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Electrochemistry of Freely Diffusing Mediators in Polyelectro-Lyte 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 millimolar concentration range. Density and charge of polyelectrolyte membranes formed on the surface of the screen-printed electrodes allow to control the diffusion of mediators. 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

Modern glucose test strips industry is rather developed field [1]. However, glucose biosensors are always in a focus of the 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 biosensors production comprises applying a numerous polymer layers with different functionality [3]. However, single-step modification of electrode supports would be preferable for test strips production.

Glucose test strips 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 to substitute oxygen, which is involved in biochemical reaction, since solubility of oxygen in water solutions is rather low. In course of chemical reaction, the reduced form of 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 the commercial glucose biosensing [4,5]. Hexaammineruthenium (III) is an interesting candidate because hexaammineruthenium (II/III) redox potential is lower than the hexacyanoferrate (II/III) one [6]. Actually, low working potential leads to reduce of interferences and increase of selectivity and accuracy.

The enzyme immobilization techniques as well as matrices, retaining enzyme activity are also the constant issue of research [7–9]. Since it seems promising to immobilize both mediator and the enzyme in one polymer matrix the electrochemical behavior of 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 drop-casting of membrane-forming mixture, containing enzyme and mediator in 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 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 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).

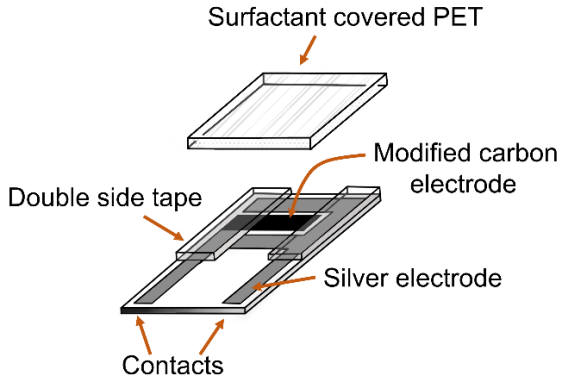

Figure 1. Fabrication of the test strip based on the screen-printed two-electrode support, with capillary formed after modification of working electrode.

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, 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. 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 5mM 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 Randles-Sevcik equation.

Mediator release rate measurement. 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. 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 sensor. A PET capillary cap pretreated with Triton-X100 solution (0.02%) was applied on the top of the electrode (Figure 1).

Chronoamperometry. 1–3 μl of the standard glucose solution was put into capillary and chronoamperometric response was recorded under constant potential (0.1–0.3 V vs. printed Ag electrode). Current reading at 5^{th} second was taken 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

Choice of matrix for immobilization of both enzyme and mediator should provide 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), as well as 0.2–0.3% chitosan solutions are preferable. PFSI is negatively charged polymer ($-SO_3^-$). In contrast to it, chitosan is positively charged in neutral solutions, due to protonation of $-NH_2$-groups. We additionally used polysiloxane derived from (γ-aminopropyl)triethoxysilane as an example of electrically neutral matrix. For single-step modification, 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 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 sweep rate according to 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.

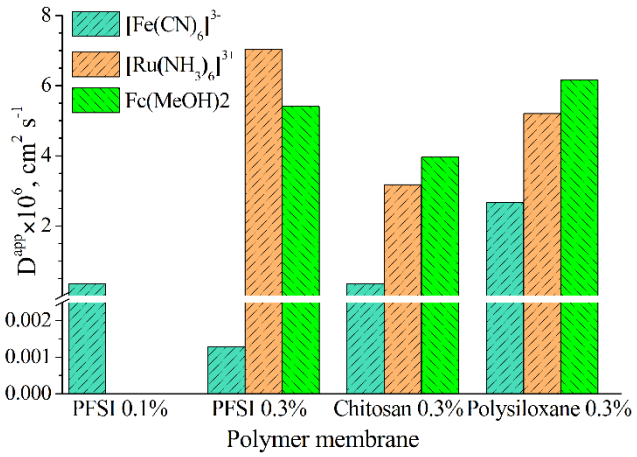

Figure 2. Apparent diffusion coefficients for $[Fe(CN)_6]^{3-}$, $[Ru(NH_3)_6]^{3+}$ and $Fc(MeOH)_2$ in the membranes (formed by drop-casting 0.3% polymer solutions on the electrode), 50 mM K_2HPO_4/KH_2PO_4, 180 mM NaCl, pH 7.4.

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 chitosan membrane may be attributed to the formation of positively charged oxidation product $Fc(MeOH)_2^+$, which diffusion is hindered (Figure 2).

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

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

In contrast to PFSI, increase in chitosan amount on the electrode surface resulted in higher apparent diffusion coefficient of 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, 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 preparation of membrane-forming mixtures, containing water-soluble mediators and enzyme. Moreover, it seems possible to manage the diffusion of mediator by changing polymer content in the membrane.

3.2. Mediator Leakage from Chitosan Membranes

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 mediator, 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 1st order reactions:

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

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

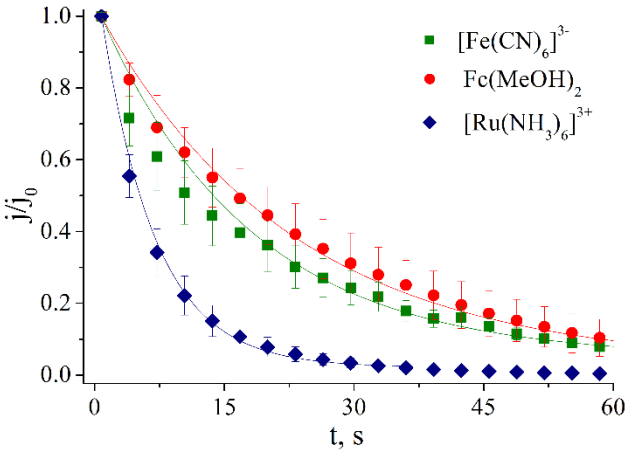

Figure 3. The relative peak current registered upon release of mediators ($[Ru(NH_3)_6]^{3+}$ (◆), $[Fe(CN)_6]^{3-}$ (■), and $Fc(MeOH)_2$ (●) from the membrane deposited on the electrode via drop-casting 5 mM of mediator in 0.1% chitosan membrane-forming mixtures, cyclic voltammetry, 500 mV s^{-1},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

Figure 3 illustrates the decrease of peak current registered upon 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, increase of chitosan content in the membrane-forming mixture hinders the release of hexacyanoferrate (III) from the membrane due to electrostatic binding of negatively charged $[Fe(CN)_6]^{3-}$ to the charged $-NH_3^+$-groups in polymer. In pH 7.4 approximately 6% of chitosan amino groups is protonated. That is why small amount of chitosan deposited on the electrode does not affect the release rate of the mediators.

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

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

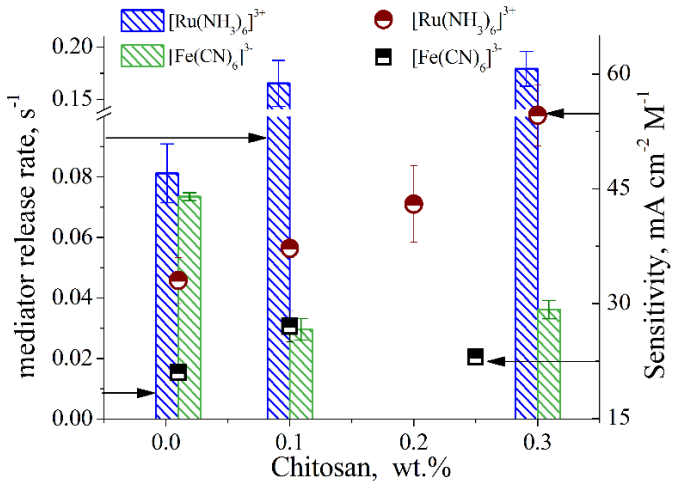

Figure 4. Release rates of $[Ru(NH_3)_6]^{3+}$ and $[Fe(CN)_6]^{3-}$ (blue and green bars respectively) from chitosan membranes (formed by drop-casting of mediator in polymer solutions on the electrode) and sensitivity of the glucose test strips based on GOx (10 mg Ml^{-1}) and 100 mM $[Ru(NH_3)_6]^{3+}$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

3.3. Single-Step Glucose Test Strips Preparation

Glucose test strips were prepared using three-component membrane forming mixtures, containing 100 mM mediator, 10 mg ml^{-1} GOx in 0.01–0.3% chitosan solutions. The current was recorded at 5th 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 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·M^{-1}·cm^{-2}). However, leakage rate is not the only reason. The activity of GOx immobilized in chitosan membranes is also known to improve with increase of chitosan content (up to 0.3% in immobilizing mixtures) [10]. Unfortunately, higher sensitivity is accompanied by 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 mediator, the gain in enzyme activity makes a minor impact on analytical performance. The use of 0.1% instead of 0.01% chitosan in membrane-forming solution for preparation of hexacyanoferrate-based test strips resulted in slightly higher sensitivity (27 ± 2 mA·M^{-1}·cm^{-2}), but the upper detection limit dropped down to 20 mM. Worth to mention, that lower sensitivity of the test strips based on ferrocenedimethanol maybe due to 3-fold lower release rate of this mediator (Table 1).

Table 1. Analytical performance of the glucose test strips.

Mediator	Chitosan Content, %	Sensitivity, mA·M^{-1}·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 mediator in polymer membranes used for enzyme immobilization is an important issue, that should be considered for test strips production. The mediator release rates depend on the density and charge of the polyelectrolyte and mediator. Moreover, polymer content in the membrane-forming mixtures allows to manage sensor performance. Another advantage of single-step approach is simple production of the test strips with an extended upper limit of the linearity. Biosensor response linear range from 1 to 30–50 mM glucose meets the requirements for glucose detection in whole blood.

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