Evaluation of the interaction between SARS-CoV-2 spike glycoproteins and the molecularly imprinted polypyrrole
Vilma Ratautaite, Raimonda Boguzaite, Ernestas Brazys, Deivis Plausinaitis, Simonas Ramanavicius, Urte Samukaite-Bubniene, Mikhael Bechelany, Arunas Ramanavicius

To cite this version:
Vilma Ratautaite, Raimonda Boguzaite, Ernestas Brazys, Deivis Plausinaitis, Simonas Ramanavicius, et al.. Evaluation of the interaction between SARS-CoV-2 spike glycoproteins and the molecularly imprinted polypyrrole. Talanta, 2023, 253, pp.123981. 10.1016/j.talanta.2022.123981. hal-03851760

HAL Id: hal-03851760
https://hal.umontpellier.fr/hal-03851760
Submitted on 14 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Evaluation of the interaction between SARS-CoV-2 spike glycoproteins and the molecularly imprinted polypyrrole

Vilma Ratautaite¹,³, Raimonda Boguzaite¹, Ernestas Brazys², Deivis Plausinaitis², Simonas Ramanavicius²,³, Urte Samukaite-Bubniene¹,², Mikhael Bechelany⁴, Arunas Ramanavicius¹,².∗

¹ Department of Nanotechnology, Center for Physical Sciences and Technology, Sauletekio av. 3, Vilnius LT-10257, Lithuania;
² Department of Physical Chemistry, Institute of Chemistry, Faculty of Chemistry and Geosciences, Vilnius University, Naugarduko str. 24, Vilnius LT-03225, Lithuania;
³ Department of Electrochemical Material Science, Center for Physical Sciences and Technology, Sauletekio av. 3, Vilnius LT-10257, Lithuania;
⁴ Institut Européen des Membranes, IEM, UMR 5635, University of Montpellier, CNRS, ENSCM, 34090 Montpellier, France.

* Corresponding author: Prof. habil. dr. Arūnas Ramanavičius (Vilnius University) arunas.ramanavicius@chf.vu.lt

Abstract:
The SARS-CoV-2 spike glycoprotein (SARS-CoV-2-S) was used as the template molecule and polypyrrole (Ppy) was applied as the electro-generated conducting polymer, which was acting as a matrix for the formation of molecular imprints. Two types of Ppy-layers: molecularly imprinted polypyrrole (MIP-Ppy) and non-imprinted polypyrrole (NIP-Ppy) were electrochemically deposited on the working platinum electrode. The performance of electrodes modified by MIP-Ppy and NIP-Ppy layers was evaluated by pulsed amperometric detection (PAD). During PAD experiments through MIP-Ppy and NIP-Ppy layers passed charge was calculated using the integrated Cottrell equation (Anson plot). The interaction between SARS-CoV-2 spike glycoproteins and molecularly imprinted polypyrrole (MIP-Ppy) was assessed by the Anson plot-based calculations. This assessment reveals that SARS-CoV-2-S glycoproteins are interacting with MIP-Ppy more strongly than with NIP-Ppy.

Keywords: Molecularly imprinted polymers (MIPs); Polypyrrole (Ppy); integrated Cottrell equation; Anson plot; SARS-CoV-2 spike glycoprotein.
1. Introduction

Molecularly imprinted polymers (MIPs) are artificially designed systems suitable for the design of highly specific, selective, and sensitive assays. For electrochemical applications, the development of MIPs is based on conducting polymers. The elegant design of the electrochemical MIP manufacturing process and the ability to modify, and ameliorate the parameters of the MIP with dopants have attracted significant attention from scientists. However, the comprehensibility of the interaction principles between the molecule of interest and the imprint in the MIP is still relevant. Molecularly imprinted polymers (MIPs) are used in the recognition process because the way they operate can be described as synthetic analogs to the natural, biological antibody-antigen, or receptor-ligand-based systems. The process of MIP fabrication usually involves such steps as the polymerization of monomers in the presence of a template molecule, extraction of the template molecules after polymerization, and afterward MIP they are ready for application as electrochemical MIP-based sensors [1-3]. During the polymerization, the template molecules vacate an imprint in the polymer, which is a complementary cavity that allows the selective recognition/binding of template molecules.

MIPs can be successfully fabricated for recognition of various types of small molecular weight template molecules [1, 4-7] imprinted in different polymers, like polypyrrole (Ppy) [4, 5, 8], polyaniline [8-10] and others, in purpose to use it with different analytical techniques [4, 11-13]. The challenges and limitations of such MIPs are well discussed in several recently published review articles [2, 3, 14-18]. But among the different template molecules, the imprinting of macromolecules has some special features [14, 19, 20]. Kan et al. [20] identified three limitations that still make it challenging for protein imprinting. In particular, it is difficult to remove the template protein from the bulk polymer matrix after polymerization. Furthermore, organic solvents are often used to produce MIP, although, they are unsuitable for protein synthesis due to the poor solubility of the protein molecule in them and possible transformation processes. In view of this shortage, electrochemical polymerization is a better choice as it can be performed in aqueous solutions. In this case, the environment is less polluted, so it perfectly meets the principles of the green chemistry [21]. Finally, the size and structural complexity of the protein result in a more non-specific binding [20].

Some conducting polymers have been molecularly imprinted with various high molecular mass biomolecules including proteins [22-33], DNA [34, 35], or even bacteria [36-38]. Polypyrrole has previously been molecularly imprinted by virus glycoproteins [33], bovine hemoglobin [20, 39, 40], bovine serum albumin [41], prostate-specific antigen [42],
cardiac troponin-I [43], and cardiac troponin T [44]. It is noteworthy that cyclic voltammetry (CV) [20, 41-44], differential pulse voltammetry (DPV) [20, 39-41, 43, 44], and electrochemical impedance spectroscopy (EIS) [20, 39-41, 43] in presence of redox probe [Fe(CN)₆]³⁻/[Fe(CN)₆]⁴⁻ were the most frequently used electrochemical methods for the evaluation of the MIPs with protein imprints. The MIPs based on polypyrrole with bovine leukemia virus glycoproteins imprints [33] and SARS-CoV-2 spike glycoproteins [45] were evaluated by pulsed amperometric detection (PAD) method in absence of ferrocyanide-ferricyanide system as a redox probe. With reference to the review of Faria et al. [46], such an analytical system should be classified as a type of non-faradaic sensor. In such a case, charging and discharging of the double-layer capacitance are playing the “main role”. At the same time, it is important to keep in mind that by changing the potential, the charging and discharging of the conducting polymers occur. This effect was well discussed by Heinze et al. [47]. The electrochemical charging process of conducting polymers should be described by a sequence of discrete but overlapping redox steps. By changing the potential, there are more effects observed and the change in volume is one of them. These effects are induced by the oxidation and the reduction of the polymer [48-50].

SARS-CoV-2 protein plays a key role in the cell receptor recognition/binding and penetration of the SARS-CoV-2 virus through the cell membrane. Aspects of electrochemical determination methods of proteins employing MIPs are especially interesting due to the recent widespread of the SARS-CoV-2 virus causing the COVID-19 disease. Recently some studies employing MIP-technology for SARS-CoV-2 were published [45, 51-56]. The nucleocapsid, envelope, spike, and membrane proteins of SARS-CoV-2 virus could be used as template macromolecules during the development of MIPs. Among the here mentioned proteins, the spike protein SARS-CoV-2-S plays a key role in the cell receptor recognition/binding and the penetration of SARS-CoV-2 virus through the cell membrane.

In this work, the polypyrrole-based MIP (MIP-Ppy) was developed and applied for the determination of SARS-CoV-2-S spike glycoprotein to examine in detail complex and little-studied advantages/peculiarities of pulsed amperometric detection as the non-faradaic method. The novelty of the article was based on the application of total charge for the evaluation of the interaction between electrode and analyte. This total charge was calculated according to the integrated Cottrell equation and was plotted as it was first demonstrated in articles of F. C. Anson’s research group for studies of reactants adsorption on the electrode [57-59]. In honor of this scientist now the plot of the total charge vs square of time is called the Anson plot. So, the key idea of this work was based on the application of the Anson plots for
elucidation of the interaction between SARS-CoV-2 spike glycoproteins and the molecularly imprinted polypyrrole.

2. Materials and methods

2.1. Chemicals and instrumentation

All chemicals were used as received without further purification. The chemicals were purchased as follows: pyrrole 98% – from Alfa Aesar (Germany), Sulfuric acid (H₂SO₄; 96 %) from Lachner (Czech Republic), nitric acid (HNO₃, 63%), sodium hydroxide (NaOH, 98%), and chloroplatinic acid (H₂PtCl₆, 40% Pt) from Merck (Germany), potassium phosphate (KH₂PO₄, 98%) from Honeywell Riedel-de Haen (Germany), sodium chloride (NaCl, 99,5%), potassium chloride (KCl, 99,5%), and disodium hydrogen phosphate (Na₂HPO₄, 99%) from Carl Roth (Germany). SARS-CoV-2 spike glycoproteins were purchased from UAB Baltymas (Lithuania).

The experiment was performed using potentiostat/galvanostat Metrohm-Autolab model µAutolabIII/FRA2 µ3AUT71079 controlled by NOVA 2.1.3 software (EcoChemie, The Netherlands). All measurements were done in a homemade cell. The total volume of the cell was 250 µL. Three-electrode system consisted of a working electrode (WE) – Pt disk with 1 mm diameter sealed in glass, reference electrode (RE) – Ag/AgCl, and counter electrode (CE) – Pt disk of 2 mm diameter.

2.2. Pretreatment of the working electrode

The working electrode was pretreated according to the procedure described in previous studies [33, 60]. Solutions were degassed with a stream of nitrogen (N₂) before use. According to this procedure: 1) the Pt electrode was rinsed with concentrated HNO₃ solution in an ultrasonic bath for 10 min, 2) then rinsed with water, and 3) polished with alumina paste. Later on, 4) it was rinsed with water again and then 5) with 10 M of NaOH, thereafter – 6) with 5 M of H₂SO₄ in an ultrasonic bath for 5 min. 7) Electrochemical cleaning of the electrode was carried out in 0.5 M H₂SO₄ by cycling the potential 20 times in the range between −100 mV and +1200 mV vs. Ag/AgCl at a sweep rate of 100 mV s⁻¹. The bare electrode surface was indicated by a stable cyclic voltammogram. A layer of ‘platinum black’ was deposited over the working electrode to improve the adhesion of the Ppy layer to the electrode surface [60]. ‘Platinum black’ was deposited from a 5 mM solution of H₂PtCl₆, 0.1 M of KCl by 10 potential cycles in the range between +500 and −400 mV vs. Ag/AgCl at a sweep rate of 10 mV s⁻¹.
2.3. Electrochemical deposition of MIP and NIP layers. Sensor signal evaluation

The electrochemical deposition of the polypyrrole layer was performed in the same electrochemical cell. Non-imprinted polypyrrole (NIP-Ppy) was electrochemically deposited from the polymerization solution containing 0.5 M of pyrrole in PBS with 0.1 M of KCl, pH 7.4. Deposition of molecularly imprinted polypyrrole (MIP-Ppy) was carried out from the polymerization solution containing 0.5 M of pyrrole and 50 μg/mL of SARS-CoV-2 spike glycoprotein in the same PBS with 0.1 M of KCl, pH 7.4. The polymeric layers were formed by a sequence of 20 potential pulses of +950 mV vs. Ag/AgCl for 1 s, between these pulses 0 V vs. Ag/AgCl potential for 10 s was applied [33, 60]. MIP-Ppy or NIP-Ppy modified electrodes were immersed in 0.05 M H₂SO₄ solution for 10 min and so the template molecules were extracted. Next, MIP-Ppy and NIP-Ppy modified electrodes were analyzed with the PAD method: the sequence of 10 potential pulses of +600 mV vs. Ag/AgCl for 2 s, between these pulses 0 V vs. Ag/AgCl was applied for 2 s (Fig. S1 and Fig. S2) [33].
3. Results and discussions

As it was described in the experimental part, the MIP-Ppy and NIP-Ppy modified electrodes were evaluated with the PAD method. The obtained amperograms are presented in figure 1.

![Fig. 1. Pulsed amperometry-based evaluation of MIP-Ppy and NIP-Ppy modified electrodes performed by the potential pulse sequence. Amperograms were obtained at Pt electrode modified: A) with MIP-Ppy modified electrode and B) with NIP-Ppy modified electrode in PBS with 0.1 M of KCl, pH 7.4 in the absence of SARS-CoV-2 spike glycoprotein or in the presence of SARS-CoV-2 spike glycoprotein concentrations from 5 μg/mL up to 25 μg/mL.](image)

The concentration of SARS-CoV-2 spike glycoprotein varied from 0 μg/mL to 25 μg/mL. Figures 1A and B demonstrate the dependence of the amperometric response of MIP-Ppy and NIP-Ppy modified Pt electrodes were incubated in SARS-CoV-2 spike glycoprotein containing PBS solution, pH 7.4. The change in the amperometric response is related to the adsorption of less conductive protein molecules on the MIP-Ppy or NIP-Ppy layer. When SARS-CoV-2 spike glycoprotein concentration in solution increased, the registered amperometric response of both MIP-Ppy and NIP-Ppy-modified Pt electrodes decreased. Before the incubation of the electrode in the SARS-CoV-2 spike glycoprotein-containing solution, higher currents were registered. This effect is determined by the presence of water molecules and electrolyte ions in the places where molecular imprints were formed. After the incubation in the solution containing SARS-CoV-2 spike glycoprotein, the molecules of SARS-CoV-2 spike glycoprotein have replaced the ions of solvent and the electrolyte and thus the current at the potential of +600 mV was decreased. The amperometric response of the last pulses of +600 mV and 0 V
from a 10 potential pulses-based sequence applied to MIP-Ppy and NIP-Ppy modified electrodes was selected for a more detailed evaluation (Fig. 2).

**Fig. 2.** Presented amperometric responses were registered during the last (10th) potential pulse of the applied potential pulse sequence (+600 mV and 0 V potentials), for **A** – MIP-Ppy modified electrode and **B** – NIP-Ppy modified electrode in the absence of SARS-CoV-2 spike glycoprotein and in the presence of SARS-CoV-2 spike glycoprotein from 5 μg/mL up to 25 μg/mL in PBS with 0.1 M of KCl, pH 7.4. The insets (a) represent the change of charge (Q, mC vs. t, s) of the corresponding amperograms.

The relation of the cumulative charge passed and time in Ppy based electrochemical sensors obeys the integrated Cottrell equation (1) [57, 61]:

$$Q = 2nFAC \sqrt{\frac{D}{\pi t}} + Q_{\text{d.l.}} + Q_{\text{ads.}} = k\sqrt{t} + Q_{\text{d.l.}} + Q_{\text{ads.}} \quad (1)$$

where: $Q$ – total charge (C); $n$ – number of electrons; $F$ – Faraday constant (96485 C/mol); $A$ – area of the electrode (cm²); $C$ – concentration (M); $D$ – diffusion coefficient (cm²/s); $t$ – time (s); $Q_{\text{d.l.}}$ – the charge of the electrical double layer; $Q_{\text{ads.}}$ – charge induced by adsorbed ions.

The cumulative charge in the Cottrell equation corresponds to the charge passed associated with redox activity leading to Faradic charges ($Q_F$), charging, and discharging of
electrode-electrolyte double-layer capacitive charges \( (Q_{dl}) \), and charge changes associated with the adsorbed species \( (Q_{ads}) \) [62]. Hence, the plot of \( Q \) vs. \( t^{1/2} \) has a linear correlation with the slope \( k \) and the intercept corresponding to \( Q_{ads} + Q_{dl} \). As it was described previously, the absence of specific adsorption of analytes on the surface of the unmodified mercury electrode was indicated by two straight lines with equal slopes that intersect each other at the \( Q = 0 \) axis [57]. Several studies are appointed to evaluate the adsorption process on the electrode. As was stated in a previous study, [59] the expected effect of the adsorbed analyte is inducing the increase of the intercept, although the slope remains unchanged. In the other two former articles, [59, 63] the adsorption process was evaluated by using mercury and the screen-printed electrodes (printed using a carbon-graphite ink). In these studies, it was demonstrated that the slope is proportional to the concentration and the intercept reflects the increasing adsorption. Confirmation that there is no analyte adsorbing on the electrode is based on the \( Q \) vs. \( t^{1/2} \) plots, which were registered during potential pulses of different polarity, with equal (but with opposite signs) slopes and intercepts [58]. It was stated, that this might usually be taken as good evidence for the absence of reactant (or product) adsorption. Another study was appointed to determine the type of adsorbate (reactant or product) on the electrode and the evidence of reactant or product adsorption was based on changes in slope and intercept [64].

The effect described in this study is incomparably more complex than it was described in here mentioned works [57, 59, 63]. In these studies, a plain electrode without any modification was used. Therefore, the simplified reaction of the analyte on the electrode is possible to describe in several steps: diffusion of the analyte from the solution to the electrode, electrochemical oxidation-reduction reaction, and then diffusion of the reaction products from the electrode to the solution. Analysis of the slopes and intercepts in the Anson plot may be used for the identification of the adsorption either of the analytes or of the reaction products on the electrode. In this study, the Pt electrode was modified with the conducting polymer Ppy that was further imprinted or non-imprinted with glycoprotein. It is noteworthy that by changing the potential, the Ppy layer itself is able to take part in charging and discharging during the electrochemical oxidation and reduction reaction [47]. On the other hand, the evaluation of charge carrier transfer mechanisms between glucose oxidase and organic semiconductors gives serious insights into the potential transfer of charge carriers (holes and electrons) [65]. This makes the analysis of the amperograms complex but still possible to describe in the terms suggested by Anson.
Fig. 3. A and D represent the Anson plots ($Q$, mC vs. $t^{1/2}$, s$^{1/2}$) derived from the amperometric response presented in figure 2 for A – MIP-Ppy modified electrode and D – NIP-Ppy modified electrode. B and E represent the slope values vs. the concentration of SARS-CoV-2 spike glycoprotein (concentration, μg/mL). C and F represent the intercept values vs. the concentration of SARS-CoV-2 spike glycoprotein (μg/mL).
Table 1. Linear regression parameters of the Anson plot ($Q$, mC vs. $t^{1/2}$, s$^{1/2}$) (derived from figure 3) on the MIP-Ppy and NIP-Ppy modified Pt electrodes for the last (10th) pulse of the potential pulse sequence.

| C, μg/mL | y = ax + b | MIP-Ppy modified electrode | NIP-Ppy modified electrode |
|----------|------------|---------------------------|-----------------------------|
|          |            | a  | b  | R² | a  | b  | R² |
| 0        | +600 mV    | 16.35 | 0.46 | 0.99 | 10.26 | 1.22 | 0.99 |
| 0        | 0 V        | -8.09 | -2.87 | 0.99 | -4.17 | -2.22 | 0.99 |
| 5        | +600 mV    | 13.99 | -1.39 | 0.99 | 9.70 | -0.19 | 0.99 |
| 5        | 0 V        | -10.48 | 0.095 | 0.99 | -5.56 | -1.74 | 0.99 |
| 10       | +600 mV    | 9.84 | -2.27 | 0.99 | 7.37 | -1.29 | 0.99 |
| 10       | 0 V        | -8.39 | 1.25 | 0.99 | -5.10 | -0.46 | 0.99 |
| 15       | +600 mV    | 8.61 | -2.22 | 0.99 | 6.83 | -1.32 | 0.99 |
| 15       | 0 V        | -7.57 | 1.40 | 0.99 | -4.97 | -0.20 | 0.99 |
| 20       | +600 mV    | 7.65 | -1.91 | 0.99 | 6.12 | -1.26 | 0.99 |
| 20       | 0 V        | -6.68 | 1.14 | 0.99 | -4.61 | 0.03 | 0.99 |
| 25       | +600 mV    | 6.85 | -1.69 | 0.99 | 5.47 | -1.13 | 0.99 |
| 25       | 0 V        | -5.98 | 1.05 | 0.99 | -4.22 | 0.12 | 0.99 |

The Anson plots are depicted (Figs. 3A, 3D, and Fig. S3) for the data from the amperograms in figure 2. The relationship of $Q$ vs. $t^{1/2}$ was fitted by linear regression, and the parameters of the corresponding linear equations are listed in Table 1. The obtained $R^2$ values indicate that there is a linear correlation in the plot of $Q$ vs. $t^{1/2}$ (Table 1). According to the experimental conditions, it was not expected that oxidation of SARS-CoV-2 spike glycoprotein can occur at the potential of +600 mV. However, the interaction of SARS-CoV-2 spike glycoprotein with carboxyl, carbonyl, and hydroxyl groups complementary arranged in imprinted cavities was playing an important role in the recognition of SARS-CoV-2 spike glycoprotein and the formation of Ppy/ SARS-CoV-2 spike glycoprotein complex, similarly, as it was described for the small molecular weight molecules [66, 67]. Due to the replacement of water molecules by SARS-CoV-2 spike glycoprotein, changes in registered current are observed after the incubation of MIP-Ppy modified electrode in SARS-CoV-2 spike glycoprotein-containing solutions.

Figures 3A (for MIP-Ppy modified electrode) and 3D (for NIP-Ppy modified electrode) demonstrate the plots of $Q$ vs. $t^{1/2}$ of +600 mV and 0 V pulses. The linear dependence of the slope on the concentration of SARS-CoV-2 spike glycoprotein is represented in figures 3B (for MIP-Ppy modified electrode) and 3E (for NIP-Ppy modified electrode). The plots represented
in figure 3 illustrate that slope values are very different for MIP-Ppy and NIP-Ppy modified electrodes under the same experimental conditions. The linear dependence of the slope value on the concentration of the analyte for the MIP-Ppy modified electrode (−0.387 for +600 mV pulse and 0.1303 for 0 mV pulse (Fig. 3B)) is steeper than for the NIP-Ppy modified electrode (−0.2015 for +600 mV pulse and 0.0156 for 0 mV pulse (Fig. 3E)). This confirms that the analyte tends to interact stronger with the MIP-Ppy than with the NIP-Ppy. There is one point falling out of the general trend in the absence of SARS-CoV-2 spike glycoprotein that is still not explained (Fig. 3B and 3E).

A positive value of the intercept was calculated only in the case where MIP-Ppy and NIP-Ppy modified Pt electrodes were incubated in SARS-CoV-2 spike glycoprotein-free PBS solution (Table 1, Fig. 3C and 3F). When the concentration of SARS-CoV-2 spike glycoprotein in the solution was increased, all obtained intercept values became negative. A plausible explanation of the observed effect was given by Plausinaitis et al. [61]. The mentioned work describes the quartz crystal microbalance-based evaluation of the electrochemical formation of an aggregated polypyrrole layer. With reference to some other reports, [68, 69] it was assumed that the pyrrole oxidation process is occurring according to the principles described by heterogeneous kinetics. Here described study is based on the operating principles of the MIP. This means the formation of the complementary cavities in the polymer in the stage of polymerization and the attendance of these cavities in the specific interaction with the analyte during the evaluation step. Thus, molecules adsorbed on the Ppy surface hinder the heterogeneous kinetics of the pyrrole oxidation and reduction reaction.
Fig. 4: Interaction of NIP-Ppy and MIP-Ppy with SARS-CoV-2 spike glycoprotein and anions (PO$_4^{3-}$, HPO$_4^{2-}$, or H$_2$PO$_4^-$ and Cl$^-$). A – NIP-Ppy in a solution containing 0 µg/L of SARS-CoV-2 spike glycoprotein; B – NIP-Ppy in a solution containing >0 µg/L of SARS-CoV-2 spike glycoprotein; C – MIP-Ppy in a solution containing 0 µg/L of SARS-CoV-2 spike glycoprotein; D – MIP-Ppy in a solution containing >0 µg/L of SARS-CoV-2 spike glycoprotein.

Data presented in figures 3B and 3E illustrate that the slope $k$ of the Anson equation, which was calculated according to equation 1, decreases by increasing the concentration of SARS-CoV-2 spike glycoprotein. At electrode potential of +600 mV, this effect is much more distinct in comparison to that at 0 mV potential. The slope $'k'$, which is indicated by the equation 1, is related to (i) the equivalent number of electrons $'n'$, which is transferred during the electrochemical reaction; (ii) electrochemically active area $'A'$; concentration of material $'C'$ and diffusion coefficient $'D'$. In our case, this opposite dependence of slope $'k'$ on the concentration $'C'$ of glycoprotein is related to the decrease of electrochemically active area $'A'$. This conclusion is based on the estimation that values of $n$ and $D$ parameters are constant at all here evaluated concentrations of SARS-CoV-2 spike glycoprotein because the concentration of all other components in the solution and, therefore, physicochemical characteristics (e.g., density and viscosity) are the same. It should be noted that in the case of the MIP-Ppy modified
electrode the dependence of ‘k’ on the concentration of SARS-CoV-2 spike glycoprotein is more significantly expressed in comparison to that of the NIP-Ppy modified electrode. At the first glance, taking into account that the dependence of ‘k’ on the concentration of SARS-CoV-2 spike glycoprotein ‘C’ is linear, it was determined that the dependence of ‘k’ value on C is 1.9 times more expressed in the case MIP-Ppy modified electrode in comparison to that value of NIP-Ppy modified electrode at +600 mV. This fact enables us to conclude that according to the slope the active area of MIP-Ppy \((A_{\text{MIP-Ppy}})\) is 1.9 times larger in comparison to that area of NIP-Ppy \((A_{\text{NIP-Ppy}})\).

A schematic representation of action, which represent results determined by chronoamperometry, is presented in figure 4. As it was discussed above, the decrease of surface charge at higher concentrations of SARS-CoV-2 spike glycoprotein is related to the blocking of MIP-Ppy surface by non-conducting material – SARS-CoV-2 spike glycoprotein. All electrical charge passing during this chronoamperometric experiment is determined by adsorption/desorption of ions on MIP-Ppy or NIP-Ppy layers and by doping/dedoping of Ppy-based layers by anions such as \(\text{PO}_4^{3-}\), \(\text{HPO}_4^{2-}\), \(\text{H}_2\text{PO}_4^-\) and \(\text{Cl}^-\). Therefore, SARS-CoV-2 spike glycoprotein adsorbed on the Ppy surface forms a significant barrier for adsorption/desorption of ions on the MIP-Ppy layer and by doping/dedoping of the MIP-Ppy layer by anions. Figures 4A and 4C well represent results that could be determined by experiment when in solution and on the surface of modified electrode SARS-CoV-2 spike glycoprotein concentration is equal to 0 \(\mu\text{g/L}\). Therefore, in this case, adsorption/desorption of ions on MIP-Ppy or NIP-Ppy layers and doping/dedoping of Ppy-based layers is more intensive in comparison to the MIP-Ppy layer. On the contrary, schematics represented in figures 4B and 4D illustrate the interaction of Ppy-based layers with SARS-CoV-2 spike glycoprotein and, respectively, partial blocking of the Ppy surface.

In this context, according to the Anson plot-based evaluation, a SARS-CoV-2 spike glycoprotein molecule is an analyte that is interacting with MIP-Ppy and nonspecifically adsorbing on NIP-Ppy layers.
Fig. 5. Calibration curves of MIP-Ppy and NIP-Ppy modified electrodes presented as $\Delta Q$ vs logarithm of the concentration of SARS-CoV-2 spike glycoprotein.

Figure 5 represents the calibration plot based on $\Delta Q$ values vs. logarithm of the concentration of SARS-CoV-2 spike glycoprotein in PBS solution, pH 7.4. The calibration plot (Fig. 6B) of the described system has a linear relationship.

$$y = Ax + B$$

The obtained functions of the linear relationships were as follows: $y=24.3599x-11.0234$, with $R^2 = 0.9497$ (for MIP-Ppy modified electrode) and $y=14.3361x-8.4771$ with $R^2 = 0.9591$ (for NIP-Ppy modified electrode). The linear relationship, in the PBS in absence of SARS-CoV-2 spike glycoprotein ($c(SARS-CoV-2-S) = x = 0 \mu g/mL$), represents the lowest change of charge (mC). The charge passed through MIP-Ppy at the initial point was higher than that through NIP-Ppy. This effect is explained by the complementary cavities in the polymer. The presence of the complementary cavities enables the fluent charge transfer after extraction of the SARS-CoV-2 spike glycoprotein from the MIP-Ppy layer. After the MIP-Ppy and NIP-Ppy modified electrodes were re-incubated in the SARS-CoV-2 spike glycoprotein containing PBS, a significant current drop was observed. The change of charge within the MIP-Ppy layer was observed higher than on the NIP-Ppy layer. This means that the MIP-Ppy modified electrode was more sensitive towards SARS-CoV-2 than the NIP-Ppy modified electrode. The registered charge variations of MIP-Ppy and NIP-Ppy modified electrodes at the highest concentrations of SARS-CoV-2 spike glycoprotein ($c(SARS-CoV-2-S) = 25 \mu g/mL$) were very similar. This should indicate that the protein covers the surface of both MIP-Ppy and NIP-Ppy layers, however, differences between MIP-Ppy and NIP-Ppy layers were observed.
The described method applied for the evaluation of the interaction between SARS-CoV-2 spike glycoproteins and the MIP-Ppy modified electrode has significant advantages and most important is those insights about interaction are drawn from PAD results. This means that no additional analytical method is needed for making a conclusion. Therefore, we believe, that the Anson plot is a simple and handy method for quick insight into the adsorption process on the electrode regardless it is unmodified or modified with polymer.

Conclusions
After the MIP-Ppy and NIP-Ppy modified electrodes were incubated for the first time in the SARS-CoV-2 spike glycoprotein containing PBS, a significant current drop was observed. On basis of the total charge vs square of time plots (the Anson plots), the interaction between SARS-CoV-2 spike glycoproteins and the molecularly imprinted polypyrrole was evaluated. Based on the dependence of the Anson plot’s slope values on the concentration of the glycoproteins, we declare that glycoproteins adsorb more strongly on MIP-Ppy than on NIP-Ppy. Moreover, it was concluded, that according to the Anson plot-based evaluation a SARS-CoV-2 spike glycoprotein molecule is an analyte that is interacting with MIP-Ppy and nonspecifically adsorbing on NIP-Ppy. Further, the calibration curve of current values was depicted for glycoprotein concentrations in the range from 0 μg/mL to 25 μg/mL. The calibration plot of the described system has a form of exponential decay. The current drop registered by the MIP-Ppy modified electrode was significantly higher than that determined by the NIP-Ppy modified electrode. That means the MIP-Ppy modified electrode was more sensitive than the NIP-Ppy modified electrode. The current value observed in the calibration curve for the MIP-Ppy modified electrode at the initial point (glycoprotein concentration was 0 μg/mL) was 1.36 times higher in comparison with the NIP-Ppy modified electrode. This phenomenon occurred due to the presence of the complementary cavities in Ppy that were formed after extraction of SARS-CoV-2 spike glycoprotein from MIP-Ppy. This observation can also serve as the validation for the formation of MIP-Ppy.

Declaration of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement:
**Vilma Ratautaite** – Methodology, investigation, data analysis, writing – original draft, writing – review & editing, interpretation of data;

**Raimonda Boguzaite** – Methodology, investigation, data analysis, writing – original draft, interpretation of data, data analysis;

**Ernestas Brazys** – Methodology, investigation, data analysis, writing – original draft, interpretation of data, data analysis;

**Deivis Plausinaitis** – data analysis, interpretation of data, writing – review & editing;

**Simonas Ramanavicius** – Writing – review & editing, data analysis, interpretation of data;

**Urte Samukaite-Bubniene** – Writing – review & editing; data analysis, interpretation of data;

**Mikhael Bechelany** – Writing - review & editing;

**Arunas Ramanavicius** – Interpretation of data, data analysis, supervision, conceptualization, writing – review & editing, funding acquisition.

**Acknowledgment**

This project has received funding from the Research Council of Lithuania (LMTLT), GILIBERT 2021 program agreement No S-LZ-21-4 and was co-founded by Campus France grant No. 46593RA (PHC GILIBERT 2021).

**References:**

[1] V. Ratautaite, U. Samukaite-Bubniene, D. Plausinaitis, R. Boguzaite, D. Balcunius, A. Ramanaviciene, G. Neunert, A. Ramanavicius, Molecular Imprinting Technology for Determination of Uric Acid, Int. J. Mol. Sci., 22 (2021) 5032. [https://doi.org/10.3390/ijms22095032](https://doi.org/10.3390/ijms22095032)

[2] S. Ramanavicius, A. Jagminas, A. Ramanavicius, Advances in Molecularly Imprinted Polymers Based Affinity Sensors (Review), Polymers (Basel), 13 (2021) 974. [https://doi.org/10.3390/polym13060974](https://doi.org/10.3390/polym13060974)

[3] S. Ramanavicius, A. Ramanavicius, Conducting Polymers in the Design of Biosensors and Biofuel Cells, Polymers, 13 (2021) 49. [https://doi.org/10.3390/polym13010049](https://doi.org/10.3390/polym13010049)

[4] V. Ratautaite, S.D. Janssens, K. Haenen, M. Nesládek, A. Ramanaviciene, I. Baleviciute, A. Ramanavicius, Molecularly Imprinted Polypyrrole Based Impedimentric Sensor for Theophylline Determination, Electrochimica Acta, 130 (2014) 361-367. [https://doi.org/10.1016/j.electacta.2014.03.035](https://doi.org/10.1016/j.electacta.2014.03.035)

[5] V. Ratautaite, M. Nesladek, A. Ramanaviciene, I. Baleviciute, A. Ramanavicius, Evaluation of Histamine Imprinted Polypyrrole Deposited on Boron Doped Nanocrystalline Diamond, Electroanalysis, 26 (2014) 2458–2464. [https://doi.org/10.1002/elan.201400294](https://doi.org/10.1002/elan.201400294)

[6] V. Ratautaite, D. Plausinaitis, I. Baleviciute, L. Mikoliunaite, A. Ramanaviciene, A. Ramanavicius, Characterization of caffeine-imprinted polypyrrole by a quartz crystal microbalance and electrochemical impedance spectroscopy, Sens. Actuat. B-Chem., 212 (2015) 63-71. [https://doi.org/10.1016/j.snb.2015.01.109](https://doi.org/10.1016/j.snb.2015.01.109)

[7] M. Peeters, F.J. Troost, B. van Grinsven, F. Horemans, J. Alenus, M.S. Murib, D. Keszthelyi, A. Ethirajan, R. Thoelen, T.J. Cleij, P. Wagner, MIP-based biomimetic sensor for the electronic
detection of serotonin in human blood plasma, Sens. Actuat. B-Chem., 171–172 (2012) 602-610. https://doi.org/10.1016/j.snb.2012.05.040
[8] G. Bagdžiūnas, Theoretical design of molecularly imprinted polymers based on polyaniline and polypyrrole for detection of tryptophan, Mol. Syst. Des. Eng., 5 (2020) 1504-1512 https://doi.org/10.1039/D0MSE0089B
[9] A.K. Roy, N.V. S, C. Dhand, B.D. Malhotra, Molecularly imprinted polyaniline film for ascorbic acid detection, Journal of Molecular Recognition, 24 (2011) 700-706. https://doi.org/10.1002/jmr.1104
[10] Y. Kong, J.H. Ni, W.C. Wang, Z.D. Chen, Enantioselective recognition of amino acids based on molecularly imprinted polyaniline electrode column, Electrochimica Acta, 56 (2011) 4070-4074. https://doi.org/10.1016/j.electacta.2011.01.120
[11] Z. Mazouz, S. Rahali, N. Fourati, C. Zerrouki, N. Aloui, M. Seydou, M.M. Chehimi, A. Othmane, R. Kalfat, Highly Selective Polypyrrole MIP-Based Gravimetric and Electrochemical Sensors for Picomolar Detection of Glyphosate, Sensors (Basel), 17 (2017) 2586. https://doi.org/10.3390/s17112586
[12] J.M. Kim, J.C. Yang, J.Y. Park, Quartz crystal microbalance (QCM) gravimetric sensing of theophylline via molecularly imprinted microporous polypyrrole copolymers, Sens. Actuat. B-Chem., 206 (2015) 50-55. https://doi.org/10.1016/j.snb.2014.09.047
[13] D. Plausinaitis, L. Sinkevicius, U. Samukaiite-Bubniene, V. Ratautaite, A. Ramanavicius, Evaluation of Electrochemical Quartz Microbalance Based Sensor Modified by Uric Acid-imprinted Polypyrrole, Talanta, 220 (2020) 121414. https://doi.org/10.1016/j.talanta.2020.121414
[14] R.D. Crapnell, N.C. Dempsey-Hibbert, M. Peeters, A. Tridente, C.E. Banks, Molecularly imprinted polymer based electrochemical biosensors: Overcoming the challenges of detecting vital biomarkers and speeding up diagnosis, Talanta Open, 2 (2020) 100018. https://doi.org/10.1016/j.talo.2020.100018
[15] J.W. Lowdon, H. Diliën, P. Singla, M. Peeters, T.J. Cleij, B. van Grinsven, K. Eersels, MIPs for commercial application in low-cost sensors and assays – An overview of the current status quo, Sens. Actuat. B-Chem., 325 (2020) 128973. https://doi.org/10.1016/j.snb.2020.128973
[16] A. Yarman, F.W. Scheller, How Reliable Is the Electrochemical Readout of MIP Sensors?, Sensors (Basel), 20 (2020) 2677. https://doi.org/10.3390/s20092677
[17] E.N. Ndunda, Molecularly imprinted polymers—A closer look at the control polymer used in determining the imprinting effect: A mini review, Journal of Molecular Recognition, 33 (2020) e2855. http://doi.org/10.1002/jmr.2855
[18] P.S. Sharma, A. Garcia-Cruz, M. Cieplak, K.R. Noworyta, W. Kutner, ‘Gate effect’ in molecularly imprinted polymers: the current state of understanding, Curr. Opin. Electrochem., 16 (2019) 50-56. https://doi.org/10.1016/j.coelec.2019.04.020
[19] J. Erdössy, V. Horváth, A. Yarman, F.W. Scheller, R.E. Gyurcsányi, Electrosynthesized molecularly imprinted polymers for protein recognition, TRAC-Trends Anal. Chem., 79 (2016) 179-190. https://doi.org/10.1016/j.trac.2015.12.018
[20] X. Kan, Z. Xing, A. Zhu, Z. Zhao, G. Xu, C. Li, H. Zhou, Molecularly imprinted polymers based electrochemical sensor for bovine hemoglobin recognition, Sens. Actuat. B-Chem., 168 (2012) 395-401. https://doi.org/10.1016/j.snb.2012.04.043
[21] R. Viveiros, S. Rebocho, T. Casimiro, Green Strategies for Molecularly Imprinted Polymer Development, Polymers (Basel), 10 (2018) 306. https://doi.org/10.3390/polym10030306
[22] Q. Zeng, X. Huang, M. Ma, A molecularly imprinted electrochemical sensor based on polypyrrole/carbon nanotubes composite for the detection of S-ovalbumin in egg white, International Journal of Electrochemical Science, 12 (2017) 3965-3981. https://doi.org/10.20964/2017.05.61
[38] Z. Iskierko, P.S. Sharma, K. Bartold, A. Pietrzyk-Le, K. Noworyta, W. Kutner, Molecularly imprinted polymers for separating and sensing of macromolecular compounds and microorganisms, Biotechnol. Adv., 34 (2016) 30-46. https://doi.org/10.1016/j.biotechadv.2015.12.002

[39] Z. Wang, F. Li, J. Xia, L. Xia, F. Zhang, S. Bi, G. Shi, Y. Xia, J. Liu, Y. Li, L. Xia, An ionic liquid-modified graphene based molecular imprinting electrochemical sensor for sensitive detection of bovine hemoglobin, Biosensors and Bioelectronics, 61 (2014) 391-396. https://doi.org/10.1016/j.bios.2014.05.043

[40] L. Li, L. Yang, Z. Xing, X. Lu, X. Kan, Surface molecularly imprinted polymers-based electrochemical sensor for bovine hemoglobin recognition, Analyst, 138 (2013) 6962-6968. https://doi.org/10.1039/C3AN01435E

[41] H.-J. Chen, Z.-H. Zhang, L.-J. Luo, S.-Z. Yao, Surface-imprinted chitosan-coated magnetic nanoparticles modified multi-walled carbon nanotubes biosensor for detection of bovine serum albumin, Sens. Actuat. B-Chem., 163 (2012) 76-83. https://doi.org/10.1016/j.snb.2012.01.010

[42] Z. Mazouz, M. Mokni, N. Fourati, C. Zerrouki, F. Barbault, M. Seydou, R. Kalfat, N. Yaakoubi, A. Omezzine, A. Boulema, A. Othmane, Computational approach and electrochemical measurements for protein detection with MIP-based sensor, Biosensors and Bioelectronics, 151 (2020) 111978. https://doi.org/10.1016/j.bios.2019.111978

[43] M.L. Yola, N. Atar, Development of cardiac troponin-I biosensor based on boron nitride quantum dots including molecularly imprinted polymer, Biosensors and Bioelectronics, 126 (2019) 418-424. https://doi.org/10.1016/j.bios.2018.11.016

[44] B.V.M. Silva, B.A.G. Rodríguez, G.F. Sales, M.D.P.T. Sotomayor, R.F. Dutra, An ultrasensitive human cardiac troponin T graphene screen-printed electrode based on electropolymerized-molecularly imprinted conducting polymer, Biosensors and Bioelectronics, 77 (2016) 978-985. https://doi.org/10.1016/j.bios.2015.10.068

[45] V. Ratautaite, R. Boguzaite, E. Brazys, A. Ramanaviciene, E. Ciplys, M. Juozapaitis, R. Slibinskas, M. Bechelany, A. Ramanavicius, Molecularly Imprinted Polypyrrole based Sensor for the Detection of SARS-CoV-2 Spike Glycoprotein, Electrochimica Acta, 403 (2022) 139581. https://doi.org/10.1016/j.electacta.2021.139581

[46] R.A. Dorledo de Faria, L.G. Dias Heneine, T. Matencio, Y. Messaddeq, Faradaic and non-faradaic electrochemical impedance spectroscopy as transduction techniques for sensing applications, Int. J. Bios. Bioelectron, 5 (2019) 29-31. https://doi.org/10.15406/ijbsbe.2019.05.00148

[47] J. Heinze, B.A. Frontana-Uribe, S. Ludwigs, Electrochemistry of Conducting Polymers— Persistent Models and New Concepts, Chemical Reviews, 110 (2010) 4724-4771. https://doi.org/10.1021/cr900226k

[48] M.F. Suárez, R.G. Compton, In situ atomic force microscopy study of polypyrrole synthesis and the volume changes induced by oxidation and reduction of the polymer, Journal of Electroanalytical Chemistry, 462 (1999) 211-221. https://doi.org/10.1016/S0022-0728(98)00414-8

[49] M.R. Gandhi, P. Murray, G.M. Spinks, G.G. Wallace, Mechanism of electromechanical actuation in polypyrrole, Synthetic Metals, 73 (1995) 247-256. https://doi.org/10.1016/0379-6779(95)80022-0

[50] T.F. Otero, E. Angulo, Oxidation-reduction of polypyrrole films. Kinetics, structural model and applications, Solid State Ion., 63-65 (1993) 803-809. https://doi.org/10.1016/0167-2738(93)90200-M

[51] O.I. Parisi, M. Dattilo, F. Patitucci, R. Malivindi, V. Pezzi, I. Perrotta, M. Ruffo, F. Amone, F. Puoci, “Monoclonal-type” plastic antibodies for SARS-CoV-2 based on Molecularly Imprinted Polymers, bioRxiv, (2020) 2020.05.28.120709. http://doi.org/10.1101/2020.05.28.120709
[52] A. Raziq, A. Kidakova, R. Boroznjak, J. Reut, A. Öpik, V. Syritski, Development of a portable MIP-based electrochemical sensor for detection of SARS-CoV-2 antigen, Biosensors and Bioelectronics, 178 (2021) 113029. https://doi.org/10.1016/j.bios.2021.113029
[53] M.A. Tabrizi, J.P. Fernández-Blázquez, D.M. Medina, P. Acedo, An ultrasensitive molecularly imprinted polymer-based electrochemical sensor for the determination of SARS-CoV-2-RBD by using macroporous gold screen-printed electrode, Biosensors and Bioelectronics, 196 (2021) 113729. https://doi.org/10.1016/j.bios.2021.113729
[54] A.G. Ayankojo, R. Boroznjak, J. Reut, A. Öpik, V. Syritski, Molecularly imprinted polymer based electrochemical sensor for quantitative detection of SARS-CoV-2 spike protein, Sens. Actuat. B-Chem., 353 (2022) 131160. https://doi.org/10.1016/j.snb.2021.131160
[55] Z. Bognár, E. Supala, A. Yarman, X. Zhang, F.F. Bier, F. Scheller, R.E. Gyurcsányi, Peptide epitope-imprinted polymer microarrays for selective protein recognition. Application for SARS-CoV-2 RBD protein, Chem. Sci., 13 (2022) 1263-1269. https://doi.org/10.1039/D1SC04502D
[56] S.A. Hashemi, S. Bahrani, S.M. Mousavi, N. Omidifar, N.G.G. Behbahan, M. Arjmand, S. Ramakrishna, K.B. Lankarani, M. Moghadami, M. Firoozsani, Graphene - Based Femtogram - Level Sensitive Molecularly Imprinted Polymer of SARS - CoV - 2, Adv. Mater. Interfaces, 8 (2021) 2101466. https://doi.org/10.1002/admi.202101466
[57] F.C. Anson, Innovations in the Study of Adsorbed Reactants by Chronocoulometry, Anal. Chem., 38 (1966) 54-57. https://doi.org/10.1021/ac60233a014
[58] F.C. Anson, R.A. Osteryoung, Chronocoulometry: A convenient, rapid and reliable technique for detection and determination of adsorbed reactants, Journal of Chemical Education, 60 (1983) 293. https://doi.org/10.1021/ed060p293
[59] F.C. Anson, J.H. Christie, R.A. Osteryoung, A study of the adsorption of cadmium(II) on mercury from thiocyanate solutions by double potential-step chronocoulometry, J. Electroanal. Chem. Interf. Electrochem., 13 (1967) 343-353. https://doi.org/10.1016/0022-0728(67)80037-8
[60] A. Ramanavicius, Y. Oztekin, A. Ramanaviciene, Electrochemical formation of polypyrrole-based layer for immunosensor design, Sens. Actuat. B-Chem., 197 (2014) 237-243. https://doi.org/10.1016/j.snrb.2014.02.072
[61] D. Plausinaitys, V. Ratautaite, L. Mikoliunaite, L. Sinkevicius, A. Ramanaviciene, A. Ramanavicius, Quartz Microbalance-Based Evaluation of the Electrochemical Formation of an Aggregated Polypyrrole Particle-Based Layer, Langmuir, 31 (2015) 3186-3193. https://doi.org/10.1021/la504340u
[62] K. Zhou, H. Wang, Y. Zhang, J. Liu, H. Yan, An Advanced Technique to Evaluate the Electrochromic Performances of NiO Films by Multi-Cycle Double-Step Potential Chronocoulometry, Journal of The Electrochemical Society, 163 (2016) H1033-H1040. https://doi.org/10.1149/2.1011610jes
[63] A. Garcia-Miranda Ferrari, C.W. Foster, P.J. Kelly, D.A.C. Brownson, C.E. Banks, Determination of the Electrochemical Area of Screen-Printed Electrochemical Sensing Platforms, Biosensors (Basel), 8 (2018) 53. https://doi.org/10.3390/bios8020053
[64] J. Puy, M. Pla, F. Mas, F. Sanz, Adsorption in double potential step chronocoulometry, J. Electroanal. Chem. Interf. Electrochem., 241 (1988) 89-104. https://doi.org/10.1016/0022-0728(88)85118-0
[65] G. Bağdžiūnas, A. Ramanavicius, Towards direct enzyme wiring: a theoretical investigation of charge carrier transfer mechanisms between glucose oxidase and organic semiconductors, Phys. Chem. Chem. Phys., 21 (2019) 2968-2976. https://doi.org/10.1039/C8CP07233G
[66] V. Syritski, J. Reut, A. Menaker, R.E. Gyurcsányi, A. Öpik, Electrosynthesized molecularly imprinted polypyrrole films for enantioselective recognition of l-aspartic acid, Electrochimica Acta, 53 (2008) 2729-2736. https://doi.org/10.1016/j.electacta.2007.10.032

[67] T.-C. Tsai, H.-Z. Han, C.-C. Cheng, L.-C. Chen, H.-C. Chang, J.-J.J. Chen, Modification of platinum microelectrode with molecularly imprinted over-oxidized polypyrrole for dopamine measurement in rat striatum, Sens. Actuat. B-Chem., 171-172 (2012) 93-101. https://doi.org/10.1016/j.snb.2011.07.052

[68] J.H. Christie, G. Laurer, R.A. Osteryoung, Measurement of charge passed following application of a potential step-application to the study of electrode reactions and adsorption, J. Electroanal. Chem., 7 (1964) 60-72. https://doi.org/10.1016/0022-0728(64)80005-X

[69] J.H. Christie, G. Lauer, R.A. Osteryoung, F.C. Anson, Determination of Charge Passed Following Application of Potential Step in Study of Electrode Processes, Anal. Chem., 35 (1963) 1979-1979. https://doi.org/10.1021/ac60205a003