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Short communication

# Measuring the impact on impedance spectroscopy of pseudo-reference electrode accumulations

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ABSTRACT

Silver is widely used on screen-printed electrodes (SPEs) to provide a reference potential for electrochemical measurements such as impedance spectroscopy (EIS). The redox activity between silver and electrolyte in SPE-based experiments is rarely reported in detail, despite the critical reliance of potentiometric measurement accuracy on the predictability of reactions at reference electrodes. In this work, potassium ferrous cyanides typically used as electrolyte components were shown to adsorb to the silver surface when applied to SPEs. EIS was performed in a two-electrode configuration to demonstrate redox instability at the silver electrode, highlighting the need for evaluation of similar systems to prevent reference potential drift caused by side reaction depositions.

## 1. Introduction

Reference electrodes are necessary for electrochemical equipment to detect and generate relevant potentials [1]. Unlike a true reference electrode, a pseudo-reference electrode (pRE) is not chemically isolated from other electrodes, allowing soluble surface reaction products to diffuse into electrolyte [2]. This risk of side reactions is inherent to the design of many SPE products, where it may be outweighed by practical factors such as size and portability [3]. The specific reactions originating surface redox products can be determined by thoroughly characterizing an electrochemical system employing a pRE [4], but such an investigation may be complex in the case of multiple electrode materials [5].

To simplify electrochemical processes, some EIS applications employ a two-electrode system, which can be achieved in conventional electrochemical equipment by connecting the reference electrode (RE) directly to the counter electrode (CE) [6]. In this configuration, the real-time voltage output by the potentiostat to the CE also drives the reference potential.

One disadvantage of a two-electrode system is ambiguity of working electrode potential, precluding its usefulness in most current vs. voltage measurements [7]. In EIS, potential at the working electrode (WE) surface must remain near redox equilibrium to produce valid impedance results in the system's linear range [8]. This potential can be predicted in a system with known electrode characteristics at redox equilibrium. Provided that stability and reversibility exists, while the WE potential is symmetrical to the known sinusoidal signal at the CE, output current analysis is valid in a two-electrode cell [9]. In contrast, degradation or instability at the RE may induce drifting potential when using a 3-electrode cell [10,11].

In electrochemical biosensing, biological test samples vary in conductivity and ion composition, which can compromise the reliability of electrochemical analysis unless replaced before measurement with a standard electrolyte [12]. These electrolytes often contain an electroactive species, or “redox probe”, to promote redox activity at the WE surface, which is in addition to a supporting electrolyte to conduct current through the cell. The concentration of each electrolyte component must be standardized to establish known background electrode redox activity and consequent potential [13].

Ferrocyanide, $[Fe(CN)_6]^{4-}$, and/or ferricyanide, $[Fe(CN)_6]^{3-}$ are commonly used as a ferro/ferricyanide redox probe for efficient heterogeneous charge transfer across electrodes [14–16]. In immuno-biosensing applications phosphate buffered saline (PBS), which contains both potassium chloride (KCl) and sodium chloride (NaCl), is used as the supporting electrolyte as it simultaneously stabilizes the biological elements (such as antibodies) in the sensing system. Both these redox probe and supporting electrolyte solutions contain compounds that can potentially react with materials at the electrode surfaces.

Adsorption or dissolution of reaction products could also result in reduced stability of the system [17] which could then have a negative

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impact on EIS measurements. In this work we characterize Ag pRE surface changes following EIS measurements to determine any reactions occurring on the pRE silver surface, together with experiments aiming at quantifying the impact of silver surface changes on the reliability of electrochemical measurements.

## 2. Materials and methods

### 2.1. Consumables and equipment

Screen-printed electrodes (SPEs) with silver pRE, carbon WE and CE, were procured from Dropsens (DRP-110). Potassium hexacyanoferrate (II) trihydrate (ferrocyanide), Potassium hexacyanoferrate (III) (ferricyanide), hexaammineruthenium (III) chloride and PBS sachets, 0.0027 M KCl and 0.137 M NaCl at pH 7.4, were purchased from Sigma Aldrich. Deionized water from a Millipore purification system was used for rinsing and as PBS solvent.

Electrolyte solution was prepared in PBS buffer by combining 5.0 mM of $K_3[Fe(CN)_6]$ and 5.0 mM of $K_4[Fe(CN)_6]$. Containers were shielded from light when not in use by wrapping in aluminum foil. Alternative electrolyte solution was also prepared in PBS, by dissolving 10.0 mM of $[Ru(NH_3)_6]Cl_3$.

A PGSTAT302N workstation, FRA32M frequency response module, boxed connector DRP-DSC and Nova 2.0 interface software were all supplied by Metrohm UK.

### 2.2. Electrode exposure

To determine whether reactions occurred spontaneously, three SPEs were exposed to 100 µL of either PBS, electrolyte solution or alternative electrolyte solution. After two hours in a temperature and humidity-controlled environment, electrodes were rinsed with deionized water and analyzed.

### 2.3. Scanning electron microscopy (SEM)

SEM was conducted using a Hitachi High-Technologies S-4800 SEM, applying 10.0 kV at 10.0 µA, at 10,000× magnification. An Oxford Instruments X-Max probe provided energy-dispersive x-ray (EDX) spectra with current set to 15 µA.

Qualitative data of the morphology and topography was obtained by SEM on three areas across the Ag electrode of PBS-exposed SPEs, on ten regions across the Ag electrode of electrolyte-exposed SPEs and across three regions across alternative electrolyte-exposed SPEs. EDX analysis was repeated for three regions on Ag electrodes. A pristine, unexposed SPE was analyzed with the same parameters, as a reference control.

### 2.4. Raman spectroscopy

Raman spectroscopy data was obtained using a Renishaw InVia Raman Spectrometer at 20× magnification, with incident energy applied at 532 nm and 10% power. A pristine silver pRE was compared to a silver pRE subjected to repeated EIS in ferrocyanide/ferricyanide electrolyte (total 27 measurements over four months). Spectral capture repeats were performed on three regions across each pRE.

### 2.5. EIS procedure

Hardware was connected following manufacturer's recommendations for 3-electrode and standard 2-electrode tests (Fig. 1a and b). An alternative 2-electrode configuration consisted of connecting the cables for both CE and RE to the WE lead of the SPE and the WE cable to the lead for the silver electrode (Fig. 1c). A potentiometric impedance procedure applied a $0.01V_{RMS}$ amplitude wave at 25 frequencies logarithmically distributed between 0.05 Hz and 1 kHz, with a 5 s delay

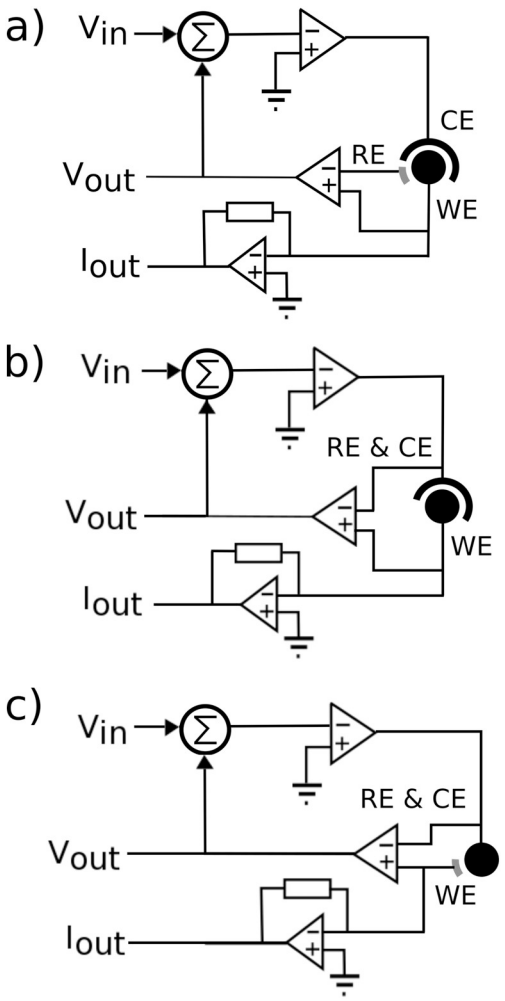


**Fig. 1.** Circuit diagrams of potentiostat connections to SPEs, based on supplier's simplified line diagram [6]; a) standard 3-electrode cell; b) standard 2-electrode cell; and c) alternative 2-electrode cell.

between "cell on" and start of the signal input. All other impedance measurement parameters were left in default settings (max integration time 0.125 s, max integration cycles 1, lowest bandwidth high stability, number of cycles to reach steady state 10, max time to reach steady state 1 s, minimum fraction of a cycle 0, automatic amplitude correction, automatic resolution correction iterative, minimum resolution 34%, maximum amount of re-measurements 25, automatic current ranging). The procedure added an open-circuit potential (OCP) to the input sinusoid, which was recorded following a 120 s OCP measurement, conducted before each set of 3 EIS measurements.

The SPE was connected to the measurement instrument before 100 µL of electrolyte was pipetted onto its surface, covering all electrodes. SPEs were tested in standard 3-electrode, standard 2-electrode and alternative 2-electrode configuration.

## 3. Results and discussion

To assess changes caused by deposition/reaction of redox probe components on pRE surface, we first determined any topographical alterations to the pRE surface by high resolution SEM. While the Ag topography appeared unchanged following PBS and hexaammineruthenium chloride exposure, ferrocyanide/ferricyanide electrolyte exposure consistently resulted in transformation of the surface from smooth swellings to a sharp, crystalline morphology (Fig. 2a). Similar morphology has been previously observed when silver composites

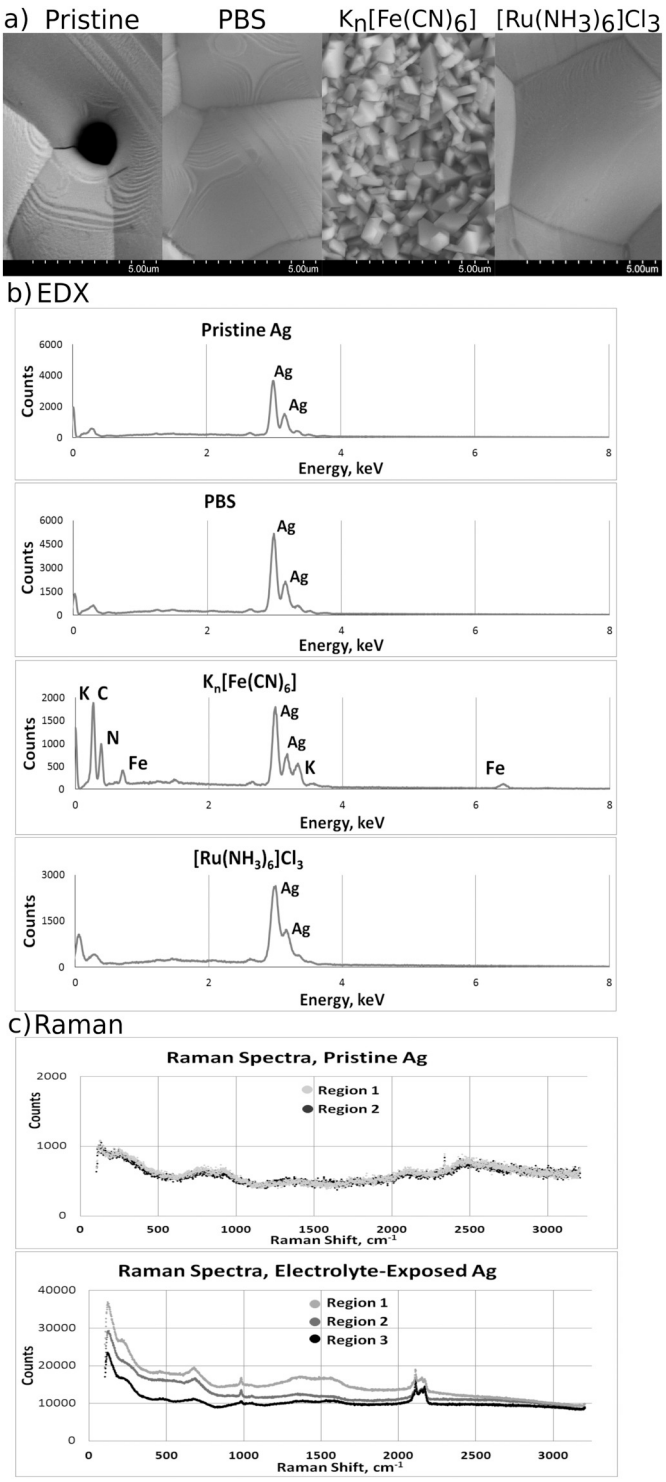


**Fig. 2.** Surface characterization of silver pREs; a) representative SEM imagery; b) EDX spectra; and c) Raman spectra.

electrodeposited onto electrode surfaces were characterized using SEM [18,19]. These observations, and the absence of any visible surface change when salt buffers were used, suggest that it is the ferrocyanide/ferricyanide redox probes that are responsible for the topological changes.

EDX spectra identified potassium and carbon peaks on ferrocyanide/ferricyanide exposed pREs, which were absent from silver exposed to $[Ru(NH_3)_6]Cl_3$ and PBS (Fig. 2b). The presence of potassium was likely the result of liberated redox probe cations, since PBS exposure alone caused no potassium peak. The carbon peak would be expected for adsorbed cyanides (CN), however, while nitrogen peaks might be expected, measured values did not exceed noise levels. Despite this their presence even at low levels suggested the presence of CN, and is consistent with previous reports where nitrogen identification by EDX has been shown to be disproportionally affected by noise due to binding arrangements within crystalline cyanide formation on silver surfaces [20,21].

Raman scans for both ferrocyanide/ferricyanide exposed and pristine pREs contained peaks at 100–300 $cm^{-1}$ (Fig. 2c) as is expected for any silver composite [22]. In the electrolyte-exposed pRE, a cluster of peaks was also detected at 2100–2200 $cm^{-1}$ corresponding to cyanide deposition which is seen in the region of 2130 $cm^{-1}$ [23–26]. No such peaks were found on the unexposed electrode, suggesting that the deposition of cyanide products occurred on electrodes exposed to ferrocyanide/ferricyanide electrolyte due to CN in the electrolyte solution.

These data support the notion of spontaneous adsorption of cyanide-based redox probe side reaction compounds onto pREs. Since adsorption of reaction products on silver pREs could block redox-active area, we predicted that this phenomenon could affect the potential stability of the electrode.

To determine whether the adsorption measurably impacts redox efficiency at the pRE, a 2-electrode cell was tested, using the silver pRE as the WE and the larger carbon electrode as the CE and RE. Due to the smaller surface area of the silver electrode (1/6 length of the carbon electrode designed as CE), higher starting impedance values were expected, however these should not change between measurements if CN compounds adsorption had a negligible impact on electrode redox activity.

Alternative 2-electrode systems differed from standard 2-electrode systems by applying the silver electrode as the WE and the circular carbon electrode as both CE and RE (Fig. 1). In contrast to 3-electrode and standard 2-electrode measurements, real and imaginary impedance progressively increased after repeated measurements in ferrocyanide/ferricyanide electrolyte with the alternative 2-electrode arrangement (Fig. 3a). While the decrease in the mean imaginary impedance was not significant for the standard 2- and 3-electrode systems (56 Ω and 31 Ω respectively, $p > 0.05$), it increased significantly in the alternative 2-electrode system (9545 Ω, $p < 0.05$). This increase in measured impedance might be caused by a less redox-active compound progressively altering interfacial charge transfer at the silver surface.

To verify the link between ferrocyanide/ferricyanide redox probes and impedance increases in the alternative 2-electrode arrangement, all EIS measurements were repeated with new SPEs in either alternative electrolyte or PBS alone. Measurements in PBS with and without $[Ru(NH_3)_6]Cl_3$ produced higher starting impedances and greater inter-repeat variation, reflecting lower efficiency of charge transfer between the carbon electrode and these ionic species (Fig. 3b & c). Nonetheless, higher inter-repeat stability of impedance was observed compared to the ferrocyanide/ferricyanide electrolyte for the alternative 2-electrode arrangement. Mean real impedance changes at 0.05 Hz between sets of 3 EIS repeats reveal significant differences ($p = 0.019$) only with the ferrocyanide/ferricyanide electrolyte in the alternative 2-electrode arrangement (Fig. 3d). Increases in real impedance at 0.05 Hz with this electrode arrangement were insignificant when using PBS ($p = 0.633$) and the hexaammineruthenium chloride redox probe ($p = 0.122$).

Non-significant increases in real impedance at 0.05 Hz were also observed in 3-electrode measurements with PBS and the alternative electrolyte, but not in standard 2-electrode measurements. These increases could be attributed to the formation of a silver chloride layer at the silver electrode, which is expected due to chloride ions in PBS. However, the formation of an AgCl layer at the silver electrode was not

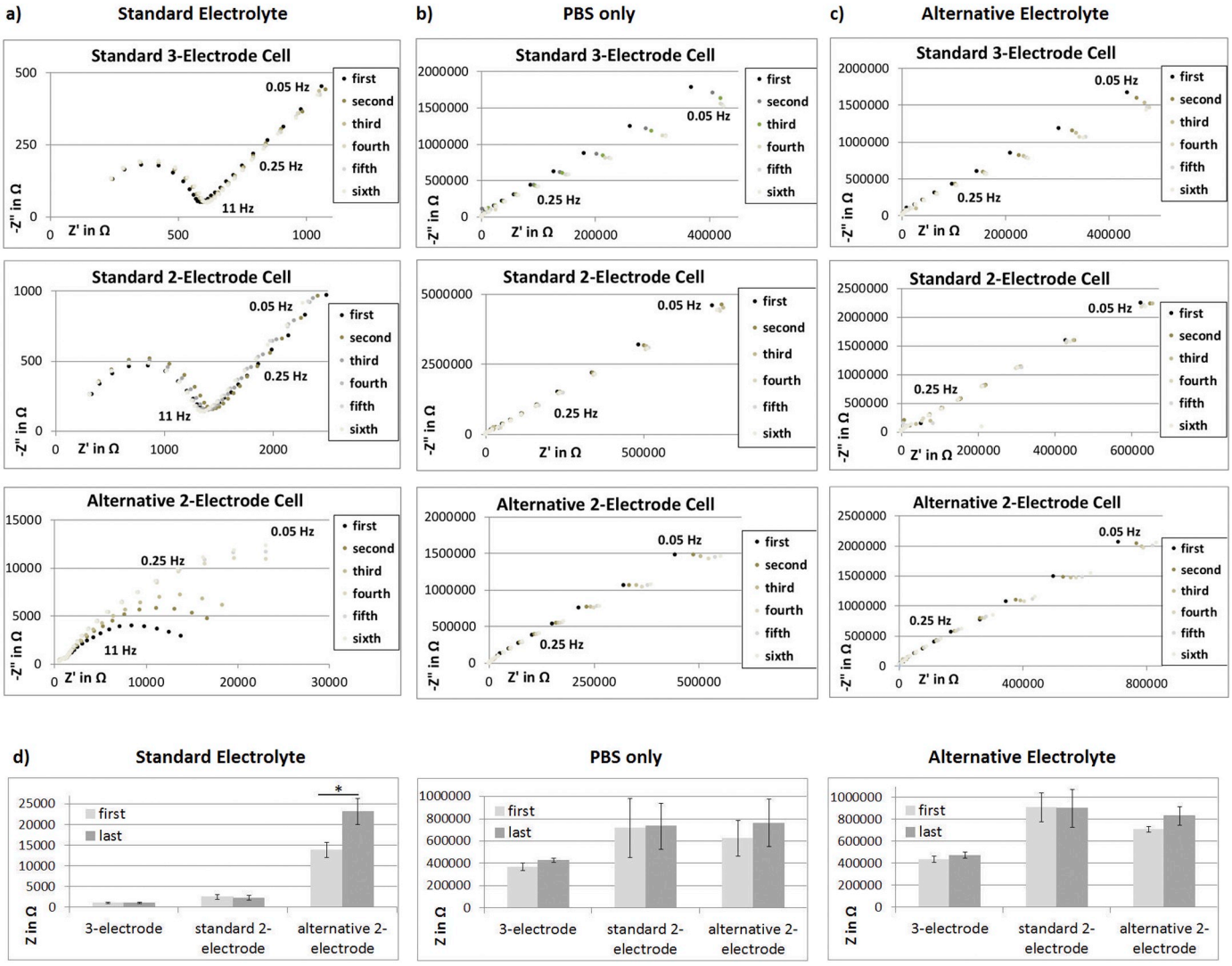


**Fig. 3.** Characterization of adsorption by EIS; Nyquist plots showing six successive measurements in a) ferrocyanide/ferricyanide based electrolyte, b) PBS alone and c) hexaaminеruthenium chloride based electrolyte by applying a 3-electrode (top panel), standard 2-electrode(middle panel) and alternative 2-electrode cell (bottom panel); d) mean real impedance for each set at 0.05 Hz, bars represent standard deviation. Statistical significance is indicated with the symbol * ($p < 0.05$) and determined using the Student's *t*-test.

observed with EDX characterization. Any effects of AgCl formation on inter-repeat impedance stability were negligible, while significant instabilities observed with the standard electrolyte could be attributed to a cyanide-based reaction. The CN compound adsorbed to the silver electrode could also have participated in a separate redox process by reacting further with electrolyte compounds [27].

Despite apparent EIS repeatability in a 3-electrode SPE system when applying a cyanide-based electrolyte, a continuous adsorption reaction at the pRE was shown to occur. This non-equilibrium reaction compromises the reliability of the reference potential during an ideally steady-state measurement, and could influence the validity of impedance results. To avoid the impact of the observed adsorption phenomenon on the reference potential, a 2-electrode arrangement can be used, producing a response similar to the 3-electrode system while improving real and imaginary impedance stability. While a 2-electrode system cannot apply an optimized equilibrium potential to WE relative to CE, it can provide a practical and effective alternative in some biosensing systems [28]. Since silver readily reacts with compounds in solution other than chloride ions [29], applying this material as a working electrode served only as a diagnostic tool and would not be recommended for biosensor development.

## 4. Conclusions

Ferrocyanide and ferricyanide electrolytes are widely used in electrochemistry as efficient redox promoters, and their application with pseudo-reference electrodes is often assumed to be stable without experimental verification at each use. The findings reported in this work, together with previous studies of silver reactions with cyanides present in literature, recommend against employing cyanide-based redox probes with silver pREs in impedance-sensing systems due to the risk of inaccuracy and instability resulting from spontaneous side reaction and deposition of by-products on silver surfaces.

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