ChemComm

Designing a cross-linked redox network for a mediated enzyme-based electrode

Journal:	*ChemComm*
Manuscript ID	CC-COM-03-2021-001707.R2
Article Type:	Communication

COMMUNICATION

Designing a cross-linked redox network for a mediated enzyme-based electrode

Motaher M. Hossain,[a] Jannatul Morshed,[a] and Seiya Tsujimura*[a]

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

A bio-conjugated redox network matrix based on glucose dehydrogenase, thionine (diamine-containing mediator), and poly(ethylene glycol) diglycidyl ether (crosslinker) is developed on a glassy carbon electrode through covalent bonding with one-pot crosslinking. Electrons from the enzyme diffuse through the network producing 400 μA cm^{-2} of glucose oxidation current at 25 °C.

Enzyme electrodes have applications in biofuel cells and biosensors for medical diagnostic functions, such as blood glucose measurements. In many cases, the enzymatic reaction is coupled with an electrode reaction using a redox mediator. For glucose-oxidising electrodes, glucose oxidase (GOx) is the most used enzyme owing to its high specificity and stability; however, GOx utilises both oxygen and an artificial electron acceptor, resulting in measurement errors. Alternatively, flavin adenine dinucleotide-dependent glucose dehydrogenase (FAD-GDH) has been developed as an enzyme electrode for electrochemical glucose biosensors.[1-3]

In typical designs, co-immobilisation of an enzyme and a redox mediator on the electrode surface is required for the continuous operation of biosensors and biofuel cells. To date, several methods of achieving this co-immobilisation have been developed, including physical adsorption,[4] entrapment,[5] grafting,[6, 7] and crosslinking.[8–9] Among these, crosslinking is a promising immobilisation technique used to attach mediators and enzymes to the electrode surface, and to facilitate the shuttling of electrons between them. For instance, Heller et al. developed wiring technology for enzymatic electrodes through poly(ethylene glycol) diglycidyl ether (PEGDGE) epoxide crosslinking between redox enzymes and polymers containing mediators to an electrode surface.[10-12] Several metal-based mediators have been investigated with FAD-GDH for glucose oxidation, namely osmium[9, 13, 14] and ruthenium[15, 16] complexes. Minteer et al. investigated naphthoquinone (NQ)/FAD-GDH-based immobilisation of a redox hydrogel, where NQ was covalently wired to a linear polyethyleneimine backbone and directly bound to the FAD-GDH enzyme molecule.[17] Hou et al. tailored an NQ derivative with an electron-withdrawing nitrogen group and water-soluble redox polymer, and cross-linked this with FAD-GDH.[18] Pöller et al. immobilised polymer-bound phenothiazine derivatives with FAD-dependent cellobiose dehydrogenase.[19] However, the current production efficiencies were low (see Table S1). The apparent electron diffusion coefficient for a mediator-bound diffusion model can be predicted as a function of the mediator concentration, electron-hopping distance, distance which the mediator can actually move, and mediator self-exchange rate.[20] A plausible strategy to improve the electron transfer efficiency through the redox polymer is to extend the spacer length.[20, 21] While the catalytic current could be improved using a tailor-made redox polymer, this would require complex synthesis with multiple steps, and a specific design depending on the enzyme used, considering the polymer/enzyme interaction.[22]

Herein, we report the development of a novel one-pot method for the immobilisation of an amine-containing redox mediator and FAD-GDH bearing a free amine (on lysine) using PEGDGE to avoid polymer tethering and other chemical modifications. Further, it is possible to increase the density of the enzyme and mediator by omitting the backbone polymer. These covalent bonds form a network-like structure and improve electron shuttling from the redox centre to the electrode surface during glucose oxidation through electron hopping. In this study, we tested a

[a] *Division of Materials Science, Faculty of Pure and Applied Science, University of Tsukuba, 1-1-1, Tennodai, Tsukuba, Ibaraki 305-5358, Japan.*

Electronic Supplementary Information (ESI) available: [Midpoint potential vs pH, CVs on the GOx/PEGDGE/TH and FAD-GDH/TH electrodes, optimization of the electrode fabrication (wt% ratio, cutting time, loading), dependence of pH and temperature, storage stability, table of Covalent-immobilisation of organic redox mediator and FAD-GDH]. See DOI: 10.1039/x0xx00000x

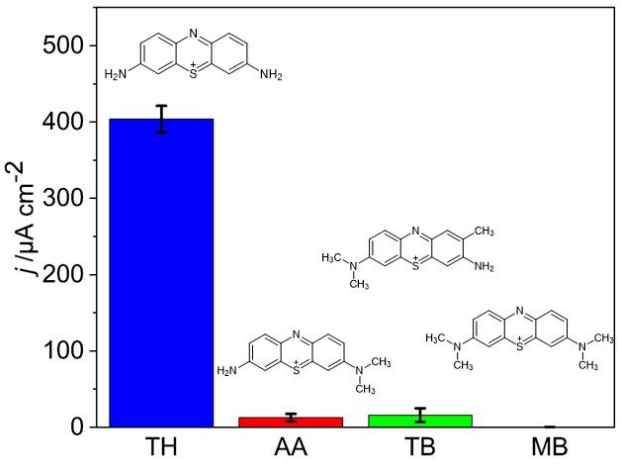
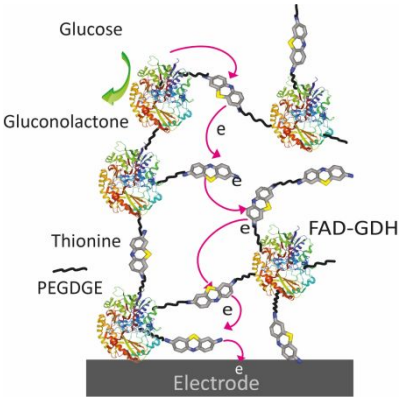


Fig. 1. Amperometric glucose oxidation current densities at 500 s of FAD-GDH/PEGDGE/PT-modified electrodes. 200 mM glucose, 100 mM phosphate buffer pH 7, 25 °C, 0.2 V vs. Ag|AgCl.

family of phenothiazines (PT), including thionine (TH), toluidine blue (TB), methylene blue (MB), and azure A (AA) as proof-of-concept amine-containing redox mediators. Before applying the composite to a glassy carbon (GC) surface, the GC (0.071 cm^2) electrode was polished with an alumina (0.3 μm) slurry, sonicated for 2 min to remove the physically adsorbed alumina, and finally dried in air. A composite solution was prepared with weight ratios of 45% FAD-GDH (5 μL from 25.2 mg/mL in water), 25% PEGDGE (3 μL from 25.0 mg/mL in 100 mM of phosphate buffer), and 30% PT (36 μL from 2.5 mg/mL in 100 mM of phosphate buffer). Subsequently, 7 μL (45 μg per electrode, equivalent to 635 μg/cm^2) of this composite was loaded onto the electrode surface. Then, the modified electrode was allowed to cure for 30 h in a drying cabinet (1% humidity, 26 °C) to permit covalent interaction between the PT and FAD-GDH through the PEGDGE. This modified electrode is represented as the FAD-GDH/PEGDGE/PT electrode. Prior to electrochemical measurements, the electrode was washed with phosphate buffer to remove loosely bound or free molecules from the surface. A standard three-electrode cell was fabricated, consisting of the modified GC as the working electrode, Ag|AgCl|KCl (saturated) as the reference electrode, and Pt wire as the counter electrode. Electrochemical experiments were performed using a CHI 1000C & 1020A potentiostat (ALS, US) and MultiEmStat3$^+$ (Palmsens, Netherlands). Each experiment was repeated three times, and the error bars were determined using Student's t-distribution at a 68% confidence level.

Figure 1 shows the catalytic effects of the phenothiazines (TH, AA, TB, and MB)-modified with FAD-GDH. These phenothiazines were previously reported to exhibit high bimolecular rate constants with respect to FAD-GDH (the logarithms of the bimolecular rate constant/M^{-1} s^{-1} for TH, AA, TB, and MB are 7.0, 6.2, 6.2 and 6.5, respectively).[23] However, AA and TB provided very low catalytic currents (13 and 16 μA cm^{-2}, respectively) because they contain only one amino group and cannot interlink the enzyme. MB displayed the lowest catalytic current (1 μA cm^{-2}) because its structure does not include a



Scheme 1. Schematic representation of immobilised mediator and enzyme electrode, where thionine and FAD-GDH are crosslinked via poly(ethylene glycol) diglycidyl ether (PEGDGE).

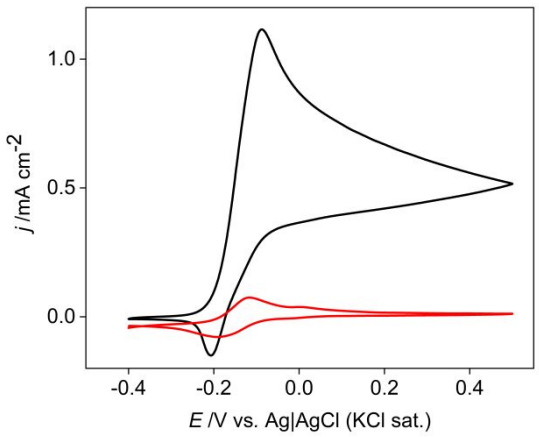


Fig. 2. Cyclic voltammograms of FAD-GDH/PEGDGE/TH electrode in the absence (red) and presence (black) of 200 mM glucose; scan rate, 5 mV/s; 100 mM phosphate buffer, pH 7.0.

primary amine group to allow crosslinking with the enzyme. In contrast, the TH-modified electrode exhibited a considerable catalytic current in a 200 mM glucose solution at 25 °C. This is because TH has two primary amine groups, so it can covalently crosslink with the enzyme via the PEGDGE. This allows the creation of a cross-linked redox network structure (**Scheme 1**). This configuration may enhance electron transfer and glucose diffusion through the FAD-GDH/PEGDGE/TH-modified film. In contrast, the lone primary amine groups in the AA and TB structures do not allow the formation of cross-linked networks. The performance of electrodes fabricated with PEGDGE and glutaraldehyde (GA) were compared for a TH-based redox network (Fig. S1). The catalytic current of the PEGDGE crosslinker electrode was three times greater than that of the GA cross-linked electrode with the same enzyme and mediator loading. The lower current of the FAD-GDH/GA/TH electrode could be attributed to the inactivation of FAD-GDH by GA, or to a different cross-linked network structure. GA has a much faster crosslinking reaction rate than PEGDGE; and the rapid network formation between TH and GA may inhibit incorporation of the

enzyme into the network structure.[24, 25] The TH-PEGDGE based structure is more hydrophilic, more flexible, and softer than the GA-TH based structure.[24] Moreover, the flexible structure is beneficial for collecting electrons from the active enzyme site and promotes electron hopping between bounded TH molecules.[20]

The electrochemical characteristics of the FAD-GDH/PEGDGE/TH electrode were investigated in the absence and presence of glucose. In the absence of glucose, a well-defined surface redox reaction was observed (**Fig. 2, red curve**). The pH dependence of the midpoint potential of free TH in solution and modified TH was investigated (**Fig. S2**). The midpoint potential of FAD-GDH/PEGDGE/TH electrode decreases approximately linearly in this pH range, with a slope of roughly 30 mV pH^{-1}. This indicates that the redox reaction of immobilised TH on the FAD-GDH/PEGDGE/TH electrode involves two electrons and one proton.[26,27] The midpoint potential of modified TH was almost the same as that of free TH across the pH range of 6–8. In the presence of glucose (**Fig. 2, black curve**), the catalytic glucose oxidation current was clearly observed. The peak-shaped CV curve indicates glucose depletion in the TH-enzyme layer on the electrode. The steady-state current of 404 μA cm^{-2} was limited by the mass-transfer of glucose from the bulk solution to the TH layer or within the matrix layer. Compared to the hydrogel electrode, wherein the redox mediator is bound to the backbone polymer, the mass-transfer through the enzyme-TH layer was impeded due to its dense structure. This limitation can be overcome by using hierarchically structured three-dimensional porous electrode materials with mesopore and macropores for smooth mass-transfer of electrolyte ions, protons, and glucose, and will allow the formation of a thinner enzyme-TH layer.

We also prepared GOx-and-TH-immobilised electrodes using PEGDGE as the crosslinker and found that the catalytic current on the GOx/PEGDGE/TH electrode was nine times less than that of the FAD-GDH/PEGDGE/TH-immobilised electrode (**Fig. S3, green curve**). This result may be attributed to the slow reaction rate between TH and GOx: the bimolecular rate constant between TH and GOx is 2.5 orders of magnitude lower than that between TH and FAD-GDH.[23] This suggests that kinetic considerations during mediator selection are an important factor in the realisation of efficient reaction systems. The catalytic current without the crosslinker was negligible compared to the FAD-GDH/PEGDGE/TH-modified electrode (**Fig. S3, orange curve)**. TH had a positive charge in neutral pH conditions, whereas FAD-GDH had a negative charge (pI = 4.4). However, the electrostatic interaction was not sufficiently dominant to allow redox network formation in the presence of the electrolyte (high ionic strength condition), which is required for the electrochemical reaction. This suggests that the formation of crosslinks between TH and FAD-GDH through covalent bonding creates a cross-linked redox network that results in a higher degree of mediator and enzyme activity on the electrode surface.

The catalytic efficiency of the FAD-GDH/PEGDGE/TH electrode depended on the optimisation of the weight ratio, curing time, and loading amount. **Figure S4 [A]** shows the wt% ratio of TH and PEGDGE with a fixed amount of FAD-GDH. It is evident that a molar ratio of TH:PEGDGE = 2.1:1.0 produced the highest current density, which may be attributed to sufficient TH availability for both the glucose oxidation reaction and optimal crosslinking. In contrast, the catalytic current increased as the TH content increased until the molar ratio of TH:PEGDGE = 2.1:1.0, due to the increasing reaction rate between bound TH molecules, and between TH and FAD-GDH within the redox network. At low crosslinker concentrations (5 wt% of crosslinker, that is a molar ratio of TH:CL = 17.4:1.0), the total bound enzyme and mediator were limited by the amount of PEGDGE, suggesting that the glucose catalytic response was inadequate (22 μA cm^{-2}). Therefore, the optimum wt% ratio was 45:30:25 (FAD-GDH:TH: PEGDGE), as this resulted in the highest catalytic current. The loading amount was also optimised (**Fig. S4 [B]**). The current increased with increased loading up to 635 μg/cm^2, after which it plateaued. We observed a hyperbolic relationship between the oxidation current and loading, indicating that the catalytic current depends on the film thickness: at a low loading range, the film is thinner than the reaction layer.[28] With a thick layer, the current remains stable with further increases in the loading amount. The crosslinking intensity of the FAD-GDH/PEGDGE/TH electrode depended not only on the weight ratio and loading amount but also on the curing time.[29] The dependence of catalytic current on the curing time of the modified electrode was investigated with the optimised loading and wt% ratio (**Fig. S4 [C]**). The modified electrode showed the highest current when it was cured for 24–30 h. This indicates that the crosslinking reaction occurs within 24 h at room temperature under low humidity (1%). Indeed, previous studies have shown that the epoxy crosslinker PEGDGE requires 24 h for the crosslinking reaction to achieve the highest activity toward glucose oxidation.[29-31]

The FAD-GDH/PEGDGE/TH-immobilised electrode was further investigated at various pH values and temperatures to examine its robustness. **Figure S5 [A]** illustrates the dependence of glucose oxidation current on the pH of the solution. A pH range of 5–8 was investigated, as this is the generally accepted operable pH range of FAD-GDH; however, this range varies depending on the immobilisation method. The FAD-GDH/PEGDGE/TH-modified electrode shows the highest catalytic current at pH 7.0, which indicates that the electrode remains sensitive at neutral pH. The catalytic current was measured in the temperature range of 15–55 °C (**Fig. S5 [B]**). The temperature profile of the FAD-GDH-immobilised electrode was very similar to that reported in a previous study.[2,32] The activation energy for the electrochemical oxidation of glucose on the FAD-GDH/PEGDGE/TH-immobilised film was calculated at temperatures between 15 and 55 °C as 31 kJ/mol from the ln(j/μA cm^{-2}) vs. 1/K plot, according to the Arrhenius model, where j is the catalytic current density and K is the absolute temperature. This value agrees with the supposition that the enzymatic reaction is the rate-limiting step.[8, 33] The storage stability of FAD-GDH/PEGDGE/TH-modified electrode was investigated at 4 and 25 °C **(Fig. S6)**. When stored at 25 °C, the response current decreased as the storage period increased. The further crosslinking of the redox composite can make

a more rigid redox film, which might inhibit the mass transfer, electron diffusion, or enzymatic activity. In contrast, the modified electrode stored at 4°C to suppress further crosslinking showed a slight decrease of glucose oxidation current over 8 days.

In summary, we have developed a facile method for the immobilisation of enzymes and mediators on an electrode surface. Covalent binding between FAD-GDH and TH occurs via the PEGDGE crosslinker to form a cross-linked redox network for the diffusion of electrons to the electrode during the oxidation of glucose. Although our modified electrode did not provide a significant current density and offered low stability in long-term applications, it provides a basis for developing an improved glucose sensor. With improved stability, such a device would be applicable for low-cost implantable or wearable sensors for monitoring glucose levels.

CRediT authors statement: Hossain M. Motaher: Investigation, Writing-Original draft preparation. Jannatul Morshed: Writing-Reviewing and Editing. Seiya Tsujimura: Conceptualisation, Writing-Reviewing and Editing.

Conflicts of Interest: There are no conflicts to declare.

Notes and references

1. S. Ferri, K. Kojima and K. Sode, *Journal of diabetes science and technology*, 2011, **5**, 1068-1076.
2. S. Tsujimura, S. Kojima, K. Kano, T. Ikeda, M. Sato, H. Sanada and H. Omura, *Bioscience, Biotechnology, and Biochemistry*, 2006, **70**, 654-659.
3. S. Tsujimura, *Bioscience, Biotechnology, and Biochemistry*, 2019, **83**, 39-48.
4. A. J. Gross, S. Tanaka, C. Colomies, F. Giroud, Y. Nishina, S. Cosnier, S. Tsujimura and M. Holzinger, *ChemElectroChem*, 2020, **7**, 4543-4549.
5. A. Navaee and A. Salimi, *J. Electroanal. Chem.*, 2018, **815**, 105-113.
6. N. Tsuruoka, S. S. Soto, A. B. Tahar, A. Zebda and S. Tsujimura, *Colloids Surf B Biointerfaces*, 2020, **192**, 111065.
7. M. Pellissier, F. Barrière, A. J. Downard and D. Leech, *Electrochem. Commun.*, 2008, **10**, 835-838.
8. B. A. Gregg and A. Heller, *Anal. Chem.*, 1990, **62**, 258-263.
9. K. Murata, W. Akatsuka, T. Sadakane, A. Matsunaga and S. Tsujimura, *Electrochim. Acta*, 2014, **136**, 537-541.
10. T. J. Ohara, R. Rajagopalan and A. Heller, *Anal. Chem.*, 1993, **65**, 3512-3517.
11. T. J. Ohara, R. Rajagopalan and A. Heller, *Anal. Chem.*, 1994, **66**, 2451-2457.
12. A. Heller, *Curr. Opin. Chem. Biol.*, 2006, **10**, 664-672.
13. M. N. Zafar, X. Wang, C. Sygmund, R. Ludwig, D. Leech and L. Gorton, *Anal. Chem.*, 2012, **84**, 334-341.
14. P. Ó Conghaile, D. MacAodha, B. Egan, P. Kavanagh and D. Leech, *J. Electrochem. Soc.*, 2013, **160**, G3165-G3170.
15. M. Okurita, N. Suzuki, N. Loew, H. Yoshida, W. Tsugawa, K. Mori, K. Kojima, D. C. Klonoff and K. Sode, *Bioelectrochemistry*, 2018, **123**, 62-69.
16. R. Sakuta, K. Takeda, T. Ishida, K. Igarashi, M. Samejima, N. Nakamura and H. Ohno, *Electrochem. Commun.*, 2015, **56**, 75-78.
17. R. D. Milton, D. P. Hickey, S. Abdellaoui, K. Lim, F. Wu, B. Tan and S. D. Minteer, *Chemical science*, 2015, **6**, 4867-4875.
18. C. Hou, Q. Lang and A. Liu, *Electrochim. Acta*, 2016, **211**, 663-670.
19. S. Pöller, M. Shao, C. Sygmund, R. Ludwig and W. Schuhmann, *Electrochim. Acta*, 2013, **110**, 152-158.
20. F. Mao, N. Mano and A. Heller, *Journal of the American Chemical Society*, 2003, **125**, 4951-4957.
21. A. Ruff, P. Pinyou, M. Nolten, F. Conzuelo and W. Schuhmann, *ChemElectroChem*, 2017, **4**, 890-897.
22. A. Suzuki, N. Mano and S. Tsujimura, *Electrochim. Acta*, 2017, **232**, 581-585.
23. N. Tsuruoka, T. Sadakane, R. Hayashi and S. Tsujimura, *Int J Mol Sci*, 2017, **18**.
24. O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues and R. Fernandez-Lafuente, *RSC Advances*, 2014, **4**, 1583-1600.
25. T. de Lumley-Woodyear, P. Rocca, J. Lindsay, Y. Dror, A. Freeman and A. Heller, *Anal. Chem.*, 1995, **67**, 1332-1338.
26. P. K. Koochana, A. Mohanty, B. Subhadarshanee, S. Satpati, R. Naskar, A. Dixit and R. K. Behera, *Dalton T*, 2019, **48**, 3314-3326.
27. M. A. Zanjanch, S. Sohrabnezhad, M. Arvand and M. F. Mousavi, *Russ. J. Electrochem.*, 2007, **43**, 758-763.
28. P. N. Bartlett and K. F. E. Pratt, *J. Electroanal. Chem.*, 1995, **397**, 61-78.
29. J. Lehr, B. E. Williamson, F. Barrière and A. J. Downard, *Bioelectrochemistry*, 2010, **79**, 142-146.
30. R. Bennett, E. Blochouse and D. Leech, *Electrochim. Acta*, 2019, **302**, 270-276.
31. R. Bennett and D. Leech, *Bioelectrochemistry*, 2020, **133**, 107460.
32. H. Iwasa, K. Ozawa, N. Sasaki, N. Kinoshita, A. Hiratsuka and K. Yokoyama, *Electrochemistry*, 2016, **84**, 342-348.
33. S. Tsujimura, K. Murata and W. Akatsuka, *Journal of the American Chemical Society*, 2014, **136**, 14432-14437.

COMMUNICATION