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Electrochemistry of Freely Diffusing Mediators in Polyelectrolyte Membranes Used for Blood Glucose Test Strips with a High Upper Limit of the Linear Range †

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Abstract: Co-immobilization of low-molecular-weight mediators and glucose oxidase in polyelectrolyte membranes results in glucose test strips operating in the millimolar concentration range. The density and charge of polyelectrolyte membranes formed on the surface of the screen-printed electrodes allow the diffusion of mediators to be controlled. Negatively charged perfluorosulfonated ionomer (PFSI) hampers the diffusion of the commonly used ferricyanide (III) ion, while the hexammine ruthenium (III) cation apparent diffusion coefficient in PFSI membrane remains the same as without the membrane. In contrast to PFSI, electrode modification with positively charged chitosan leads to additional adsorption of potassium hexacyanoferrate on the membrane. Additionally, the rate of mediator leakage from the membrane was found to govern the sensitivity of the resulting biosensors. The leakage rate also depends on the density and charge of the polyelectrolyte and mediator. However, the main advantage of the proposed simple approach of single-step deposition of three-component membrane-forming mixtures on the screen-printed electrodes is the extended upper limit of the linearity: 30–50 mM glucose. Hence, the obtained test strips are suitable for glucose detection in undiluted blood.

Keywords: glucose; test strip; biosensor; mediator; diffusion; membrane; polymer; chitosan

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1. Introduction

The modern glucose test strips industry is quite a developed field [1]. However, glucose biosensors are always a focus of scientific research as the most widespread tool for express analysis of one of the most valuable metabolites [2]. The mass-production requires simple approaches and technologies along with minor losses in quality and accuracy. Commercial biosensor production comprises applying numerous polymer layers with different functionalities [3]. However, single-step modification of electrode supports would be preferable for test strip production.

Glucose test strip action is based on biochemical recognition of glucose by specific enzymes, and further signal transduction to electrochemical or optical. For electrochemical biosensors, amperometry under constant potential is the most widespread technique. The common compounds involved in biochemical to electrical signal transformation are called mediators. These electroactive molecules are aimed at substituting oxygen, which is involved in the biochemical reaction, since the solubility of oxygen in water solutions is rather low. In the course of the chemical reaction, the reduced form of the mediator proportional to glucose concentration is produced and detected on the electrode.

The mediator may be directly impregnated into the printing material, admixed in the enzyme layer, physically adsorbed at the electrode surface or covalently bound with the

polymer matrix [4]. The advantage of mediated biosensors is low dependence on oxygen fluctuations and interfering electroactive species concentration.

Numerous organic and inorganic compounds were proposed as mediators for biosensors: ferricyanide, ferrocene, phenazine, phenothiazine, methylene green/blue, tetrathiafulvalene, quinone, osmium and ruthenium complexes [5]. However, ferricyanide is still the most used mediator in commercial glucose biosensing [4,5]. Hexaammineruthenium (III) is an interesting candidate because the hexaammineruthenium (II/III) redox potential is lower than the hexacyanoferrate (II/III) one [6]. The low working potential leads to low interferences and increase in selectivity and accuracy.

The enzyme immobilization techniques, as well as matrices, retaining enzyme activity are also a constant issue in research [7–9]. Since it seems promising to immobilize both the mediator and the enzyme in one polymer matrix, the electrochemical behavior of the mediator in this matrix is an important factor.

In this work, we studied the electrochemical behavior of mediators immobilized in polymer matrices on the electrode surface. We compared different membranes and three water-soluble freely diffusing mediators. The performance of the corresponding test strips produced in a single step via the drop-casting of the membrane-forming mixture, containing the enzyme and mediator in a polymer solution, is presented.

2. Materials and Methods

2.1. Materials

Experiments were carried out with Millipore Milli-Q water (resistivity 18.2 MΩ·cm at room temperature). Inorganic salts (K_2HPO_4, KH_2PO_4, NaCl) were obtained from Reachim (Moscow, Russia). $K_3[Fe(CN)_6]$, $[Ru(NH_3)_6]Cl_3$, 1,1′-ferrocenedimethanol, D-glucose, chitosan, Triton X-100, (3-aminopropyl)triethoxysilane, and glucose oxidase (GOx) (EC 1.1.3.4, 248 IU mg^{-1}) from Aspergillus niger (type VII, lyophilized powder) were obtained from Sigma-Aldrich (Germany). Ionomer MF4 SK (perfluorosulfonated ionomer (PFSI)) was purchased from Plastpolymer (Saint Petersburg, Russia).

Planar three-electrode screen-printed structures with a carbon working electrode (Ø = 1.8 mm) were produced by Rusens Ltd. (Moscow, Russia). Flexible 250 µm thin polyethylene terephthalate film (PET) obtained from Vladimirskii Khimicheskii Zavod (Vladimir, Russia) was used as the substrate. Planar two-electrode structures with a carbon working electrode (Ø = 2.25 mm) and Ag-reference electrode were produced using silver polymer paste (NPP "Delta-Paste", Zelenograd, Russia), carbon paste (C2030519P4, Sun Chemical, South Normanton, UK) and UV curable insulating paste (UNICA, Ternat, Belgium).

2.2. Instrumentation

A SCF–300 (Technical Industrial Co., Ltd., Hong Kong) screen printer was used for electrode structure production. Electrochemical measurements were carried out using a Palmsens 4 potentiostat (PalmSens BV, Houten, The Netherlands).

2.3. Methods

Screen-printed electrode fabrication. The printing process involves applying a layer of silver paste and two layers of graphite paste to a PET substrate and drying after each step.

Chitosan solution preparation. Chitosan polymer water mixtures were prepared from 1% chitosan solution in 1% acetic acid; 0.01–0.3% chitosan solutions were prepared from 1% via dilution with water.

Apparent diffusion coefficient determination. The working electrode of screen-printed three-electrode structures was modified with water or isopropanol solution of chitosan, PFSI or polysiloxane and dried at room temperature (+25 °C). Cyclic voltammograms of modified electrodes were recorded in phosphate buffer solution, containing 5 mM of $K_3[Fe(CN)_6]$, $[Ru(NH_3)_6]Cl_3$ or $Fc(MeOH)_2$ at different scan rates: 10–1000 $mV^{-1}s^{-1}$. Apparent diffusion coefficients were found from the dependence of the anodic peak current on the square root of the sweep rate, using the Randles–Sevcik equation.

Mediator release rate measurement. The working electrode of screen-printed three-electrode structures was modified with water chitosan solution, containing 5 mM of $K_3[Fe(CN)_6]$, $[Ru(NH_3)_6]Cl_3$ or $Fc(MeOH)_2$, dried at room temperature (+25 °C). Cyclic voltammograms of modified electrodes were recorded in mediator-free phosphate buffer solution.

Preparation of the test strips. The mediator ($K_3[Fe(CN)_6]$, $[Ru(NH_3)_6]Cl_3$ or $Fc(MeOH)_2$) was dissolved in chitosan water solution and added to glucose oxidase. The resulting mixture (1 μL) was cast onto the surface of screen-printed electrodes with subsequent drying at room temperature (+25 °C). Then, a capillary (1.5 mm width) was formed using 100 μm double-sided adhesive on the surface of the sensor. A PET capillary cap pretreated with Triton-X100 solution (0.02%) was applied on the top of the electrode (Figure 1).

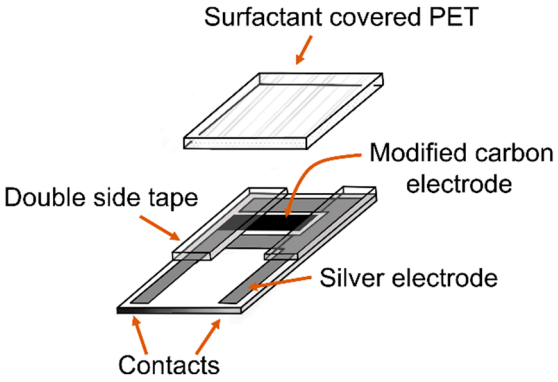

Figure 1. Fabrication of the test strip based on the screen-printed two-electrode support, with a capillary formed after modification of the working electrode.

Chronoamperometry. In total, 1–3 μL of the standard glucose solution was put into the capillary, and the chronoamperometric response was recorded under constant potential (0.1–0.3 V vs. printed Ag electrode). The current reading was taken at fifth second of measurement for calibration.

Statistics. All experiments were repeated at least three times, and the data is represented by mean value ± standard deviation (S.D.).

3. Results and Discussion

The choice of matrix for immobilization of both enzyme and mediator should provide a suitable environment for the enzyme. Our previous studies on the optimal polymer content in the membrane-forming mixture have shown that, for glucose oxidase, 0.3% perfluorosulfonated ionomer (PFSI) and 0.2–0.3% chitosan solutions are preferable. PFSI is a negatively charged polymer ($-SO_3^-$). In contrast, chitosan is positively charged in neutral solutions, due to the protonation of $-NH_2$-groups. We also used polysiloxane derived from (γ-aminopropyl)triethoxysilane as an example of an electrically neutral matrix. For single-step modification, the mediator and enzyme should be applied to the electrode surface in one matrix. Thus, we studied the diffusion of mediators with different charges through the above-mentioned membranes and their release rate from these membranes.

3.1. Polymer Membrane as Diffusion Barrier for Mediator

The membrane-forming mixtures containing 0.3% polymer were deposited on the screen-printed electrode surface and dried in air. Screen-printed electrodes modified with polymer membranes were studied using cyclic voltammetry in 5 mM potassium hexacyanoferrate (III) solution, hexaammineruthenium (III) chloride and 1,1′-ferrocenedimethanol ($Fc(MeOH)_2$. Mediators diffuse from the bulk solution through the membrane to the electrode surface, where they are reduced or oxidized. The peak current found from cyclic voltammogram is proportional to the sweep rate according to the Randles–Sevcik equation:

$$j = 2.69 \cdot 10^5 \, n^{3/2} A D^{1/2} C v^{1/2} \quad (1)$$

Using Equation (1), the apparent diffusion coefficients were found from the slope of the dependence of the anodic peak current on the square root of the sweep rate.

We expected that the diffusion of charged mediators depends on the charge of polyelectrolyte. Indeed, neutral 1,1′-ferrocenedimethanol ($Fc(MeOH)_2$) is characterized by comparable apparent diffusion coefficients in all the membranes. A slightly lower value in the chitosan membrane may be attributed to the formation of positively charged oxidation product $Fc(MeOH)_2^+$, the diffusion of which is hindered (Figure 2).

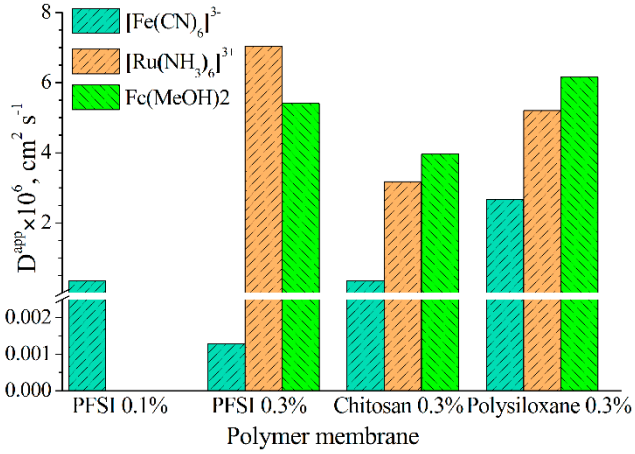

Figure 2. Apparent diffusion coefficients for $[Fe(CN)_6]^{3-}$, $[Ru(NH_3)_6]^{3+}$ and $Fc(MeOH)_2$ in the membranes (formed by drop-casting 0.3% polymer solutions on the electrode), 50 mM K_2HPO_4/KH_2PO_4, 180 mM NaCl, pH 7.4.

The positively charged $[Ru(NH_3)_6]^{3+}$ ion was found to freely diffuse through the negatively charged PFSI membrane, while polysiloxane and chitosan membranes hampered its diffusion. Thus, for the PFSI-coated electrode, the apparent diffusion coefficient of the hexammineruthenium (III) cation remains the same as without the membrane (Figure 2).

Significantly lower apparent diffusion coefficients were obtained for the hexacyanoferrate anion. The negatively charged perfluorosulfonated ionomer dramatically hampered the diffusion of the ferricyanide ion (Figure 2). However, an effective diffusion coefficient in the case of 0.1% PFSI in the membrane-forming mixture is comparable to that found for the 0.3% chitosan mixture (Figure 2).

In contrast to PFSI, an increase in the chitosan amount on the electrode surface resulted in a higher apparent diffusion coefficient of the potassium ferricyanide ion. The effect of additional adsorption of this mediator on the oppositely charged membrane has been studied in our previous work [10].

Therefore, the chitosan membrane seems to create diffusion restrictions for all the mediators studied. However, in contrast to PFSI and polysiloxane, chitosan is soluble in water. It is an advantage for the preparation of membrane-forming mixtures, containing water-soluble mediators and enzymes. Moreover, it seems possible to manage the diffusion of mediators by changing the polymer content in the membrane.

3.2. Mediator Leakage from Chitosan Membranes

The response of glucose test strips used with portable electrochemical devices (glucose meters) is usually recorded within the first 5–7 s of measurement [11]. Therefore, for the test strips based on freely diffusing mediators, the rate of mediator release from the membrane should be considered. For this purpose, screen-printed electrodes were modified with 0.01, 0.1, and 0.3% chitosan solutions, containing mediators, and dried in air.

The rate of mediator leakage from the membrane was studied via cyclic voltammetry. The decreasing anodic peak current has been plotted as a function of time (Figure 3). The obtained curves were fitted to the exponential equation for reversible first-order reactions:

$$j = A \cdot \exp\{-kt\} + B \quad (2)$$

The experimental data and the fitting results are shown in Figure 2.

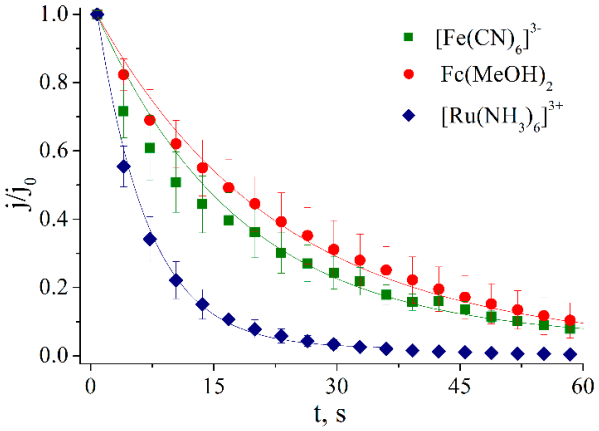

Figure 3. The relative peak current registered upon release of mediators ($[Ru(NH_3)_6]^{3+}$ (♦), $[Fe(CN)_6]^{3-}$ (■) and $Fc(MeOH)_2$ (●) from the membrane deposited on the electrode via drop-casting 5 mM of mediator in 0.1% chitosan membrane-forming mixtures, cyclic voltammetry, 500 mV s^{-1}, 50 mM K_2HPO_4/KH_2PO_4 with 180 mM NaCl, pH 7.4.

Figure 3 illustrates the decrease in peak current registered upon the cycling of electrodes modified with different mediators and chitosan in buffer solution. One can consider the mediator release rate, which is proportional to the first derivative: $dj/dt = A \cdot k$ (see Equation (2)). The rate of mediator release from the chitosan membranes was also found to be dependent on the density of the membrane (Figure 4). As expected, the increase in chitosan content in the membrane-forming mixture hinders the release of hexacyanoferrate (III) from the membrane due to the electrostatic binding of negatively charged $[Fe(CN)_6]^{3-}$ to the charged $-NH_3^+$-groups in polymer. At pH 7.4, approximately 6% of the chitosan amino group is protonated. That is why a small amount of chitosan deposited on the electrode does not affect the release rate of the mediators.

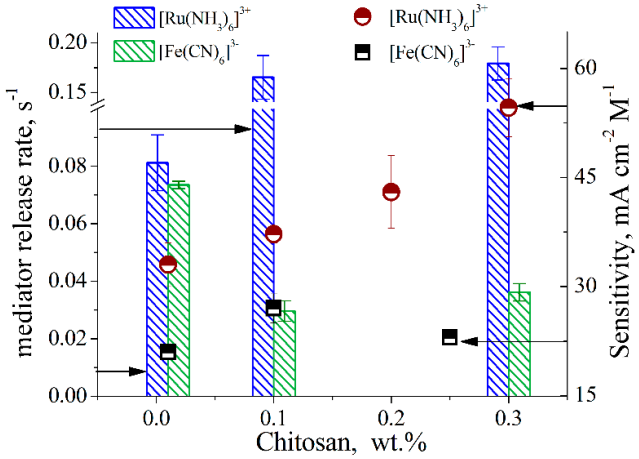

Figure 4. Release rates of $[Ru(NH_3)_6]^{3+}$ and $[Fe(CN)_6]^{3-}$ (blue and green bars, respectively) from chitosan membranes (formed by drop-casting the mediator in polymer solutions on the electrode) and sensitivity of the glucose test strips based on GOx (10 mg Ml^{-1}) and 100 mM $[Ru(NH_3)_6]^{3+}$ or $[Fe(CN)_6]^{3-}$ (red and black dots, respectively), 50 mM K_2HPO_4/KH_2PO_4, 180 mM NaCl, pH 7.4.

An opposite effect was observed for the positively charged hexaminеruthenium ion. Its release rates increase for the membranes prepared from membrane-forming mixtures with a higher chitosan content, due to the charge repulsing effect.

Thus, the chitosan membrane limits the release of the negatively charged hexacyanoferrate (III) ion, while the positively charged hexaamminruthenium (III) ion release rate is enhanced.

3.3. Single-Step Glucose Test Strips Preparation

Glucose test strips were prepared using three-component membrane forming mixtures, containing 100 mM mediator, and 10 mg mL^{-1} GOx in 0.01–0.3% chitosan solutions. The current was recorded at fifth second after potential had been applied and was plotted as a function of glucose concentration in the sample. Figure 3 also illustrates the sensitivity of the test strips based on different membrane compositions. We found that, in the case of hexaamminruthenium (III) chloride, sensitivity increases simultaneously with the release rate. As it was shown, $[Ru(NH_3)_6]^{3+}$ is characterized by the highest release rate from chitosan membranes (0.1–0.3%). Indeed, the highest sensitivity is achieved for test strips based on this mediator (55 ± 4 $mA \cdot M^{-1} \cdot cm^{-2}$). However, the leakage rate is not the only reason. The activity of GOx immobilized in chitosan membranes is also known to improve with the increase in chitosan content (up to 0.3% in immobilizing mixtures) [10]. Unfortunately, higher sensitivity is accompanied by a narrow linear range (Table 1), while test strips for blood glucose analysis should operate in a wide millimolar range: 1–30 mM glucose. If hexacyanoferrate is used as the mediator, the gain in enzyme activity has a minor impact on analytical performance. The use of 0.1% instead of 0.01% chitosan in membrane-forming solution for the preparation of hexacyanoferrate-based test strips resulted in slightly higher sensitivity (27 ± 2 $mA \cdot M^{-1} \cdot cm^{-2}$), but the upper detection limit dropped down to 20 mM. It is worth mentioning that the lower sensitivity of the test strips based on ferrocenedimethanol may be due to the 3-fold lower release rate of this mediator (Table 1).

Table 1. Analytical performance of the glucose test strips.

Mediator	Chitosan Content, %	Sensitivity, $mA \cdot M^{-1} \cdot cm^{-2}$	Linear Range, mM
$[Ru(NH_3)_6]^{3+}$	0.01	33 ± 3	1–30
$[Ru(NH_3)_6]^{3+}$	0.1	37 ± 1	1–30
$[Ru(NH_3)_6]^{3+}$	0.2	43 ± 5	5–20
$[Ru(NH_3)_6]^{3+}$	0.3	55 ± 4	5–15
$[Fe(CN)_6]^{3-}$	0.01	21 ± 1	1–50
$[Fe(CN)_6]^{3-}$	0.1	27 ± 2	1–20
$[Fe(CN)_6]^{3-}$	0.25	23 ± 1	1–30
$Fc(MeOH)_2$	0.01	11 ± 1	1–30
$Fc(MeOH)_2$	0.1	8 ± 1	1–20

Thus, we conclude that the behavior of the mediator in polymer membranes used for enzyme immobilization is an important issue that should be considered for test strip production. The mediator release rates depend on the density and charge of the polyelectrolyte and mediator. Moreover, the polymer content in the membrane-forming mixtures allows sensor performance to be managed. Another advantage of the single-step approach is the simple production of the test strips with an extended upper limit of the linearity. The biosensor response linear range from 1 to 30–50 mM glucose meets the requirements for glucose detection in whole blood.

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