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In-situ electrochemical synthesis of prussian blue composite with gold nanoparticles and its application in hydrogen peroxide biosensor

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ABSTRACT

This manuscript presents *in-situ* electrochemical synthesis of Prussian Blue-gold nanoparticles (PB- AuNPs) composite for application in hydrogen peroxide (H₂O₂) biosensor. The SEM image clearly showed the presence of AuNPs of size in range of 50 to 200 nm spread on PB matrix. UV-Visible spectra showed absorbance peak at 530 nm corresponding to AuNPs and a hump in 690-740 nm region for PB, confirming the synthesis of composite. The cyclic voltammetry (CV) showed the surface coverage of 3.65 x 10⁻⁹ mol/cm² for pure PB film and 4.33 x 10⁻⁹ mol/cm² for PB-AuNPs film, with diffusion coefficient of 1.19 x 10⁻⁹ cm²/s, and 5.64 x 10⁻⁹ cm²/s respectively. The film thickness is found to be 2.4 x 10⁻¹² cm for PB and 2.9 x 10⁻¹² cm for PB-AuNPs composite. The concentration of redox active centers (Fe^{+3/+2}) is 3.5 moles/cm³ for ITO/PB and 4.1 moles/cm³ for ITO/PB-AuNPs respectively. The CV of ITO/PB showed one redox couple at 0.118 V and 0.215 V, whereas with ITO/PB-AuNPs electrode, two sets of well-defined redox peaks; (i) 0.095 V & 0.135 V and (ii) 0.74 V & 0.78 V were obtained. The faradic current obtained with ITO/PB was 3.6 x 10⁻³ A and 7.3 x 10⁻³ A for ITO/PB-AuNPs composite film, respectively. The faradic current was almost double in presence of gold nanoparticles, as compared to pure PB. For H₂O₂ biosensor, the horse radish peroxidase (HRP) was immobilized on composite film and was used for H₂O₂ detection. The linearity was obtained from 10 to 90 nM, with sensitivity of 0.73µA/nM and the apparent K_m value was 45 nM. The response time of reported biosensor is 20 sec and is stable for about three months. Copyright © 2015 VBRI Press.

Keywords: Prussian blue; gold nanoparticles; hydrogen peroxide; biosensor; electrochemical.

Introduction

The low level detection of hydrogen peroxide (H_2O_2) finds application in various fields like environmental control, textile industries, food industries and health care [1, 2]. It is also produced as by-products in many automatic or enzymatic oxidation processes such as oxidation of glucose and catechin. At present, several techniques like chemiluminescence, fluorescence are being used but these tend to be complex and time-consuming [3, 4]. Thus, rapid and reliable methods for measuring H_2O_2 are required, among which electrochemical H_2O_2 biosensors proved to be excellent source.

The concept of electrochemical biosensors sparked after Clark and Lyons disclosed biosensing of dissolved oxygen using glucose oxidase (GOx) enzyme in 1962 [5]. In electrochemical biosensors, the analytical capability of electrochemical techniques is combined with the specificity of biological recognition processes. The configuration involves immobilization of a bio-specific agent on the surface of an electrode, which converts the biological recognition process into a quantitative amperometric or potentiometric response [6]. Electrochemical biosensors have improved performance over conventional analytical tools, use less expensive reagents, and are portable and simple-to-operate [7, 10].

Prussian blue (PB) modified electrodes represent attractive sensor for hydrogen peroxide detection owing to its low reduction potential (0.0 V), where no interfering species get reduced or oxidized. PB is also denoted as iron (III) hexacyanoferrate (II) and can readily form thin solid films on electrode substrates. It is characterized by fast redox reactions and, in the presence of potassiumions in supporting electrolyte, can be reversibly oxidized to Prussian Yellow (PY) or reduced to Prussian White (PW). The possibility for selective detection of hydrogen peroxide by its reduction in the presence of oxygen on PB modified electrodes was first demonstrated in 1994 by Karyakin [11]. Due to its selectivity toward hydrogen peroxide reduction, it has been applied in the development of H₂O₂ biosensors for clinical, environmental and food applications [12, 13]. However in-spite of highly selective for H2O2, PB film suffers the problem of instability at high pH [14, 15].

The composite formation of PB with another electroactive material can support its stability [16, 23]. Currently, concentration on use of metal nanoparticles for

composite formation is at alarming rate. PB film embedded with nanoparticles has the advantage of large surface to volume ratio, better adhesion and enhanced surface activity [24]. Presence of nanoparticles with Prussian blue matrix not only provides stability but also contributes to signal enhancement. Moreover, morphology of nanosized material is well suited for biosensor applications as they are conductive, biocompatible, and can be functionalized. Among others, gold nanoparticles (AuNPs) are actively being used for composite formation with PB due to their high stability, good electronic properties, biocompatibility and their electrocatalytic activity [24, 27].

In present work, PB-AuNPs composite has been synthesized electrochemically on ITO surface and synthesis involved potentiodynamic cycling between two potential limits in a supporting electrolyte containing both metal ion $(M^{n+}; M=Fe)$ and ferricyanide species. Potentiodynamic cycling then activates in situ precipitation of the insoluble PB on an inert substrate [24]. The composite film has been its morphology. characterized for optical and electrochemical behavior using field emission scanning microscope (FE-SEM), **UV-Visible** electron spectrophotometer and cyclic voltammetry (CV). The composite has further been used for enzymatic detection of hydrogen peroxide which showed linearity in the range 10 to 110 nM. The characteristic of the reported work is fast synthesis of composite and fast response of the biosensor.

Experimental

Materials and instrumentation

Potassium ferricyanide $[K_3Fe(CN)_6]$, ≥ 99 %), KCl, ethanol, mercapt-propionic acid (MPA) were purchased from Loba Chem and tetra chloro auric acid (HAuCl₄.x H₂O) was purchased from Spectrochem. The horseradish peroxidase (HRP) enzyme was obtained from Sigma. All chemicals were of analytical grade and used without further purification. The deionized water (DI) was used throughout the experiments for preparing stock solutions and dilutions. Before using, ITO electrodes were cleaned by sonication in detergent solution followed by washing with ethanol and DI water.

The UV-Vis absorption spectra of the PB and PB-AuNPs composite were recorded on UV-Visible spectrophotometer (Model No: U-3900 H, Hitachi, Japan). FE-SEM images of PB and PB-AuNPs composite films were acquired on FE-SEM microscope (Model No: S-4300 SE/N, Hitachi, Japan). Electrochemical synthesis and characterization was done on CH Instruments, USA (Model No. CH 1140 B) using three electrode cell configuration; ITO plate as working, platinum wire as auxiliary and Ag/AgCl as reference electrode.

Method

For the synthesis of PB, the presence of two oxidation states of iron is important. Thus, generally the PB is synthesized using mixture of ferric salt (Fe^{III}) with ferrocyanide (Fe^{II}), or ferrous salt (Fe^{II}) with ferricyanide (Fe^{III}). In present work, for electrochemical synthesis of PB, the solution consisting of 5.0 mM of K_3 [Fe(CN)₆], and 5.0 mM of FeCl₃ in 1:1, and 0.2 M KCl as supporting electrolyte was used. For the synthesis of PB-AuNPs

composite, 1 mM tetra chloro auric acid was added to the above mentioned solution. Various combinations of cycling potential and concentration ratios of $K_3[Fe(CN)_6]$, and FeCl₃ were tried. And the best films were obtained when potential was cycled between -0.2 V to 5.0 V at a scan rate of 50 mV/s, using equimolar concentrations of $K_3[Fe(CN)_6]$, and FeCl₃. The equimolar concentration is required to maintain the electroneutrality. Many researchers have reported successful synthesis of PB using equimolar concentration, electrodes were washed with deionized water and air dried. Cyclic voltammetry studies for PB and PB-AuNPs have been carried out in 0.1 M KCl solution.

Prior to the immobilization of HRP on composite film, the film was modified with 3-mercapto propionic acid (MPA). The MPA has sulfur group to bind with gold and acidic groups to bind with protein. The modification was performed by dipping the ITO/PB-AuNPs electrode in ethanolic solution of MPA for 24 h. The electrode was taken out, washed with ethanol and water and was air dried. The HRP enzyme was then physically adsorbed on composite film and was allowed to air dry. After air dry, the electrode was washed with buffer and stored in refrigerator.



Scheme 1. Pictorial representation of fabrication of ITO/PB-AuNPs/HRP bioelectrode.

Results and discussion

Electrochemical synthesis of PB and PB-AuNPs

Fig. 1 shows electrochemical deposition curves for PB and PB-AuNPs composite. In present work, during the electrodeposition, in both the cases, initially current increased linearly for about 15-18 cycles and thereafter, it reached to almost constant value, indicating the completion of deposition process. The surface coverage ITO/PB and ITO/PB-AuNPs is found to be 3.65×10^{-9} and 4.33×10^{-9} mol/cm² respectively. As evident from surface coverage, in case of PB film formation, the decomposition of ferricyanide to free ferric ion seems to be a slow process in absence of HAuCl₄ in solution. The film thickness has also been estimated using relationship; Film Thickness (in cm) = Γ x Molar Volume, molar volume has been taken as 677 cm³/mole from literature [**32**, **33**]. The calculated thickness is 2.4 x 10⁻¹² cm for PB and 2.9 x 10⁻¹² cm for PB-AuNPs composite. The concentration of redox active centers (Fe^{+3/+2}) has been calculated using relationship C (moles/cm³) = Γ/L , where ' Γ ' is surface coverage and 'L' is unit length of PB cubic cell unit. For ITO/PB, concentration is 3.5 moles/cm³ and for ITO/PB-AuNPs, it is 4.1 moles/cm³ respectively.



Fig. 1. Electro-deposition curve of (a) PB, (b) PB-AuNPs, (c) Overlay of (a) & (b).



Fig. 2a. CV of (a) ITO/PB (b) ITO/PB-AuNPs in 0.1 M KCl (c) Overlay of a and b.

Mechanism of PB and its composite formation

For Prussian blue film deposition, deposition is due to the charge transfer from Fe (II) to Fe (III) [**34**], according to following equation (eq 1):

$$Fe^{3}[Fe^{2}(CN)_{6}]_{3} + 4e^{-} + 4K^{+} \longrightarrow K_{4}Fe^{2}[Fe^{2}(CN)_{6}]_{3}$$
 (1)

As reported by Itaya and Uchida [**34**], it is the movement of cations that maintains electro-neutrality within the film upon cycling between Prussian Blue and Prussian White. For ITO/PB-AuNPs, the in-situ electrochemical synthesis of of Prussian blue film takes the aid of Au particles (from HAuCl₄ salt). Under potential cycling conditions, $AuCl_4$ readily undergoes electrochemical reduction to form Au^o (eq 2), rather than combining with ferricyanide [**13**].

$$\operatorname{AuCl}_{4}^{-} + 3 e^{-} \longrightarrow \operatorname{Au}^{0} + 4 \operatorname{Cl}^{-}$$
 (2)

The above reaction results in the formation of AuNPs that act as a catalyst for the conversion of ferricyanide to ferric ions in the vicinity of the Au particles. The generated Fe^{2+} and $[Fe(CN)_6]^{3-}$ interact at the interface to form Prussian blue on the electrode surface according to the equation 1. This mechanism has been reported elsewhere for the detection of hydrogen peroxide on a glassy carbon electrode coated with gold nanoparticles [13].



Fig. 2b. CV of ITO/PB (a) and ITO/PB-AuNPs (b) in 0.1 M KCl as function of scan rate (v) and their respective curves as a function of $(v)^{1/2}$

 Table 1. Electrochemical parameters obtained with for ITO/PB and ITO/PB-AuNPs electrodes.

Parameters Calculated/Obtained	ITO/PB film	ITO/PB-AuNPs film
Surface Coverage	3.65 x 10 ⁻⁹ mol/cm ²	4.33 x 10 ⁻⁹ mol/cm ²
Concentration of redox active centers (Fe ^{3+/2+})	3.5 moles/cm ³	4.1 moles/cm ³
Potentials (E _{pc} & E _{pa})	One redox couple (0.118 V & 0.215 V)	Two redox couples ((i) 0.095 V & 0.135 V (ii) 0.74 V & 0.78 V)
Diffusion coefficient	1.19 x 10 ⁻⁹ cm ² /s	5.64 x 10 ⁻⁹ cm ² /s
Capacitance	7.0 x 10 ⁻³	3.0 x 10 ⁻³
Faradic current	3.6 x 10 ⁻³	7.3 x 10 ⁻³

Electrochemical characterization

To study the electrochemical behavior of PB and PB-AuNPs composite film, the CV was run in 0.1 M KCl, using repetitive potential cycling between 1.0 V and -0.60 V vs. Ag/AgCl at scan rate of 100 mVs⁻¹ [Fig. 2 a (a, b, c)].



Fig. 3. SEM of (A) ITO/PB (B) ITO/PB-AuNPs.

With ITO/PB electrodes, one redox couple was obtained at $E_{pc} = 0.118$ V & $E_{pa} = 0.215$ V. For ITO/PB-AuNPs, the two well defined and sharp sets of redox peaks ((i) 0.095 & 0.135 V (ii) 0.74 & 0.78) appeared which seems to be facilitated by the presence of AuNPs. The redox peak at 0.095 V is due to the transformation between Prussian blue and Prussian white, while redox peak at 0.74 V is due to the transformation between Prussian blue and Prussian yellow. At lower potentials, PB exists in its reduced form which is known as Prussian white and at higher potentials, PB is converted to its oxidized state, known as Prussian yellow [5, 14, 35]. Such set of sharp peaks in CV confirms regular structure of Prussian blue with homogeneous distribution of charge and is typical characteristics of PB modified electrode [11]. The intensity of redox peaks is almost 80 % more in presence of gold nanoparticles, which suggests that presence of nanoparticles supports the charge transport within the composite film. The AuNPs are known to provide conduction pathways for accelerated electron transfer [36]. The waveform with a ΔE_p of approximately 49 mV reveals fast electrochemistry in PB-AuNPs composite. The diffusion coefficient has been calculated to be 1.19 x 10⁻⁹ cm^2/s and 5.64 x 10⁻⁹ cm^2/s , respectively for ITO/PB and ITO/PB-AuNPs composite, which again supports fast electron transfer in presence of AuNPs.

The effect of scan rate on electrochemical behavior of ITO/PB and ITO/PB-AuNPs composite films has also been studied. For both type of films, the I_a and I_c increased with

increase in scan rate (**Fig. 2 b**), indicating that the pair of redox waves originates from the surface confined molecules [**37**]. For ITO/PB, the reduction peak shifted to negative and oxidation peak shifted to more positive on moving to higher scan rates.

In ITO/PB-AuNPs composite, the peaks appeared at same potential, with increase in scan rates, which is a characteristic of electrode reactions having rapid electron transfer kinetics. The peak to peak separation is almost zero in this case thus showing ideal response behavior. This behavior can be explained in terms of high surface area and electrocatalytic effect of nanoparticles which helps to overcome any resistance barriers in the film. The capacitance and faradic current has been calculated from the graph of ΔI vs scan rate (v) (data not shown) and values obtained are 7.0 x 10^{-3} (capacitance) and 3.6 x 10^{-3} (faradic current) for ITO/PB film and 3.0 x 10⁻³ (capacitance) and 7.3 x 10^{-3} (faradic current) for ITO/PB-AuNPs composite film, respectively. Table 1 shows the electrochemical parameters obtained for ITO/PB and ITO/PB-AuNPs electrodes.

Morphological study

Scanning electron micrographs (SEM) were recorded for ITO/PB and ITO/PB-AuNPs composite film (**Fig. 3**). SEM of PB film shows plain algal surface whereas SEM of PB-AuNPs shows the presence of AuNPs on PB surface (as contrast). The approximate size of AuNPs is from 50 to 200 nm.



Fig. 4. UV-Visible spectra of (a) PB and (b) PB-AuNPs composite.

Spectroscopic studies of PB and PB-AuNPs composite

The UV-Visible spectra were recorded for pure PB and PB-AuNPs composite film (**Fig. 4**). For PB, absorption band appeared in the form of hump at about 690-740 nm, characteristic of PB films, corresponding to the mixedvalence charge-transfer absorbance of the polymeric [Fe(II)-C-N-Fe(III)], in the form of $Fe_4[Fe(CN)_6]_3$. The other forms of PB i.e., Prussian White, Everitt's salt, and Prussian Yellow, do not adsorb in the 500-800 nm range. The result is in accordance with the literature [**27**, **38**, **39**]. For PB-AuNPs composite, along with the hump in 690-740 nm range, an absorbance band appeared at 530 nm. The hump at 690-740 nm corresponds to that of PB and band at 530 nm corresponds to the gold nanoparticles. This band at 530 is due to the surface plasmon resonance which is intrinsic properties of metallic nanostructures and arises from the interaction between surface charges of metallic NPs and an electromagnetic field [40, 41].



Fig. 5. FTIR spectra of PB and PB-AuNPs composite.

FTIR studies

Fig. 5 shows FTIR spectra of PB and PB-AuNPs composite. In FTIR spectrum of PB, absorption band at 2076 cm⁻¹ is characteristics of PB and its analogues and corresponds to the stretching vibration of the CN group in cyano-metallate lattice [**42**] The absorption band at 501cm⁻¹ is due to the formation of M-CN-M' which indicates the presence of PB [**43**]. In addition, the absorption bands near 3405.31 cm⁻¹ and 1612.8 cm⁻¹ refer to the O-H stretching mode and H-O-H bending mode [**44**], respectively, indicating the presence of interstitial water in the samples [**26**].

However, in case of composite, band at 2076 cm⁻¹ got shifted to 2078.14 cm⁻¹, which indicates that some bonding took place between gold nanoparticles and PB [45]. The band at 1612.8 cm⁻¹ shifted to 1638.09 cm⁻¹, and band at 3405.3 cm⁻¹ got shifted to 3417.65 cm⁻¹. The width of these two peaks decreased after composite formation with AuNPs, the reason might be the replacement of water moiety by nanoparticles. The band at 501 cm⁻¹ however shifted to shorted wave-number of 492 cm⁻¹. The shifting of bands to higher or lower wave-numbers after composite formation confirms some linkage between PB and AuNPs. Introduction of nanoparticles results in the breakage of a large number of bonds for surface atoms, thus leading to the rearrangement of un-localized electrons on the particle surface [26]. This rearrangement induces red shift of absorption bands [46]. The shifting of bands to lower wavenumber has also been observed by Singh et al after composite formation of quantum dots with starch-silica nanobiocomposite [47].

Analytical performance of ITO/PB-AuNPs composite electrode

The PB-AuNPs composite electrodes have been used for enzymatic detection of hydrogen peroxide (H_2O_2) and result is shown in **Fig. 6a**. As compared to ITO/PB-

AuNPs/HRP bioelectrode, the response of ITO/PB/HRP electrode towards H_2O_2 was very low. The current obtained for reduction of H_2O_2 by ITO/PB-AuNPs/HRP bioelectrode increased initially for about 20 seconds thereafter it became almost constant (**Fig. 6b**). This observation suggests the completion of reaction within 20 seconds.



Fig. 6a. CV of ITO/PB-AuNPs/HRP in buffer (a) ITO/PB/HRP in buffer containing 30 nM H_2O_2 (b), ITO/PB-AuNPs/HRP in buffer containing 30 nM H_2O_2 (c).



Fig. 6b. Plot of current vs response time for ITO/PB-AuNPs/HRP for $\rm H_2O_2$ detection.



Fig. 6c. CV of ITO/PB-AuNPs/HRP in in buffer containing 30 nM $\rm H_2O_2$ as a function of buffer pH (a) pH 4.0 (b) 5.0 (c) 6.0 and (d) 8.0.

The effect of pH on the analytical response of ITO/PB-AuNPs/HRP bioelectrode towards H_2O_2 has also been studied (**Fig. 6c**). The optimum pH of HRP enzyme is in

the range 5.5 to 6.0, so the studies were carried out from 4.0 to 8.0 pH. The current increased with increase in pH from 4.0 to 6.0 with potential shift of 10 mV to 15.0 mV. At pH 8.0, decrease in current was observed, which might be due to the instability of PB film towards alkaline pH resulting in breakdown of Fe-CN-Fe bond [**48**, **49**].

Calibration curve for H₂O₂ detection was obtained for ITO/PB-AuNPs/HRP bio-electrode by successive additions of hydrogen peroxide and signal was recorded in the form of CV (Fig. 6d). The catalytic effect of PB towards oxygen and hydrogen peroxide is ascribed to its zeolitic nature which allows the diffusion of low molecular weight molecules (such as O_2 and H_2O_2) through the crystal [50-**52**]. $Fe^{III,II}$ lattice ions of PB are expected to be predominantly responsible for the film's electrocatalytic reactivity towards the hydrogen peroxide reduction. The electrode showed linear detection limit from 10 to 90 nM, thereafter exhibited sudden drop in current, with sensitivity of about 0.73 μ A/nM/cm² and apparent K_m value of 45 nM (calculated from $V_{1/2max}$ value) (Fig. 6e). The decrease in current at higher concentrations of H₂O₂ (100 and 110 nM) can be attributed to the kinetic limitations or to a possible decomposition of PB layer during its interaction with hydrogen peroxide. Higher concentration of peroxide leads to higher amounts of hydroxyl anions in the reaction layer, inducing a faster decomposition of Prussian blue.



Fig. 6d. CV of ITO/PB-AuNPs/HRP in Buffer (a), ITO/PB-AuNPs in buffer containing 30 nM H_2O_2 (b), ITO/PB-AuNPs/HRP in buffer containing H_2O_2 10 to 90 nM (c to j), 100 nM (k), 110 nM (l).

Table 2. Hydrogen peroxide detection limit achieved by other researchers and present work.

Matrix/Composite	Synthesis method used	H ₂ O ₂ Detection Limit	Sensitivity	Reference
PB-AuNPs	Chemical	1.0 x 10 ⁻⁵ to 60 x 10 ⁻³ M	5 nA/mM	[26]
Au/MPA/PAMAM/n ano-Au/PB*	Electrochemic al	4.0 x 10 ⁻⁴ to 1.0 x 10 ⁻² M	21.98 [mA/(Mcm ⁻²)]	[53]
PB-NPGF (Nanoporous gold film)	Electrochemic al	5 x 10 ⁻⁶ to 100 x 10 ⁻ ⁶ M	10.6 µА/µМст ⁻²	[17]
PB/CTAB/AuNPs	Electrochemic al	8.8 x 10 ⁻⁷ to 195 x 10 ⁻⁶ M		[18]
PB	Electrochemic al	10 ⁻⁷ to 5 x 10 ⁻⁵ M	234 µA/mMcm ⁻²	[52]
PB-AuNPs	Electrochemic al	1.0 x 10 ⁻¹⁰ to 90 x 10 ⁻⁹ M	0.73 µA/nM	Present Work

Table 2 shows the detection limits achieved by various researchers and present work for H_2O_2 sensing Electrodes showed good stability and reproducibility and could be used for about 10 times for hydrogen peroxide detection. The same electrode was continuously used after an interval of a week, which continued for about three months. After three months, electrodes showed about 60 % response, which again can be due to loss of stability of composite film with prolonged use. Electrodes were stored at 4 °C when not in use.



Fig. 6e. Linearity curve of ITO/PB-AuNPs/HRP bioelectrode towards H_2O_2 concentration (10 to 90 nM)

Conclusion

The present study demonstrates fast electrochemical synthesis of PB-AuNPs composite. The combined properties of PB and gold nanoparticles permits to produce the biosensor exhibiting wide linear range, fast response time and good operational stability. The gold nanoparticles have large surface area due to nanoscaled size, are biocompatibility and electroactive, PB on other hand is highly selective for hydrogen peroxide reduction. PB-AuNPs composite showed characteristics of good surface coverage and enhanced rate of film deposition. Cyclic voltamogram of composite is characterized by appearance of well-defined and sharp redox couples, an indication of a polycrystalline regular inorganic structure, with homogenous charge distribution. The current intensity of redox peaks increased almost 80 % in presence of gold nanoparticles, which shows that AuNPs supports charge transport within the composite film. The PB-AuNPs composite based H₂O₂ biosensor demonstrated linearity from 10 to 90 nM. Based on the present studies, it has been concluded that PB-AuNPs matrix can be extended for the detection of hydrogen peroxide generated as by products in many enzymatic and auto-oxidation processes.

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