Diffusion Behavior of Analyte Molecules in a Nanoporous Matrix Created from Polystyrene Nanoparticles

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Abstract— This study investigates the diffusion behavior of analyte molecules in a nanoporous matrix of polystyrene nanoparticles. Two approaches, steady-state current and potential step chronoamperometry were employed to calculate diffusion coefficients and compare the effect of nanoporous matrices with different diameter of pores and different method of immobilization of enzyme. Pores were created with close packing of polystyrene nanoparticles. The covalent and cross-linking immobilization were used to immobilize the enzyme. Chronoamperometric measurement was applied to detect analyte. The study found that the presence of nanoporous matrices immobilized on p-lysine affects diffusion. The potential step chronoamperometry method showed the improvement of diffusion. This study provides insight into the diffusion behavior of analyte molecules in nanoporous matrices, which can be useful in optimizing the design of amperometric biosensors.

Keywords—Diffuse coefficient, nanoporous matrix, potential step chronoamperometry, polystyrene nanoparticles, steady-state current

I. INTRODUCTION

The diffusion coefficient is a fundamental property of a material that characterizes how quickly particles or molecules can move through it under the influence of random thermal motions [1]. It is used to model the transport of solutes in porous media, is important in fields such as environmental science, geology, and biomedical engineering. It can be used to model the transport of analyte molecules to the sensing element of the nanobiosensor. The rate at which the analyte molecules diffuse to the sensing element depends on the diffusion coefficient of the molecules and the properties of the surrounding medium. By understanding the diffusion behavior of analyte molecules, the design of the sensing element can be optimized to improve the sensitivity, accuracy, and speed of the nanobiosensor [2]. In recent years, there has been a growing interest in modifying sensors using nanomaterials, particularly in the field of nanopore membranes for biosensors [3]. Nanoporous materials have unique properties, such as high surface area, tunable pore size, and the ability to selectively capture or transport molecules, that make them attractive for use in biosensors. They have numerous potential applications in areas such as medical diagnostics, environmental monitoring, and food safety. A dense planar arrangement of spherical nanoparticles (NPs) can be a viable option for creating nanopores, as the spaces between the NPs form the pores, with their width determined by the NPs’ diameter. This allows to produce a versatile system by selecting the appropriate NPs material and shape. The study presented here utilized nonconductive polystyrene NPs of uniform size to generate porous matrices on electrodes, resulting in a homogenous monolayer. Electrochemical measurements, in the presence of redox mediators, are typically used to quantify the affinity phenomena in these nanopores.

In the past, it was worked with similar systems as this paper [4][5]. We want to test, if diffusion through the pores is an important factor and whether the diffusion coefficients will decrease (a higher diffusion coefficient means better transport of substances). We also wanted to investigate if the sample without NPs, but with enzyme glucose oxidase (GOD) on poly-L-lysine hydrobromide (p-lys) will have the same diffusion. Therefore, we can distinguish only the effect of NPs. Then only the bare electrode with cross-linked enzyme was tested, to determine effect of p-lys separately.

However, it is quite challenging for our electrode arrangement to devise and conduct diffusion coefficient measurements, especially considering the presence of not only ruthenium diffusion as both Hexaamine-ruthenium(II) chloride (Ru(II)) and Hexaamine-ruthenium(III) chloride Ru(III) but also glucose and its products. Because due to enzymatic reaction with glucose the ruthenium mediator Ru(III) was reduced to Ru(II) on electrode as showed on Fig. 2A,C. Two approaches for determining diffuse coefficient were selected, both based on amperometry measurements, namely the Steady-state current approach and the Potential step chronoamperometry approach. These are not new approaches, as they have already been employed as models in other systems [2] [6].

II. THEORY

A. Steady-state current approach

Steady-state current approach can be used for determining the diffusion coefficient of electroactive species in solution. In this approach, a steady-state current is measured as a function of the concentration of the electroactive species at the electrode surface. The modified expression for the steady-state current was used:
\[ I = 4nFcDr_0 \]  
(1)

And were modified to expression for the microelectrodes with a recess depth [2]:
\[ I = \frac{4nFcDr_0}{\frac{4L}{\pi r_0} + 1} \]  
(2)

where \( n \) is the number of transferred electrons, \( F \) is the Faraday constant, \( c \) is concentration, \( D \) diffusion coefficient, \( r_0 \) is the radius of the electrode, and \( L \) is the value of the recess height. We rearranged the equation from [2] to calculate \( D \):
\[ D = \frac{\frac{4L}{\pi Nr_p} + 1}{4nFcN_r} \]  
(3)

where \( N \) is the estimated number of pores and \( r_p \) represent the radius of single pore between NPs. Therefore the \( r_0 = Nr_p \) because it is approximation of all pores in the surface area of electrode. The equation, which can be derived from Fig. 2D, for obtaining \( r_p \) is:
\[ r_p = R \cos 30^\circ - R \]  
(4)

The possibility to approximate \( r_p \) this way has already been proposed in [4]. The \( R \) is the radius of NP. The relation is derived from the assumptions of ideal monolayers formed with NPs arranged closely together. The number of pores is calculated from:
\[ N = \frac{S}{(2R)^2} \]  
(5)

where \( S \) is area of the electrode \( S = 0.378 \times 10^{-6} \text{ m} \).

**B. Potential step chronoamperometry approach**

Second principle to calculate the diffusion coefficient is to use potential step chronoamperometry approach [7][8]. It is a technique used in electrochemistry to study the kinetics of electrode reactions, particularly for studying diffusion-controlled electrode processes. The potential step is applied to an electrode, and the current response of the system is measured as a function of time. The current response is analyzed using the Cottrell equation [6]:
\[ I = \frac{FS\sqrt{D}}{\sqrt{\pi t}} [A]_{\text{bulk}} \]  
(6)

where \( D \) is the diffusion coefficient, \([A]_{\text{bulk}}\) the bulk concentration of species \( A \) in the solution, \( F \) is the Faraday constant and \( S \) the electrode area, \( t \) is the time, we can express the fitting function:
\[ I = \frac{K}{\sqrt{t}} + C \quad \text{K = } \frac{FS\sqrt{D}}{\sqrt{\pi}} [A]_{\text{bulk}} \]  
(7)

The diffusion coefficient was calculated from \( K \):
\[ D = \left( \frac{K\sqrt{\pi}}{FS[A]_{\text{bulk}}} \right)^2 \]  
(8)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
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<tbody>
<tr>
<td>([A]_{\text{bulk}})</td>
<td>the bulk concentration of species ( A ) in the solution</td>
</tr>
<tr>
<td>( c )</td>
<td>concentration</td>
</tr>
<tr>
<td>( D )</td>
<td>diffusion coefficient</td>
</tr>
<tr>
<td>( F )</td>
<td>Faraday constant</td>
</tr>
<tr>
<td>( I )</td>
<td>current</td>
</tr>
<tr>
<td>( L )</td>
<td>value of the recess height</td>
</tr>
<tr>
<td>( n )</td>
<td>number of transferred electrons</td>
</tr>
<tr>
<td>( R )</td>
<td>radius of NP</td>
</tr>
<tr>
<td>( r_0 )</td>
<td>radius of the electrode</td>
</tr>
<tr>
<td>( r_p )</td>
<td>radius of single pore</td>
</tr>
<tr>
<td>( S )</td>
<td>area of the electrode</td>
</tr>
<tr>
<td>( t )</td>
<td>time</td>
</tr>
</tbody>
</table>

**III. METHODS AND MATERIALS**

**A. Chemicals**

Carboxylated polystyrene nanoparticles (PS NPs) of diameter \( \approx 40 \) and \( 80 \) nm were purchased from Magsphere Inc. (U.S.A.). Poly-L-lysine hydrobromide (p-lys), \( N\)-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), \( N\)-Hydroxysuccinimide (NHS), glucose oxidase (GOD) from Aspergillus Niger, bovine serum albumin (BSA), Hexaammineruthenium(III) chloride (98%) (Ru\(^{3+}\)), potassium ferrocyanide and potassium ferricyanide (Fe\(^{2+}\)/Fe\(^{3+}\)), glutaraldehyde solution (GO) (25%), 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (Germany). D-(+)-Glucose monohydrate, KOH, \( \text{H}_2\text{O}_2 \) (30%), isopropyl alcohol was purchased from Penta (Czech Republic).

**B. Aparature and electrodes**

Chronoamperometry was recorded using \( \mu \text{AUTOLAB III / FRA2 (Metrohm Autolab, Netherlands) analyzer, with help of NOVA software. The commercial electrodes in a biamperometric setup (www.printed.cz, Czech Republic) of a pair of two identical gold disk electrodes were used in this work. The electrode has internal diameter 400 µm and there is no separate reference or counter electrode.**
C. Preparation of samples

The commercial electrodes in a biamperometric setup (www.printed.cz, Czech Republic) of a pair of two identical gold disk electrodes were used in this work. The electrode has internal diameter 400 µm and there is no separate reference or counter electrode. The aim was to immobilize polystyrene nanoparticles (PNPs) with negative charge, of diameters approximately 40 nm and 80 nm, onto a thin layer of positively charged poly-L-lysine (p-lys) on a gold electrode. To prepare the electrode, it was first activated by polishing with microcloth and isopropyl alcohol to remove any residual photoresist and passivation layers from the surface. It was then treated with a solution of 0.5 M KOH and 20% H₂O₂ for 10 min. Next, amino groups were introduced onto the gold surface by functionalizing the gold electrode with 50 µg/mL p-lysine in phosphate-buffered saline (PBS; 10 mM, pH 7.4) through adsorption for at least 30 minutes. Following washing and drying steps, and then the solution of 1% w/v PS NPs, dispersed by ultrasound for 20 min, with temperature lower than 35°C, was applied onto the electrode modified with positive charge and left for 40 minutes. Next objective was to immobilize the enzyme. Two different immobilization procedures of the enzyme GOD were compared. In both cases, the enzyme was applied only on one of the two electrodes. The first method involves cross-linking using a bi-functional agent, while the second method involves introducing biomolecules onto the surface of PNPs through covalent binding.

Crosslinking method (Fig. 2C): The mixture of 16 µl GOD (8 mg/ml) and 25 µl BSA (16 mg/ml) in the optimal concentrations to be cross-linked with 2.75 µl 2% glutaraldehyde (GO) solution was prepared. The microliter drop was applied to the PNP's modified electrodes and let dry. The reference sample was created as 33 µl BSA (13 mg/ml) with 2.75 µl 2% glutaraldehyde solution, without GOD. The second reference was the cross-linked GOD enzyme with BSA, but without NPs.

Covalent binding (Fig. 2A,B): Covalent binding of GOD to NPs was initiated by the activation of carboxy-groups of PNPs with a mixture of 5 mM EDC and 5 mM NHS dissolved in MES buffer (pH 6.2) for 15 min. After rinsing the electrode in MES buffer, the GOD (8 mg/ml) solution was applied to the electrode and incubated for 24 hours. Then the electrodes were rinsed in PBS buffer and used for the measurement. The reference sample was created by replacing enzyme GOD with protein BSA (8 mg/ml) and incubated for 24 hours. The second reference was the covalent bounded GOD enzyme to glutaraldehyde 2% (2.75 µl, 2%, 40 min), but without NPs.

IV. RESULTS AND DISCUSSIONS

To determine the diffusion coefficients, it was first necessary to perform amperometry. To get as much information as possible about the sensor, we performed calibration measurements, with gradually increasing glucose concentration (Fig. 3a). It also check correct function of this biosensor. Then, the most significant jumps of amperes were selected for further analysis of the diffusion coefficient. However, it turned out that although cross-linking of the enzyme was successful for these conditions, the same could not be said for covalent binding (Fig. 3b). The covalent bounding had not distinguishable distribution and characteristic only constantly increased. Therefore, only cross-linking samples were usable for this method and following text will deal more with them only.

At first, diffusion coefficient was calculated from equation (3). Current I was used from chronoamperometry as the difference between two currents for different concentration of glucose. As recess height L was used diameter of NPs. Diameters of pores was counted as: 6.2 nm and 12.4 nm. Number of pores (N) were approximated from equation (5) as 2 x 10⁸ for 40 nm NPs and 5 x 10⁹ for 80 nm NPs. Values for r_p counted from equation (4) was estimated as r_p(40) = 3.1 nm and r_p(80) = 6.2 nm. Diffuse coefficient for NPs of cross-linked method GOD bonding was calculated from averaged currents which are in Table II and results are listed in Table III. Numbers in brackets in Table III determine which equation was used to calculate them. Diffuse coefficient for the reference, electrode without NPs with crosslinked enzyme GOD on p-lys, was taken for counting D from equation (1), because there are no polystyrene nanopores. The sample with p-lys were
chosen, because then we can observe just change created by the NPs. Assumption was that D would be lower for samples containing NPs because polystyrene particles shield the electrode, which for counting from equation (3) turn out to be true. According to literature the ruthenium(II) has diffusion coefficient of about

\[ D_{Ru(II)} = 7.9 \times 10^{-10} \frac{m^2}{s} \] [9]

same as ruthenium(III) diffusion coefficient of about

\[ D_{Ru(III)} = 7.9 \times 10^{-10} \frac{m^2}{s} \] [10] and glucose in water

\[ D_G = 6 \times 10^{-10} \frac{m^2}{s} \] [11].

For our experiment the measurable diffusion is affected by traveling of glucose in liquid and by Ru(II) to the electrode not only from Ru(III), but as we see from literature, they diffuse coefficients are very similar, so the difference is neglected. Our values D without NPs are 100x higher, which may be caused by

\[
\text{TABLE III. DIFFUSION COEFFICIENTS [m}²\text{s}^{-1}\text{] OF PLYS+GO AND PLYS+40NPs COVERED ELECTRODES WITH GOD IMMOLIZED BY CROSS-LINKING FROM CALIBRATION CHRONOAMPEROMETRIC MEASUREMENTS. NUMBERS IN BRACHIT SHOWS NUMBER OF EQUATION USED FOR CAUTING.}
\]

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Diffusion coefficients from steady state current</th>
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<tbody>
<tr>
<td></td>
<td>(D_{bare})</td>
</tr>
<tr>
<td>1 mM</td>
<td>(4.4 \times 10^{-4})</td>
</tr>
<tr>
<td>10 mM</td>
<td>(1.54 \times 10^{-7})</td>
</tr>
</tbody>
</table>

the applied voltage, therefore the movement of particles is not free, but it is influenced in direction to the electrode. Secondly, the comparison with the potential step chronoamperometry approach for distinguish diffusion coefficient taken place. For measurement (Fig. 4) were used three different types of electrodes. The bear with cross-linked GOD, but this time without poly-lysine. Then GOD immobilized by the cross-linking method for 40 nm and 80 nm NPs. Covalent bonding was skipped because amperometry does not seem like reasonably sensitive method for this type of biosensor. Therefore, only samples with cross-linked enzyme were tested. The diffusion coefficient was retrieved by fitting the measured curves with equation (7) and then calculated from equation (8). The measured currents after potential increment from 0 mV to 0.5 mV were shown in Fig. 4. The diffusion coefficients are listed in Table IV. We can see that the electrode without a layer has a slightly lower value, but for a higher concentration of glucose, it is much lower. The products of glucose molecules may hinder movement and prevent a faster reaction process. In addition, since there is no complicated nanoterrain on the surface, where a larger amount of enzyme could be captured due to the increased surface area, this leads to a smaller number of ions arriving and transforming on the electrode, and therefore a diffusion coefficient is smaller. Between 40 NPs and 80 NPs, we can observe a difference in that for lower glucose concentrations (1 mM), \(D_{80}\) has twice the value, but at higher glucose concentrations, it is the opposite. It is possible that at low concentrations, 80 NPs benefit from the increased surface area for immobilization, while glucose products do not block their path. However, for higher concentrations, 40 NPs will benefit from a larger mobilization surface and balance out the poorer pore permeability. What is most important is how is it possible that the first and second method give such different trends between NPs and the non-porous electrode? If we used equation (2) instead of (3) (the values given in Table III labeled (2)) \(D\) would result into closer result. That is, if we did not consider all individual pores separately but took it as one surface and only included the height, the result would be closer between the methods. This seems more plausible, especially considering amperometry, where for lower concentrations, the 40 nm sample has lower currents than the other samples, while
for higher concentrations, it is the opposite. That is consistent with equation (2) and potential step method. The equation (3) does not have to be completely wrong, but may be missing some necessary approximation, or our surface is not adequately porous.

![Graph](image)

Fig. 4. Chronoamperometry after increment of potential from 0 to 0.5 mV. Measured separately for 1 mM and 10 mM concentration of glucose. Average of the curves were used to calculate the diffusion coefficient. Obtain only cross-linked samples.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Diffusion coefficients from Cottrell equation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$D_{bare}$</td>
</tr>
<tr>
<td>1 mM</td>
<td>$1.22 \times 10^{-7}$</td>
</tr>
<tr>
<td>10 mM</td>
<td>$1.59 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

### V. CONCLUSION

We tested two different methods for the application of the GOD enzyme for use as an amperometric biosensor with a nanoporous matrix of polystyrene particles. It was found that while there is enough enzyme left on the electrode with cross-linking and we are able to measure the response for individual increases in glucose, for covalent bonding we get a continuous increase in current, but we are not able to measure individual additions, and this increase is only in the nA range. Therefore, further investigation of the diffusion coefficient focused on cross-linking. Using two different methods: steady-state current and potential step chronoamperometry, diffusion coefficients were calculated. In comparison with an empty electrode with cross-linking GOD, we can say that NPs in conjunction with p-lys affect diffusion to the electrode, but in potential step chronoamperometry, it was shown to improve rather than worsen it. This counterintuitive result is probably due to the fact that we used amperometry as a basis on which we built the measurement of incoming Ru ions, but by increasing the surface area for enzyme immobilization, more RuII was necessarily produced, resulting in larger currents being measured. We also compared approximations from the modified expression for steady-state current. When not accounting for individual pores but considering only the height of the matrix, we obtained a result closer to the potential step method.

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### REFERENCES


