

**Original Article:****Direct electron transfer of laccase enzyme based on RGO/AuNPs/PNR****Fariba Mashayekhi Mazar<sup>1</sup>, Mahdi Alijanianzadeh<sup>1,\*</sup> Ahmad Molaei Rad<sup>1</sup>, Payam Heydari<sup>2</sup>**<sup>1</sup>Department of Bioscience and Biotechnology, Malek-Ashtar University of Technology, Tehran, Iran<sup>2</sup>Young Researchers and Elite Club, Islamic Azad University, Roudehen Branch, Roudehen, Tehran, Iran\*Corresponding author: email address: [alijanianzadeh\\_m@yahoo.com](mailto:alijanianzadeh_m@yahoo.com) (M. Alijanianzadeh)**ABSTRACT**

Biofuel cell has received much attention in recent years due to the global energy demand increase per year. This paper presents a design for *Mytheliophthora thermophila* laccase on the electrode as a biocathode for biofuel cells based on direct electron transfer (DET) between the active site of the enzyme and reduced graphene oxide-gold nanoparticles-poly neutral red (RGO/AuNPs/PNR). The RGO/AuNPs/PNR/laccase biocathode was characterized by cyclic voltammetry (CV) method. The CV experiments demonstrated the activity, direct electron transfer, and stability of immobilized enzyme on the nanocomposite. The results showed that the immobilized enzyme had good stability and performance on the nanocomposite after 10 days. Therefore, the presented method could properly be used in the design of biosensors or biocathode of biofuel cells.

**Keywords:** Direct electron transfer; Laccase; Reduced graphene oxide (RGO); Gold nanoparticles (AuNPs); Poly neutral red (PNR); Biofuel cell.

**INTRODUCTION**

Biofuel cells are energy conversion devices that use enzymes, microorganisms or organelle as bio-electro-catalysts [1-3]. Chemical reactions in enzyme based on biofuel cells is carried on by two different electron transfer methods: direct electron transfer (DET) or mediated electron transfer (MET). In DET, electrons transfer directly between enzyme and electrode surface. In MET, the mediator shuttles the electron between enzyme and electrode surface to reduce the kinetic barrier in the electron transfer. DET is desirable for efficient communication between enzyme and electrode; eliminating the mediator would simplify the construction of biofuel cells [4, 5]. The current and power density in DET systems is traditionally lower than MET systems. Due to advantages of eliminating the mediator, scientists are interested on employing direct electron transfer systems at the anode and cathode of biofuel cells [6, 7]. Laccase is a glycoprotein enzyme which catalyzes the degradation of many phenolic compounds [8, 9]. This enzyme has four copper atoms (Cu<sup>2+</sup>):

copper atom type 1 (T1) is in the enzyme surface. Likewise, a cluster of three coppers contain a copper type 2 (T2) and 2 coppers type 3 (T3) are at depth of the enzyme. Copper T1 captures electron from the substrate and transfers it to the T2/T3 copper cluster. Copper T2 is a resource of electrons and copper T3 reduces oxygen to water [10]. There are different applications of laccases in the enzymatic fuel cells and biosensors. Therefore, optimization of the performance of laccase-based biocathode seems critical [11, 12]. In the present study, laccase enzyme was immobilized on the glassy carbon electrode (GCE) based on RGO/AuNPs/PNR nanocomposite. The RGO/AuNPs/PNR biocathode was evaluated by cyclic voltammetry (CV). Laccase on the RGO/AuNPs/PNR nanocomposite showed an excellent response to reduction of syringaldazine. This new biocathode is a proper choice for practical applications in biosensor and biofuel cell.

**MATERIALS AND METHODS****Materials**

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) from *Mytheliophthora thermophila* was obtained from Novozyme Company (Portugal). NR, AuNPs and syringaldazine were obtained from Sigma-Aldrich (U.S.A).  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$ ,  $\text{CH}_3\text{COOH}$ ,  $\text{CH}_3\text{COONa}$  were purchased from Merck (Germany). All electrochemical experiments were repeated at least three times and were performed through deionized water.

#### Preparation of biocathode

The bare GCE was cleaned by alumina powder and distilled water before modification. The clean electrode was modified by dropping RGO-AuNPs, electropolymerization of NR and immobilization of 4  $\mu\text{L}$  of GOx enzyme (5  $\text{mg ml}^{-1}$ ) on the electrode surface [13]. The RGO was synthesized from graphite based on Hummers' method [14]. PNR polymer was synthesized by CV in the phosphate buffer containing 100 mM of NR, pH=5.5 and 25 °C at a scan rate of 100  $\text{mV s}^{-1}$ . The potential was carried out at 100  $\text{mV s}^{-1}$  between -1 and +0.6 V for 20 cycles [15].

#### Cyclic Voltammetry

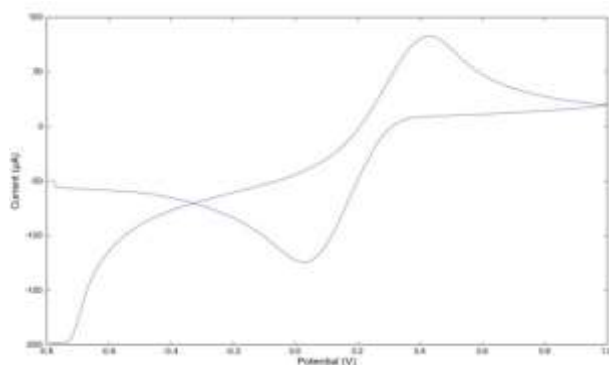
The electrochemical measurements were performed with Potentiostat Galvanostat (Drop Sens model DRP-STAT 200) at room temperature. A conventional three-electrode cell of volume 5 ml electrolyte, containing 100 mM acetate buffer pH 4.6 (included glassy carbon) was used for the purpose of the experiments. A platinum wire and Ag/AgCl

were used as the counter and the reference electrodes respectively. Similarly, electrochemical measurements were obtained by a cyclic potential scan in a potential range from 0.0 to 1.0 V and the scan rate of 100  $\text{mV s}^{-1}$ . Before all experiments, acetate buffer solution was bubbled for 30 min, using nitrogen gas.

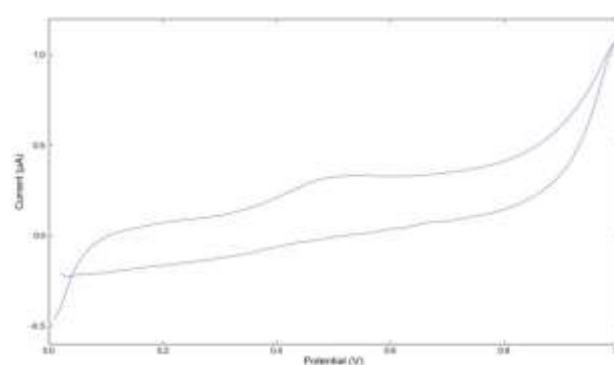
## RESULTS

#### CV characterization

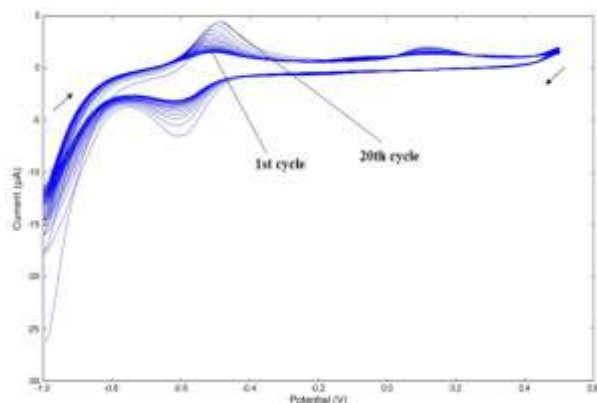
The catalytic activity of the modified biocathode was investigated by CV in different steps. The bare GCE voltammogram was characterized in 10 mM ferrocyanide at scan rate of 100  $\text{mVs}^{-1}$  at room temperature (Fig. 1). The voltammogram of RGO-AuNPs nanocomposite on the GCE in the presence of 100 mM acetate buffer, pH 4.6 and scan rate of 100  $\text{mVs}^{-1}$  was studied (Fig. 2). Figure 3 shows the electro-synthesis of PNR on the RGO-AuNPs nanocomposite. The activity of modified biocathode with laccase in buffer and presence of 10 mM syringaldazine as substrate is manifested in Figure 4. The biocathode in buffer was used as a control to show the activity of laccase in presence of syringaldazine. A pair of well-defined redox peaks shows that the RGO/AuNPs/PNR nanocomposite plays an important role in facilitating electron transfer between the laccase and GCE.



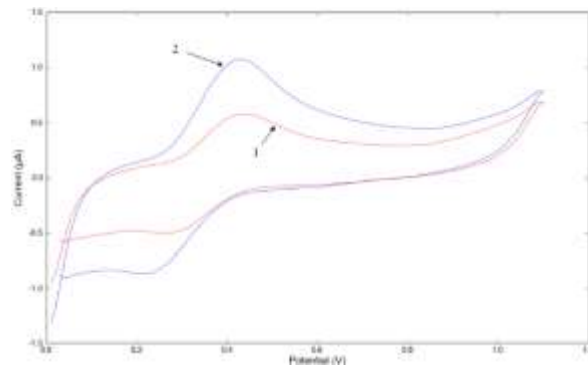
**Figure 1.** Cyclic voltammogram of the bare GCE in 10 mM potassium ferrocyanide at 25 °C. Scan rate: 100  $\text{mVs}^{-1}$ . Scan range: -0.8–1.0 V.



**Figure 2.** Cyclic voltammogram of the RGO-AuNPs nanocomposite in 100 mM acetate buffer, pH 4.6 at 25 °C. Scan rate: 100  $\text{mVs}^{-1}$ . Scan range: 0.0–1.0 V.



**Figure 3.** Cyclic voltammogram of the electro-synthesis of PNR on AuNPs/RGO in 100 mM potassium phosphate buffer, pH 5.5 at 25 °C. Scan rate: 100 mVs<sup>-1</sup>. Scan range: -1.0–0.6 V



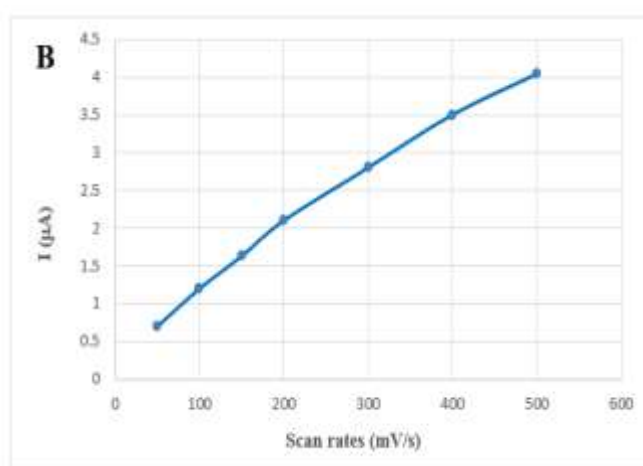
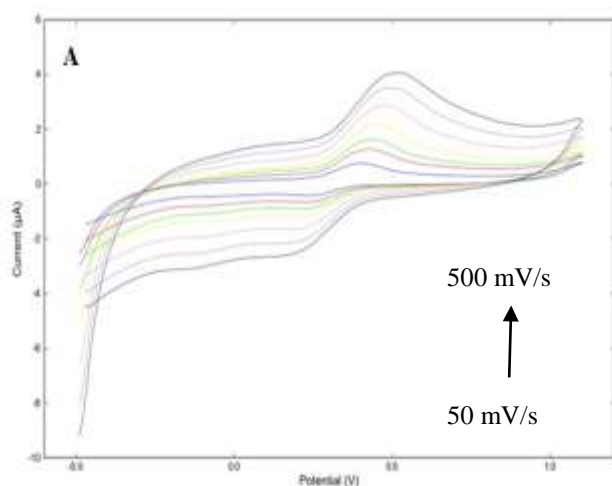
**Figure 4.** Cyclic voltammograms of the laccase immobilized using the RGO-AuNPs/PNR nanocomposite in buffer (1) and in presence of 10 mM syringaldazine (2) in 100 mM acetate buffer, pH 4.6 at 25 °C. Scan rate: 100 mVs<sup>-1</sup>. Scan range: 0.0–1.0 V

### Direct electrochemistry and Effect of scan rate

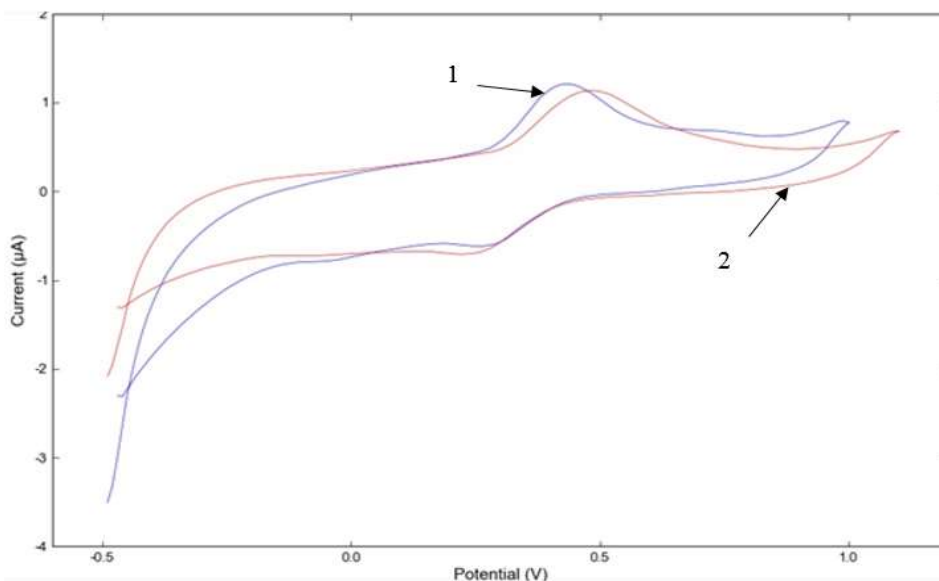
The CV of immobilized laccase on RGO/AuNPs/PNR nanocomposite at different scan rates of 50, 100, 150, 200, 300, 400 and 500 mV s<sup>-1</sup> was achieved in the potential range from 0.0 to 1.0 V in 100 mM acetate buffer pH 4.6 (Fig. 5A). Figure 5B shows linear behavior of the current changes at different scan rates. The present results demonstrated that the redox process of laccase in the nanocomposite is reversible and fast. Therefore, a direct electron transfer of laccase on RGO/AuNPs/PNR based nanocomposite has been achieved successfully.

### Stability

The stability of the presented biocathode was examined by keeping the biocathode at 4 °C for 10 days and measuring its activity. The peak current of the conserved biocathode decreased only 10% of its initial response after being stored for 10 days (Fig. 6). The results showed that the presented biocathode has good stability and biocompatibility for laccase enzyme. Table 1 shows the anodic and cathodic peak cyclic voltammograms of modified biocathode in first day and after 10 days of starvation in 4 °C.



**Figure 5.** (A) Cyclic voltammograms of the laccase immobilized onto RGO/AuNPs/PNR nanocomposite at a scan rate of 50, 100, 150, 200, 300, 400 and 500 mVs<sup>-1</sup> in 100 mM acetate buffer, pH 4.6 at 25 °C. Scan range: 0.0–1.0 V. (B) Relation between the cathodic current peaks and different scan rates of presented biocathode



**Figure 6** Cyclic voltammograms of RGO-AuNPs/PNR/laccase modified electrode after 1 day and 10 days of starvation in the 100 mM acetate buffer at pH 4.6 and 25 °C. Scan rate: 100 mVs<sup>-1</sup>. Scan range: -0.5–1.0 V.

**Table 1.** Stability of presented biocathode after 10 days of enzyme immobilization

Time	Anodic peak current (µA)	Cathodic peak current (µA)	Cathodic potential (V)
First Day	1.22	-0.59	0.25
After 10 Days	1.14	-0.70	0.23

## DISCUSSION

The most important point in constructing a biofuel cell is immobilization of enzyme onto electrode surface. RGO-AuNPs/PNR on electrode surface was a good nanocomposite for laccase immobilization. The immobilized laccase on the RGO-AuNPs/PNR keep its biologic activity and showed good response to 10 mM syringaldazine. Because, RGO and AuNPs have nanostructures that increase the surface of electrode and enhance the electron transferring between electrode and laccase. PNR is an organic dye by phenazine structure that contains electron-donating pendant amine groups and at least one ortho or para free position [16, 17]. Therefore, RGO-AuNPs/PNR nanocomposite has good electric conductivity and a high rate of electron transfer without any electron transfer mediator for new enzyme immobilization. The direct electrochemistry of enzyme using nanomaterials and omitting the toxic mediators for electron transferring in the fields of biofuel cells and biosensors is very important issue [18, 19]. This process is controlled by the redox monolayer species adsorbed on the electrode surface [20, 21]. For redox monolayer modified electrode in voltammetric studies, the peak potentials and

different parameters that calculated by Laviron method confirmed the direct and fast electron transfer of electron from laccase to electrode surface [22].

## CONCLUSION

This paper we was immobilized the laccase enzyme on GCE modified by RGO-AuNPs/PNR. The electrochemical results of biocathode showed a pair of well-defined redox peak, corresponding to direct electron transfer of immobilized laccase on the RGO-AuNPs/PNR in the presence of syringaldazine as a substrate. Furthermore, the results manifest enhanced DET and high activity and stability of modified biocathode after 10 days. As a result, the method presented here could be properly used in laccase-based biosensors or as a bioelectrode in enzymatic biofuel cells.

*“The authors declare no conflict of interest”*

## REFERENCES

1. Tender LM, Reimers CE, Stecher HA, Holmes DE, Bond DR, Lowy DA, et al. Harnessing microbially generated power on the seafloor. *Nature biotechnology*. 2002;20(8):821-5.

2. Katz E, Shipway A, Willner I. Handbook of fuel cells—fundamentals, technology and applications. Fundamentals and Survey of Systems. 2003;1:355.
3. Alijanianzadeh M, Mashayekhi Mazar F, Molaei Rad A. Design and Fabrication of Glucose/O<sub>2</sub> Enzymatic Biofuel Cell. Biomacromolecular Journal. 2016;2(2):102-7.
4. Kannan A, Renugopalakrishnan V, Filipek S, Li P, Audette G, Munukutla L. Bio-batteries and bio-fuel cells: leveraging on electronic charge transfer proteins. Journal of nanoscience and nanotechnology. 2009;9(3):1665-78.
5. Mashayekhi Mazar F, Alijanianzadeh M, Jamshidy Nia Z, Molaei Rad A. Introduction to biofuel cells: A biological source of energy. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects. 2017;39(4):419-25.
6. Topcagic S, Minter SD. Development of a membraneless ethanol/oxygen biofuel cell. Electrochimica Acta. 2006;51(11):2168-72.
7. Tasca F, Gorton L, Harreither W, Haltrich D, Ludwig R, Noll G. Direct electron transfer at cellobiose dehydrogenase modified anodes for biofuel cells. The Journal of Physical Chemistry C. 2008;112(26):9956-61.
8. Wang Y, Zhang D, He FR, Chen XC. Immobilization of laccase by Cu<sup>2+</sup> chelate affinity interaction on surface modified magnetic silica particles and its use for the removal of pentachlorophenol. Chinese Chemical Letters. 2012;23(2):197-200.
9. Betancor L, Johnson GR, Luckarift HR. Stabilized laccases as heterogeneous bioelectrocatalysts. ChemCatChem. 2013;5(1):46-60.
10. Jamshidinia Z, Mashayekhimazar F, Ahmadi M, Molaeirad A, Alijanianzadeh M, Janfaza S. Investigation of Direct and Mediated Electron Transfer of Laccase-Based Biocathode. Journal of Electrochemical Science and Technology. 2017;8(2):87-95.
11. Barton SC, Kim H-H, Binyamin G, Zhang Y, Heller A. The “Wired” laccase cathode: high current density electroreduction of O<sub>2</sub> to water at +0.7 V (NHE) at pH 5. Journal of the American Chemical Society. 2001;123(24):5802-3.
12. Freire RS, Durán N, Kubota LT. Effects of fungal laccase immobilization procedures for the development of a biosensor for phenol compounds. Talanta. 2001;54(4):681-6.
13. Mazar FM, Alijanianzadeh M, Molaeirad A, Heydari P. Development of Novel Glucose oxidase Immobilization on Graphene/Gold nanoparticles/Poly Neutral red modified electrode. Process Biochemistry. 2017;56:71-80.
14. Marcano DC, Kosynkin DV, Berlin JM, Sinitskii A, Sun Z, Slesarev A, et al. Improved synthesis of graphene oxide. ACS nano. 2010;4(8):4806-14.
15. Schlereth DD, Karyakin AA. Electropolymerization of phenothiazine, phenoxazine and phenazine derivatives: characterization of the polymers by UV-visible difference spectroelectrochemistry and Fourier transform IR spectroscopy. Journal of Electroanalytical Chemistry. 1995;395(1):221-32.
16. Bauldrey J, Archer M. Dye-modified electrodes for photogalvanic cells. Electrochimica Acta. 1983;28(11):1515-22.
17. Sun Y-x, Ye B-x, Zhou X-r. Electrochemical Characteristics and Catalytic Properties of Poly (neutral red) Film Modified Electrodes. Chinese Journal of Analytical Chemistry. 1998;26:166-9.
18. Kim J, Jia H, Wang P. Challenges in biocatalysis for enzyme-based biofuel cells. Biotechnol Adv. 2006;24(3):296-308.
19. Sales FC, Iost RM, Martins MV, Almeida MC, Crespilho FN. An intravenous implantable glucose/dioxygen biofuel cell with modified flexible carbon fiber electrodes. Lab on a Chip. 2013;13(3):468-74.
20. Wu Y, Hu S. Direct electrochemistry of glucose oxidase in a colloid Au-dihexadecylphosphate composite film and

its application to develop a glucose biosensor. *Bioelectrochemistry*.

2007;70(2):335-41.

21. Bard AJ, Faulkner LR. Fundamentals and applications. *Electrochemical Methods*.

2001;2.

22. Laviron E. General expression of the linear potential sweep voltammogram in the case of diffusionless electrochemical systems. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*.

1979;101(1):19-28.