[AMT07] Fabrication of photocurable ferrocene-containing methacrylates polymer for the use of glucose biosensors

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Introduction

Ferrocene and its derivatives heve been widely used as mediators in the construction of mediated amperometric biosensor. The incorporation of ferrocene-based mediators into polymeric films is always preferred to achieve reagentless devices and also prevent its leaching from the biosensor. Attempt to prohibit leaching has lead to the synthesis of a number of redox polymers where the ferrocene mediator are attached directly onto the polymer backbone (Armada *et al.*, 2003, Hale *et al.*, 1989, Koide & Yokoyama, 1999, Mulchadani *et al.*, 1995, Saito & Watanabe, 1998, Nagasaka *et al.*, 1995). Although these redox polymers have successfully prevented the leaching of the mediator and also demonstrated good redox behaviour, their preparation is generally complicated and did not lead to direct film deposition on a biosensor device. Other simpler approaches for immobilization of ferrocene by physical entrapment in poymers have also been reported (Tkáč *et al.*, 2002, Zhou *et al.*, 1997). However, even the immobilization of the ferrocene mediator was successful in some of these polymeric films but the enzymes used, e.g. glucose oxidase and xanthine oxidase were still required immobilization through the use of cross-linking agents such as glutaraldehyde.

In this paper, we report a novel procedure for the preparation of ferrocene-containing photopolymeric films based on poly(2-hydroxyethyl methacrylate) (polyHEMA) and poly(methyl methacrylate-co-2-hydroxyethyl methacrylate), polyMH. The novelty of the procedure is that the hydrophilicity of the photocured films produced is low and hence even without covalent attachment, leaching of both ferrocene and enzyme is prohibited. The procedure is also a single-step but rapid method for the immobilization of enzyme, mediator and *in situ* deposition of the biosensor film. The redox characteristics of these two ferrocene-containing polymers are investigated, and the usefulness of such films for biosensor application is assessed through the construction of glucose biosensors by co-immobilization of the enzyme glucose oxidase into the ferrocene-containing polymers.

Materials and Methods

Materials

Ferrocene, enzyme glucose oxidase (GOD), glucose monohydrate and phosphate buffer were obtained from Fluka; monomer 2-hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) were obtained from Sigma and Aldrich respectively. The photoinitiator 2,2-dimethoxy-2-phenylaceto phenone (DMPP) was obtained from Aldrich. Phosphate buffer was prepared in distilled water, and glucose solutions were prepared by dissolving appropriate amounts in 0.1M phosphate buffer (pH 7.0). The glucose was allowed to mutarotate for 24 h before use. Phosphate buffer (0.1M, pH 7.0) used as electrolyte in electrochemical measurements was prepared in 0.1M NaCl.

Apparatus and measurements

The electrochemical measurements were performed with an Autolab PGSTAT 12 Pontentiostat/Galvanostat. A one-compartment cell with a working volume of 5mL was used. The working electrode is a carbon paste screen-printed electrode coated with ferrocene-containing polymer film; whereas saturated calomel electrode (SCE) and a glassy carbon electrode were used as reference electrode and auxiliary electrode respectively. The electrodes used for the preparation of working electrode are carbon-paste screen-printed electrodes with active surface 4mm in diameter designed by Scrint Co. Cyclic voltammetry was studied between 0.0 and 0.70 V versus SCE. The amperometric measurements of the glucose biosensor were performed at 0.35 V and 0.25 V versus SCE respectively. All experiments were performed

in 0.1M phosphate/0.1M NaCl buffer (pH 7.0).

Preparation of ferrocene-containing polymer films

The procedure used for the preparation of photocurable methacrylic films is similar to those reported before (Heng & Hall, 2000, Heng & Hall, 2001). A mixture was prepared by mixing an appropriate amount of monomer HEMA and photoinitiator DMPP in a vial. An appropriate amount of ferrocene was then added into the mixture. A same amount of Fc was used in preparing all films. The mixture was then exposed to UV radiation to photocure to produce a polyHEMA film containing ferrocene, Fc/HEMA. Another copolymer films containing ferrocene which is Fc/MH, was prepared using same method by adding MMA into the mixture of HEMA and DMPP. These polymer films containing physically entrapped ferrocene (Fc/HEMA and Fc/MH) were prepared for the examination of ferrocene leaching behavior upon exposure to water.

Evaluation of water absorption, ferrocene and enzyme GOD leaching.

A mixture of monomer HEMA and initiator DMPP without ferrocene was prepared in a vial. The mixture was then deposited onto a supporting material with a known weight, and then exposed to UV light. The photo-copolymer formed was weighed together with the supporting material and thus the weight of the blank photopolymer (polyHEMA) was obtained. The photopolymer together with the supporting material was then exposed to 0.1M phosphate buffer. After 5 minutes, the weight was recorded and the water content of the copolymer after 5 minutes was then calculated as reported by Bayramolu *et al.* (2003). The water absorption of the photopolymer was determined with duration of exposure to water.

As for ferrocene leaching test, Fc/HEMA polymer film was exposed to 0.1M phosphate buffer and after 5 minutes a fixed volume of the phosphate buffer was taken out. This step was repeated several times in a four hours' period. The samples collected were then analysed using a Perkin-Elmer atomic absorption spectrometer (AAS) to determine the presence of iron quantitatively, which

would be related to the amount of ferrocene that leached out from the polymer into the solution.

The Fc/HEMA polymer films incorporated with enzyme GOD were prepared and also exposed to 0.1M phosphate buffer. After five minutes, a fixed volume of the phosphate buffer was taken out. This step was repeated several times in a four hours' period. The buffer was then assayed with diluted Bradford reagent and the absorbance was monitored at 595 nm using a Varian Cary 100 UV-Vis spectrophotometer (Bradford, 1976).

All the above procedures were repeated for evaluation of water absorption of copolymer, polyMH, and also ferrocene and enzyme GOD leaching from membrane Fc/MH.

Preparation of electrodes and glucose biosensors

Mixtures of HEMA, DMPP and Fc were deposited on screen-printed carbon paste electrodes. This was then exposed to UV radiation and a thin layer of film with thickness *ca.* 170 μm was formed. These electrodes were then used in the investigation of the redox behaviour of the immobilized ferrocene. Glucose biosensors based on Fc/HEMA films were constructed by including the enzyme GOD into the mixture before photocuring. The amount of Fc or GOD used in all films (Fc/HEMA-GOD) was the same. Glucose biosensors based on copolymer Fc/MH i.e. Fc/MH-GOD was also fabricated using the same method.

Results & Discussions

Water absorption of photopolymers and leaching of Fc and GOD

Figure 1 shows the result of water absorption of photocured polyHEMA and polyMH films over a period of 2 and 3½ hours, respectively. At the first 45 mins the water absorbed by the polyHEMA increased linearly and attained 11.12% at the end of 45 mins. However, the water absorbed increased further to only 2% after 90 mins to a final value of 14.90% where the polymer film reached water saturation. As for the copolymer polyMH, there is a steady rate of water absorption in the first 2 h and the amount of water absorbed reaches a maximum of approximately 12% by weight after 3 h.

This shows that the level of water absorption for the polyMH is lower and it is more hydrophobic as compared to polyHEMA. The hydrophobic nature of this copolymer is mainly due to the introduction of MMA monomer which is less polar than a HEMA monomer.
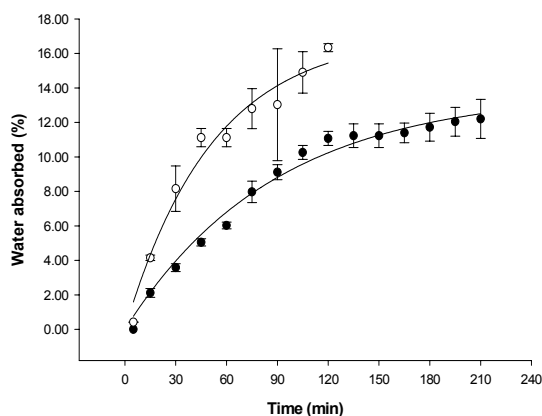

FIGURE 1 The water absorption profile of the polyHEMA (○) and polyMH (●).

Figure 2 depicts the results of leaching studies for the immobilized entrapped ferrocene (Fc) in polyHEMA and polyMH films. It can also be observed that the leaching of the entrapped Fc in Fc/HEMA increased slowly with time at the first 90 mins from ~3% to ~8.6% and finally reached ~15% at the end of 4 hours. This high leaching rate might be due to the inefficient entrapment of the Fc in polyHEMA. Besides, the hydrophilicity nature of the polyHEMA (Bayramoğlu *et al*., 2003, Schulz *et al.*, 1999) caused swelling after water absorption (Hoffman, 2002), and thus promotes the leaching of the entrapped Fc. Similar behaviour was also observed by Calvo *et al.* (1993) where leaching of ferrocene from the acrylamide-acrylic acid derived hydrogel upon a prolonged immersion in aqueous solution resulted in lower redox charge for electrodes. As for copolymer Fc/MH, no iron was detected using an atomic absorption spectrophotometer below 100 min. However, leaching of Fc began after approximately 100 min of exposure to buffer solution (Figure 2). A plot of correlation between the amount of Fc leached out and water absorbed (not shown) demonstrated that when the water absorption reached approximately 10 % (w/w), Fc leaching began. Therefore, below 10% of water absorption, it seems that Fc

could not leach out of the polyMH film and only 2.2% of Fc was found to leach out after 4 h of exposure to the buffer solution.

Leaching studies conducted on entrapped GOD in the polyHEMA and polyMH film also showed that no GOD was detectable after the polymers were exposed to water for four hours. These studies have demonstrated that by using a hydrophobic copolymer such as MH to physically immobilise Fc and GOD, the loss of the mediator Fc through leaching can be delayed and minimised whilst that of enzyme GOD leaching can be eliminated altogether.
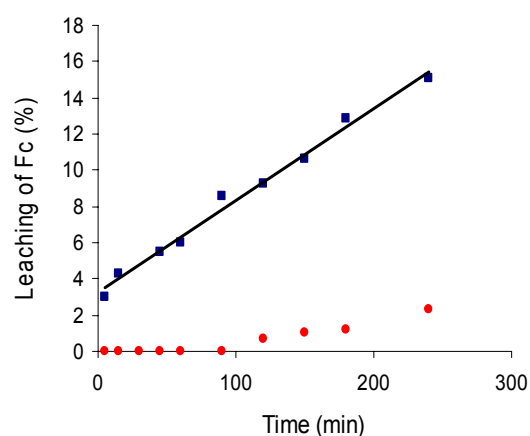

FIGURE 2 Leaching of the entrapped Fc in polyHEMA (■) and in polyMH (●) films with time.

Electrochemical behavior of the ferrocene-containing polymers at fixed scan rate

The cyclic voltammograms of Fc/HEMA film were obtained between 0.00 to +0.70 V in a phosphate buffer after the film was deposited onto a screen-printed carbon paste electrode (SPE). Altogether 20 scans were performed but only 10 of the scans were shown in Figure 3.

For Fc/HEMA film, the redox current increases during the first 16 cycles and remained constant thereafter. The increase in the redox current with repeated scanning has been reported by Dong *et al.* (1991) for ferrocene entrapped in nafion films and such increase was attributed to the building up of the charged Fc^+, which is responsible for the increase in the oxidation peak. The thickness of the film could also influence the redox current and hence the time taken for the electrode to reach steady state. At

equilibrium, the anodic (E_{pa}) and cathodic (E_{pc}) peak potentials for the steady-state cyclic voltammogram are 0.298V and 0.150V respectively. The large peak separation shown in the cyclic voltammograms is similar to that reported in previous papers for other polymer films with ferrocene entrapped or covalently bound (Armada *et al.*, 2003; Dong *et al.*,1991) and is attributed to the resistance of the polymer film and slow migration of counterions into the film. However, the redox waves of Fc entrapped in this polymer is stronger compared to that reported for polymeric films with ferrocene covalently immobilized such as methacrylate redox copolymer (Nagasaka *et al.*, 1995) and siloxane-based polymer (Armada *et al.*, 2003).
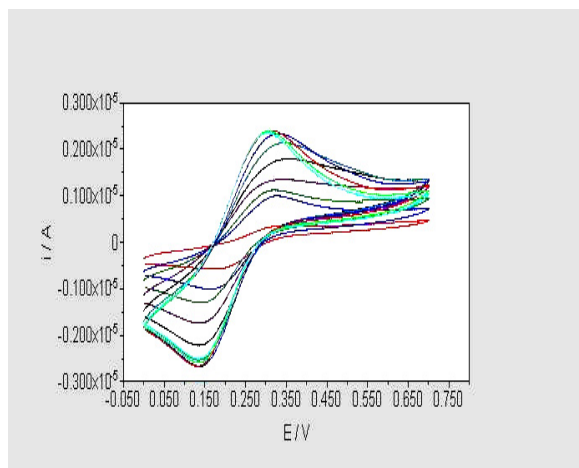

FIGURE 3 Cyclic voltammograms of Fc/HEMA-coated carbon-paste screen-printed electrode in 0.1M phosphate/0.1 M NaCl buffer (pH = 7.0). Scan rate: 5 mVs[-1].

The cyclic voltammogram of Fc/MH (not shown here) is similar to that shown in Figure 3. The anodic and cathodic currents of the Fc/MH film rise continuously with potential scans until a distinct redox couple of Fc occurred and reached steady state after 13-16 cycles. As the Fc/MH film was scanned further, increase in stability of the measured current was observed and this probably due to the reorientation of the Fc entrapped in the MH film. After equilibrium was established, the peak potentials E_{pa} and E_{pc} values remained essentially constant. The steady-state values for E_{pa} and E_{pc} obtained in cyclic voltammograms of Fc/MH are 0.294V and 0.156V respectively, which are very close to that of Fc/HEMA.

Electrochemical behavior of the ferrocene-containing polymers at different scan rates

Cyclic voltammograms of the Fc/HEMA films at different scan rates (Figure 4) show that both i_{pa} and i_{pc} are proportional to the square root of scan rate ($v^{1/2}$) for the scan rates ranged from 1 to 10 mVs[-1]. Similar phenomenon was also observed for copolymer Fc/MH. As shown in Figure 5, a plot of the current i_{pa} and i_{pc} against $v^{1/2}$ according to the Randles-Sevčik equation demonstrated a strong linear relationship for Fc/HEMA (R^2 = 0.9238 and 0.9743) and Fc/MH (R^2 = 0.9869 and 0.9761) films. The linear behavior of the current peak with scan rate indicates that the number of redox sites at the electrode surface is constant in the sweep rate interval studied and the redox behavior of the electrodes is diffusion controlled where electron transfer to and from the redox centers of the ferrocene compounds involves diffusion (Bu *et al.*, 1995). The only deviation of the Figure 5 from Randles-Sevčik equation is non-zero intercept and this may be due to non-faradaic current (Calvo *et al.*, 1993). From the electrochemical data extracted from the cyclic voltammograms of both polymer films at different scan rates, it is also observed that the E_{pa} and E_{pc} change with different scan rates. As scan rate increased, E_{pa} shifted to more positive values whilst E_{pc} became more negative. Hence, smaller ΔE_p values at low scan rate were observed. Theoretically, for a reversible system, the E_{pa} and E_{pc} are independent of the scan rate and any changes of the ΔE_p values indicate slow charge-transfer process in the polymer film and the system is moving towards a quasi-reversible state (Monk, 2001). When v = 1mVs[-1] ($v^{1/2}$ = 0.03V[1/2]s[-1/2]), the ΔE_p is in agreement with the theoretical value, which demonstrates a one-electron reversible system. The slight increase in ΔE_p with increasing scan rate may be due to the slow electron transfer of Fc/Fc[+] couple in the polymer. This observation is in accordance with that reported before by Zhou *et al.* (1997), in which ferrocene perchlorate was incorporated in nafion film. For both Fc/HEMA and Fc/MH films, the i_{pa}/i_{pc} is close to 1 (theoretically, i_{pa}/i_{pc} = 1) for almost all the scan rates used.

From the slope of the i_p against $v^{1/2}$ plot, the diffusion coefficient, D, which is the diffusion for the diffusion-like transport of charge through the polymer film, can thus be

determined. The D value for Fc/HEMA and Fc/MH are estimated to be 2.08 x10^{-11} cm^{2}s^{-1} and 1.76 x10^{-11} cm^{2}s^{-1}, rspectively. This value is much lower than that reported for Fc in a acrylamide-acrylic acid hydrogel film where the D value is in the order of 10^{-6} cm^{2}s^{-1}, which is similar to the diffusion of free ferrocene in aqueous solution [Calvo et al., 1993]. The lower diffusion characteristic of Fc in the MH copolymer may be attributed to the lower water absorption and the dense polymer network as compared to polyHEMA.
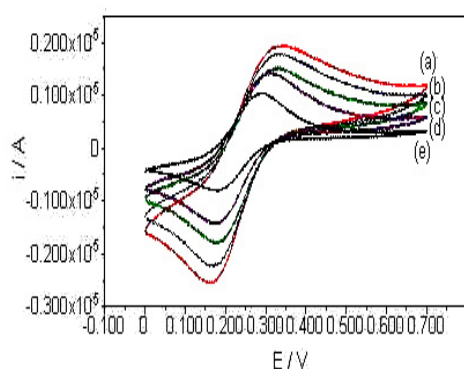

FIGURE 4 Cyclic voltammograms of a Fc/MH-coated electrode in 0.1M phosphate/0.1 M NaCl buffer at scan rate of (a) 10, (b) 7.5, (c) 5, (d) 2.5 and (e) 1 mVs^{-1}.
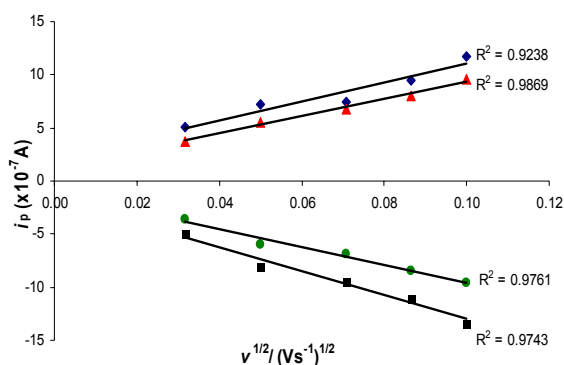

FIGURE 5 A plot of anodic and cathodic current versus square root of scan rate for a Fc/HEMA-coated (♦ and ■) and Fc/MH-coated electrode (▲ and ●), respectively.

Detection of glucose concentrations
    The current response of a Fc/HEMA-GOD and Fc/MH-GOD electrode to changes in glucose concentrations at respective applied potential 0.35V and 0.25V versus SCE is shown in Figure 6. A good linear relationship

between the glucose concentrations and the measured current was obtained in the range of 3.85-16.67 mM and 2 – 11 mM glucose for the Fc/MH-GOD electrode.
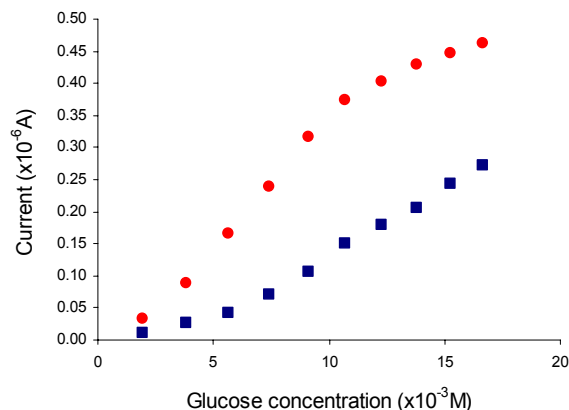

FIGURE 6 Calibration curve of current response to glucose concentration at a (■) Fc/HEMA-GOD enzyme electrode and a (●) Fc/MH-GOD enzyme electrode in 0.1M phosphate buffer/0.1M NaCl (pH = 7.0) at applied potential 0.35V and 0.25V respectively versus SCE.

Analytical performance of the glucose biosensor based on polymer films

    The repeatability and reproducibility of the Fc/HEMA-GOD and Fc/MH-GOD electrodes were evaluated and it was found that the relative standard deviation (RSD) value yielded for Fc/HEMA-GOD is higher (4.46-21.62%) as compared to Fc/MH-GOD (3.01-13.65%). This indicates that the Fc/MH-GOD fabricated giving a better reproducibility and repeatibility.

Apart from reproducibility and repeatability, a study of Fc/HEMA-GOD and Fc/MH-GOD electrodes under dry storage at 4°C for a period of 2 weeks were also carried out. The result obtained demonstrated that the Fc/MH-GOD enzyme electrodes remained stable and exhibited a 90% of the original response even after two weeks' period (Figure 7). While for Fc/HEMA-GOD, only 75% of initial response is retained.
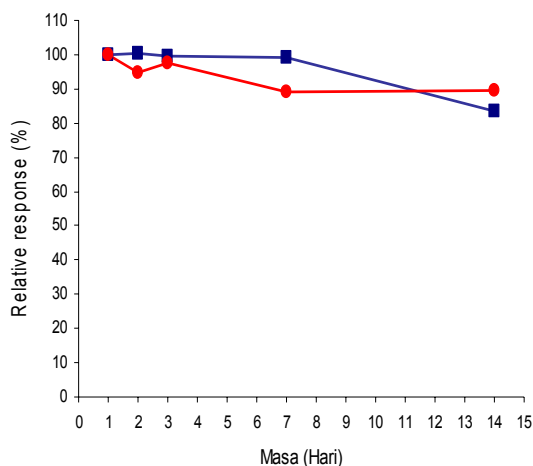
FIGURE 7 Relative response of a Fc/HEMA (■) and Fc/MH-coated electrode (●) with time, respectively.

From the cyclic voltammetry and amperometric measurements, the entrapped ferrocene compounds were proved to function as electron shuttling agent between GOD and carbon-paste screen-printed electrode. However, the electron transfer between ferrocenium ion and ferrocene was very slow. Because of the simplicity in preparation of these two ferrocene-containing films (Fc/HEMA and Fc/MH), photolithography technique can thus be applied to the fabrication of various reagentless biosensors.

Acknowledgements

References

Armada, M. P. C., Losada, J., Cuadrado, I., Alonso, B., González, B., Ramírez-Oliva, E. & Casado, C. M. (2003). Electrodes modified with a siloxane copolymer containing interacting ferrocenes for determination of hydrogen peroxide and glucose. *Sensors and Actuators B* 88: 190-197.

Bayramoğlu, G., Akgöl, S., Bulut, A., Denizli, A. & Arıca, M. Y. (2003). Covalent Immobilisation of invertase onto a reactive film composed of 2-hydroxyethyl methacrylate and glycidyl methacrylate: properties and application in a continuous flow system. *Biochemical Engineering Journal* 14: 117-126.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Bu, H. Z., Mikkelsen, S. R. & English, A. M. (1995). Characterization of a ferrocene-containing polyacrylamide-based redox gel biosensor use. *Analytical Chemistry* 67: 4071-4076.

Calvo, E. I., Danilowicz, C. & Diaz, L. (1993). Enzyme catalysis at hydrogel-modified electrodes with redox polymer mediator. *Journal of Chemical Society Faraday Trans* 89(2): 377-384.

Dong, S., Wang, B. & Liu, B. (1991). Amperometric glucose sensor with ferrocene as an electron transfer mediator. *Biosensors & Bioelectronics* 7: 215-222.

Hale, P. D., Inagaki, T., Karan, H. I., Okamoto, Y. & Skotheim, T. A. (1989). A new class of amperometric biosensor incorporating a polymeric electron-transfer mediator. *Journal of American Chemical Society* 111: 3482-3484.

Hoffman, A. S. (2002). Hydrogels for biomedical applications. *Advanced Drug Delivery Reviews* 43: 3-12.

Koide, S. & Yokoyama, K. (1999). Electrochemical characterization of an enzyme electrode based on a ferrocene-containing redox polymer. *Journal of Electroanalytical Chemistry* 468: 193-201.

Lange, M A. & Chambers, J. Q. (1985). Amperometric Determination of glucose with a ferrocene-mediated glucose oxidase/polyacrylamide gel electrode. *Analytica Chimica Acta* 175: 89-97.

Lee, Y. H. & Hall, E. A. H. (2000). Producing "self-plasticizing" ion-selective membranes. *Analytical Chemistry* 72: 42-51.

Lee, Y. H. & Hall, E. A. H. (2001). Assessing a photocured self-plasticised acrylic membrane recipe for Na^+ and K^+ ion selective electrodes. *Analytica Chimica Acta* 443: 25-40.

Monk, P. (2001). *Fundamentals of electroanalytical chemistry*. England: John Wiley & Sons Ltd.

Mulchandani, A., Wang, C.-L. & Weetall, H. H. (1995). Amperometric detection of peroxides with poly(anilinomethylferrocene)-modified enzyme electrodes. *Analytical Chemistry* 67: 94-100.

Nagasaka, H., Saito, T., Hatakeyama, H. & Watanabe, M. (1995). Preparation of novel redox copolymers: poly[ferrocenylmethyl methacrylate-co-methoxy-oligo(ethylene oxide) methacrylate], and their use as polymeric mediators in amperometric glucose sensors. *Denki Kagaku* 63 (12): 1088-1094.

Okawa, Y., Nagano, M., Hirota, S., Kobayashi, H., Ohno, T. & Watanabe, M. (1999). Tethered mediator biosensor. mediated electron transfer between redox enzymes and electrode via ferrocene anchored to electrode surface with long poly(oxyethylene) chain. *Biosensors & Bioelectronics* 14: 229-235.

Saito, T. & Watanabe, M. (1998). Characterization of poly(vinylferrocene-*co*-2-hydroxyethyl methacrylate) for use as electron mediator in enzymatic glucose sensor. *Reactive & Functional Polymers* 37: 263-269.

Schultz, B., Riedel, A. & Abel, P. U. (1999). Influence of polymerization parameters and entrapment in poly(hydroxyethyl methacrylate) on activity and stability of GOD. *Journal of Molecular Catalysis B: Enzymatic* 7: 85-91.

Tkáč, J., Voštiar, I., Gemeiner, P. & Šturdík, E. (2002). Stabilization of ferrocene leakage by physical retention in a cellulose acetate membrane. The fructose biosensor. *Bioelectrochemistry* 55: 149-151.

Zhou, D.-M., Ju, H.-X. & Chen, H.-Y. (1997). A miniaturized glucose biosensor based on the coimmobilization of glucose oxidase and ferrocene perchlorate in nafion at a microdisk platinum electrode. *Sensors and Actuators* B 40: 89-94.