosity were found. The use of PTOL film as a uricase matrix could maintain the uricase activity against the substrate (uric acid, UA) with excellent analytical characteristics. Therefore, PTOL film could be used as conducting matrices for immobilizing enzymes (e.g. biosensors).

Keywords: Conducting matrices, PTOL film, immobilizing, uricase

INTRODUCTION

It has been known that conducting polymers synthesized by either chemical or electrochemical methods have attractive properties such as good stability, high electrical conductivity, and good ability to entrap and release biomolecules (Kaur et al., 2015; Saranya et al., 2015). Recently, they have been widely used in enzyme immobilization matrices, gas separation membranes electrochromic, metal protection against corrosion, and batteries (Tanveer & Mobin, 2011; Ziadan et al., 2012).

Several conducting polymers have been synthesized, namely polyaniline (PANI), poly(phenylenevinylene), polypyrrole, polythiophene, etc. Among these polymers, PANI (Li & Wang, 2012; Mazzeu et al., 2017) has received much attention as conducting polymer with various advantages, including high conductivity, excellent electrochromic effects, and good redox reversibility (Arora et al., 2007; Mansoor et al., 2011). This is due to the presence of conjugated π electrons delocalized across the conjugated system, making the polymer easily oxidized or reduced. The simplicity of the doping process and its excellent stability in aqueous solution

and air, make it the most chosen conducting polymer. Unfortunately, the delocalized π -bonds along its backbone affected PANI insoluble in organic solvents, limiting its practical use.

Due to their unique characteristics, the PANI family polymers became potentially interesting materials for biotechnology applications, including biosensors (Bayramoglu et al., 2011). PANI as solid support has been employed for uricase immobilization (Kan et al., 2004), since uricase has great importance in medical application and biochemistry for uric acid determination in gout patients. Conducting polymers are compatible with biological molecules including an enzyme in aqueous solutions under neutral conditions. They are reversible electrochemically doped and undoped accompanied by significant changes in the conductivity and spectroscopic properties of the films so that they can be used as signals for biochemical reactions. Changes in pH and the redox potential of the environment affect the electronic conductivity of the conducting polymer (Gerard et al., 2002). Immobilization of this enzyme in appropriate matrix allows the enzyme was used several times while maintaining its catalytic activity (Hermanto et al.,

2020; Hermanto et al., 2019). In addition, immobilized enzymes on conducting polymer facilitate the electron transfer from the active site of an enzyme that redox reaction takes place to analyte solution, providing good detectability and fast response as redox reaction of the substrate. Unfortunately, the low electrochemical activity and conductivity of PANI at above pH 4 will decrease its redox activity through its electron transferability in enzymatic reaction, hence it was not appropriate for a wide pH range (Sarauli et al., 2014).

It is necessary to increase the pH dependency of PANI, via its derivatives such as poly(*o*-toluidine) (PTOL), alkyl-substituted of aniline monomer in the ortho position. PTOL has been probably the most widely studied among the ring-substituted PANI derivatives (Elmansouri et al., 2007; Hwang et al., 2017; Mahudeswaran et al., 2015). Methyl-substituted derivatives of PANI have better solubility and processability than PANI (H. Gul et al., 2018; Saharan et al., 2015). Moreover, PTOL has a faster transition time between oxidation and reduction states than PANI (Dhanavel et al., 2016). Synthesis of PTOL could be done by chemical oxidation of *o*-toluidine in presence of ammonium peroxydisulphate as a strong oxidizing agent (Cordeiro et al., 2005). It has been reported that chemically synthesized PTOL offers a polymer having very good physicochemical properties with good electrical conductivity, making it an excellent matrix for enzymes involving redox reaction (Eltaher & Khan, 2019; Melad & Jarour, 2017; Stepura et al., 2019). In previous research, PTOL serves as a good matrix for the immobilization of enzymes including an amyloglucosidase matrix. The immobilized enzyme showed greater stability to changes in pH and higher temperature compared to the free enzyme and retained high catalytic activity after multiple uses (Ashly & Mohanan, 2010). Hence, PTOL film has the potential to be an attractive material for applications in the field of biotechnology due to its excellent properties. In this study, the properties of PTOL and its impact on uricase activity when it is used as conducting matrices for immobilizing enzyme films was investigated.

EXPERIMENTAL SECTION

Materials

o-toluidine (99%) was obtained from Aldrich and was stored at 4 °C. Uricase isolated from *Aspergillus niger* (EC 1.7.3.3, type I) and ammonium peroxydisulphate ((NH₄)₂S₂O₈) were purchased from Sigma. UA, citric acid (monohydrate, ≥99.5%), hydrochloric acid (HCl) 37%, sodium hydroxide (NaOH), potassium phosphate (KH₂PO₄), sodium borate (NaB₄O₇·10H₂O) and N-methyl-2-pyrrolidone (NMP) were purchased from Merck. UA stock solution (1000 µg/mL or 6.0 mM) was prepared in an aqueous solution. The sample solution was prepared by appropriate dilution and all solutions were diluted with

distilled deionized water. The used reagents were analytical grade.

Preparation of PTOL Film

PTOL film was prepared by *o*-toluidine chemical oxidation using ammonium peroxydisulphate solution with a mole ratio of oxidizer/monomer 1.62 at room temperature under stirring conditions (300 rpm, 30 min), as described by Pron & Rannou (2002). PTOL polymerization was carried out on rectangular transparent mica attached to glass support. Once the reactants were introduced, the polymerization process would begin, and the film grew on the surface of the transparent mica. The polymerization process was carried out for 30 min, then the transparent mica was removed from the glass support. The obtained film was rinsed with 0.1 M HCl and dried at 30 °C. Mica-coated PTOL film was used for uricase immobilization.

Characterization Methods

Measurement of absorbance was conducted using the UV/VIS spectrophotometer (U-1800, Hitachi). Dissolving a certain amount of powder in NMP solvent was done for sample preparations.

The functional group of the obtained PTOL film was identified using the KBr pellet method at FT-IR spectrometer (FT-IR 1600 Perkin Elmer Co Japan) with a compression pressure of 2500 lb/m². The measurement was conducted at the resolution of 4 cm⁻¹ and wavenumber range of 400-4000 cm⁻¹.

The X-ray diffractometer (Rigaku XRD Co Japan) with Cu K α radiation (1.54060 Å) was used for the examination of sample crystallinity. The XRD diffractograms were collected at a 2 θ interval of 5-50° (step size and time were 0.017° and 10 s/step, respectively). While the film surface morphology was characterized using a scanning electron microscope (Hitachi TM-3000) with 3000 \times magnification and 15 kV accelerating voltage.

Immobilization Procedure

The droplet coating method for uricase immobilization was done by adding 0.5 mL of enzyme solution containing 2 mg/mL uricase in 5 mM phosphate buffer (pH 6.0) to the PTOL film and storing at 4 °C overnight. The film was washed out with the same buffer solution to remove non-attached uricase. For absorption measurement of uricase-PTOL film, a cell holder with the size of 9 \times 40 mm² was used.

Measurement Procedure

Firstly, the uricase-PTOL film was immersed in UA solution in phosphate buffer (pH 6) for 5 min. The film was rinsed with distilled water and dried. The absorbance of the film was measured at the maximum wavelength using the unreacted film as a reference.

RESULTS AND DISCUSSION

Conducting Matrices of the PTOL Film

In the preliminary work, the fabrication of PTOL film was carried out by mixing *o*-toluidine monomer and ammonium peroxydisulphate as oxidants. The

1. Coating process with the coating technique produces a thin layer of green PTOL film on the surface of transparent mica. The mechanism of the polymerization reaction of o-toluidine is described in Figure 1.

1. Ammonium peroxodisulphate in 1.0 M HCl solution is a strong oxidizing agent that is expected to oxidize all o-toluidine monomers in polymer solutions. The polymerization process always begins with the oxidation of o-toluidine monomer by ammonium peroxodisulphate which produces a radical cation (initiation stage). Then, radical cation pairs with other radicals or neutral monomers to form dimers by releasing H⁺ protons (propagation stage). The addition of an oxidizing agent to the reaction will oxidize the dimer to a radical and then pair it with another radical to form an oligomer, inducing dication formation. In other words, this process increases the length of the chain, whether dimer, trimer, oligomer, or longer, and is always preceded by an oxidation process which results in reacting with other radical cations to form emeraldine (termination stage). The growth mechanism of the longer polymer chain in solution causes the precipitation of poly(o-toluidine) polymer. In previous work (Cordeiro et al., 2005), oxidizing agents oxidized not only monomers but also species formed in the reaction such as neutral or radical species of dimers, trimers, and oligomers. Hence, the oxidizing agent required for PTOL formation was greater than the amount required by the reaction stoichiometry.

PTOL Film Characterization

The characteristics of PTOL film were synthesized by using the chemical oxidative polymerization technique, which is shown in Figure 2. Characterization of the obtained polymer was done through UV/Vis spectroscopy, XRD, FTIR, and SEM.

The basic structure of a conductor polymer is known through its optical properties, including the conjugation in polymer implied by its electronic spectra. Hence, spectroscopy is an appropriate detection tool to determine the occurred electronic transition of polymer conductors. As a sensitive tool, UV/Vis spectroscopy could explain protonation as well as interactions between solvents, dopants, and polymer chains. The PTOL optical absorption spectrum in NMP solvents is shown in Figure 2(a). It was illustrated that there were two characteristics of absorption around 310 nm and 615 nm which indicated the electronic transition π - π^* of the benzoic ring and n - π^* of benzoic to quinoid ring, respectively. The absence of an extended tail at a higher wavelength was observed when NMP was used as a solvent. While the absorption of the quinoid ring is indicated at the absorption of \sim 590 nm. These rings are associated with intrachain or interchain charge transport signifying the basic form of insulating emeralds from polymers as described in the literature (Chabukswar et al., 2011). According

to a previous study, this implied PTOL polymer exists as an emeraldine state (Dhanavel, 2016).

Some characteristics peak of PTOL was shown in Figure 2(b), FTIR spectra of PTOL. The spectra at 2913 cm⁻¹ indicated the C-H vibration of the methyl group. The C=C vibration of the quinoid and benzenoid rings in PTOL was shown by absorption of 1596 cm⁻¹ and 1494 cm⁻¹, respectively. The presence of quinoid and benzenoid was conformed with vibration at 1162 cm⁻¹ for the C-H plane of the quinoid ring and spectra at 573 cm⁻¹ for C-H out plane stretching of the benzenoid ring. The secondary amine group was associated with absorption at 1307 cm⁻¹ for C-N vibration and 3390 cm⁻¹ for N-H stretching. The methyl group attached to the phenyl ring was characterized by absorption at 879 cm⁻¹, while absorption at 804 cm⁻¹ was attributed to para-disubstituted aromatic rings proving polymer has formed (Kulkarni et al., 2006). The appearance of these characteristic spectra indicated that PTOL was successfully synthesized.

The properties, composition, and structure of ICPs were investigated through the resulting XRD pattern. The conductive state of conducting polymers due to their highly ordered structure was related to their crystallinity and orientation. In Figure 2(c), the XRD pattern shows that PTOL films are semi-crystalline materials (Saharan et al., 2015). This result agrees with previous work by Cordeiro et al., (2015), which indicated PTOL has a semi-crystalline structure. This work also concluded higher crystallinity was obtained in PTOL with higher MW and monomer: oxidizing ratio. Some of the intense and sharp PTOL film peaks are shown at 2 θ of 14, 16, 38, 44, 64°, and other peaks at 2 θ of 24, 38, 57, and 69°. The peak at 2 θ \approx 24° is caused by reflected/scattered radiation perpendicular to the chain (Gul et al., 2018). The high crystallinity is possible due to the substitution and hydrogen bonding between the hydrogen of one ring and the sulfonic group of the other ring of the adjacent chain.

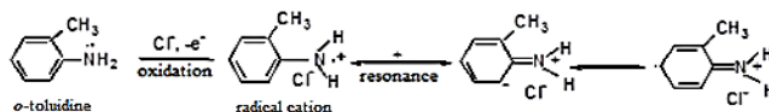
The presence of substituents in PTOL affects the morphology of the polymer. Figure 2(d) shows the micrograph of PTOL at 3000 \times magnifications. The film was uniform and no holes or porosity were found in its surface morphology. In some locations on the surface of the film, granular aggregation was found. It is possibly correlated to the steric contribution of the methyl group on the ortho position of the benzene ring which leads to distortion in the polymer chain and results in the breakdown of the polymer chain into small granular fragments (Gul et al., 2018).

Applications of PTOL Film

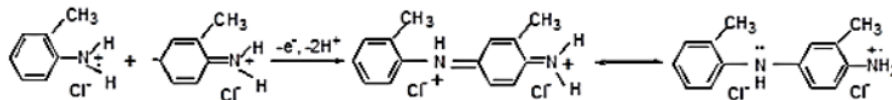
The supports coated with PTOL film are used for uricase immobilization, which was called the uricase-PTOL film. The film reacted with the substrate as soon as it contacted UA solution (in phosphate buffer, pH 6.0), which was written in Equation (1).



step 1, initiation stage



step 2, propagation stage



step 3, termination stage

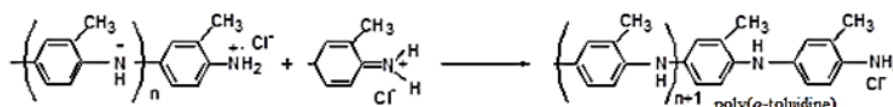


Figure 1 The mechanism polymerization reaction of o-toluidine

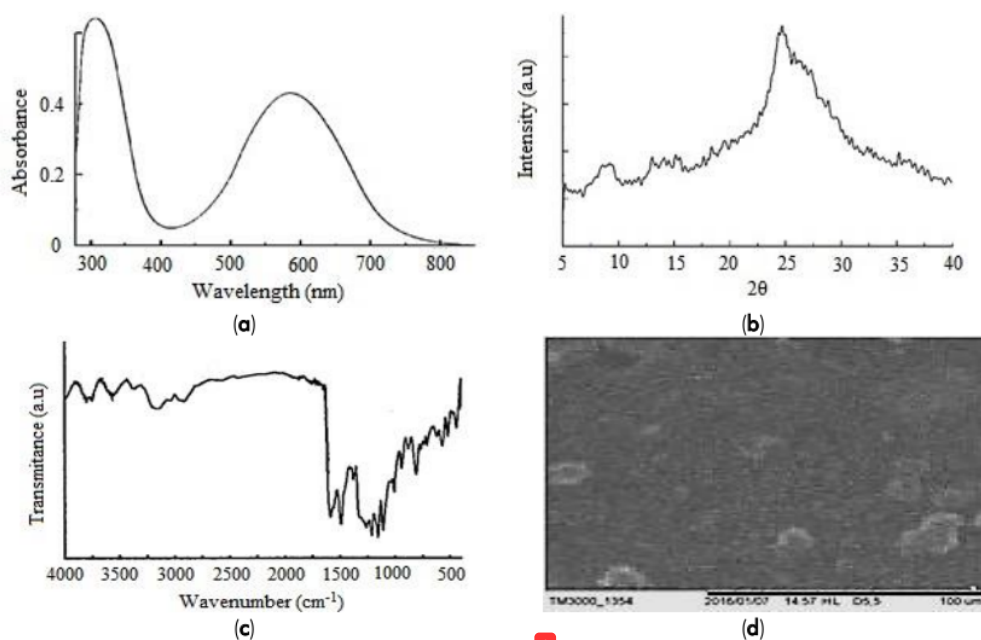


Figure 2 Characteristics of PTOL film were synthesized by mixing o-toluidine monomer and ammonium peroxydisulphate in HCl solution (1.0 M) (a) UV-vis spectra (b) XRD patterns (c) FTIR spectra (d) SEM images of surface morphology

The color change of the conductor matrix from purple to bluish green after reacting with UA indicated that uricase was well immobilized in the matrix. Immobilized uricase catalyzes the hydrolysis reaction of UA to produce hydrogen peroxide which increased the pH and changed the color of the PTOL film. The hydrogen peroxide result as given above can be easily reduced in the presence of the PTOL film. While

the PTOL film, in vice versa, can be easily oxidized and protonated, leading to color change in the film. In other words, the optical determination of UA is based on the H_2O_2 oxidation produced by the uricase-catalyzed UA oxidation reaction. To transfer the electrons involved in the H_2O_2 oxidation in the optical mode, it is necessary to use an optical membrane with

good conductivity, such as a PTOL film. In the enzymatic reaction, it could be observed optically by reducing the PTOL film. It confirms that uricase was adsorbed in the surface cavities of the PTOL film (Figure 3). The color change was measured spectrometrically in the visible region (400-800 nm), as shown in Table 1. The intensity of color change correlated to UA concentration. The response of uricase-PTOL film change merely depends on the color of the film, which is dictated by the presence of the UA solution. The absorbance maximum, in this case, was found at 590 nm. Therefore, this wavelength was used for further measurements.

Parameter of pH is determining aspect in the uricase-PTOL film optimizing which is changes in pH could affect sensor response sensitively. Therefore, the influence of matrix and other electrocatalytic interference can be minimized by using a buffer solution (Hermanto et al., 2019, Hermanto et al., 2022). In phosphate buffer solution containing 0.6 mM UA, the pH effect on the uricase-PTOL film response was measured at intervals of 4.0 to 9.0 (Table 1). It was found that with increasing pH at intervals of 4.0 to 6.0, the uricase-PTOL film response increased. It indicated that under this condition the effect of increasing enzyme activity was more dominant than decreasing polymer conductivity. Conversely, at pH 6.0 to 9.0 the effect of decreasing the polymer conductivity was dominant so the uricase-

PTOL film response decreased. Hence, obtained pH of 6.0 was the optimum pH that was used in subsequent measurements.

The effect of temperature on the uricase-PTOL film response was studied in the range of 25-45 °C at 590 nm for the UA solution in phosphate buffer at a concentration of 0.6 mM (Table 1). It was generally found that uricase-PTOL film worked well in this temperature range with activity curve changes increased in the temperature range of 25-35 °C and slightly decreased at intervals of 35-45 °C. Despite decreasing activity at 45 °C was detected, the uricase-PTOL film still maintained its activity which indicated that the immobilized matrix polymer increased uricase thermal stability. The temperature of 35 °C was chosen as the optimum temperature, then it was used in the next measurement. Optical uricase-PTOL film measurements showed that there was a large change in the uricase-PTOL film response to UA exposure at pH 6.0. In this condition, it was found that the 5 min response time was the optimum time to achieve a 90% response of uricase-PTOL film (Table 1), and was used for further measurements.

By applying the optimal parameters, the dynamic response of the urease-PTOL film to UA exposure was measured as absorbance change at 590 nm. The effect of UA concentration on absorbance was shown in Figure 4.

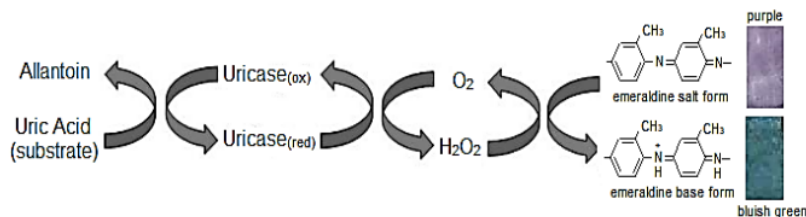


Figure 3 Proposed reaction mechanism at Uricase-PTOL film

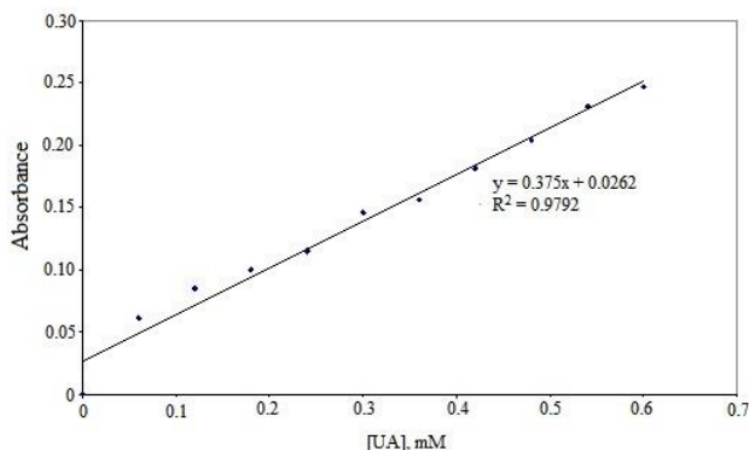


Figure 4 Analytical signal of the uricase-PTOL film at 590 nm and 0.0-0.6 mM UA concentration. As a reference, the unreacted uricase-PTOL film was used.

Table 1 Optimization of experimental parameters

Parameter	Range	Optimum
Wavelength (nm)	400–800	590
pH	4–9	6
Temperature (°C)	25–45	35
Response time (min)	0–20	5

In the range of UA concentration of 0.0-0.6 mM obtained a linear curve with a correlation coefficient (r) of 0.989 and regression equation of $A = 0.375UA + 0.0262$ (Figure 4). The limit of detection of the uricase-PTOL film is 0.063 mM UA, obtained from the mean of the blank signal plus three times the standard deviation with 10 repetitions.

The color change of the uricase-PTOL film from purple to bluish-green, when reacted with UA, showed that uricase was loaded successfully in the formed PTOL film. The uricase-PTOL film also has a linear curve at various concentrations of UA so that it can be used as an analytical tool in the determination of UA in the sample solution, it's called an optical biosensor for UA. Therefore, the PTOL film as conducting matrices has good characteristics as a matrix for uricase immobilization and has further potential applications, for example as a matrix for biosensing systems.

CONCLUSIONS

The PTOL film was successfully synthesized by using ammonium peroxodisulphate in HCl solution (1.0 M), confirmed with characteristic FTIR spectra of film. The PTOL was in an emeraldine state with a semi-crystalline structure, uniform morphology, and some granular aggregation on its surface. Immobilized uricase the PTOL film could retain its catalytic activity, indicated by the color change of the film from purple to bluish-green after interaction with UA as substrate. In addition, the analytical characteristics of the uricase-PTOL film was excellent, hence PTOL film offer conducting matrices for immobilizing enzyme in the uricase-PTOL film application.

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