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Solid reference electrode integrated with paper-based microfluidics for potentiometric ion sensing

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Abstract

A new concept for a solid reference electrode integrated with microfluidic paper-based sampling was introduced and applied in potentiometric ion sensing. The new reference electrode consisted of an Ag/AgCl reference element (reusable) that is in contact with a disposable paper substrate (DPS) containing solid KCl. Thus, no KCl reference solution was applied during the analysis step, but the applied sample solution dissolved the solid KCl in the paper providing the electrolyte for the Ag/AgCl reference element. Such reference electrode was found to give a relatively constant potential after less than ca 1 min of equilibration. Moreover, the response of the reference electrode was not affected by concentrations of background electrolytes in the sample. Thus the proposed reference electrode was integrated with a paper-based microfluidic device and solid-contact ion-selective electrodes (ISEs) sensitive to K^+ , Na^+ and Cl^- . The optimized systems were characterized with near-Nernstian sensitivities (59.1 ± 1.5 , 57.5 ± 0.5 and -56.4 ± 0.6 mV dec⁻¹) and detection limits ($10^{-4.1 \pm 0.1}$, $10^{-3.3 \pm 0.1}$ and $10^{-4.1 \pm 0.1}$ mol dm⁻³) for K^+ , Na^+ and Cl^- ions, respectively. The paper-based measurement setup was favourably used for potentiometric determination of ions in environmental (wastewater sludge) and clinically relevant (sweat) samples.

Keywords

Paper-based microfluidic sampling; solid reference electrode; μ PAD; potentiometry; clinical and environmental analysis

1. Introduction

Determination of ion concentrations in clinical and environmental samples is essential for maintaining human well-being [1-4]. For that reason, potentiometry is often used for the determination of sodium, potassium and chloride ions in clinical analysis and e.g. heavy metal cations in environmental samples [5,6]. Many of these samples may have limited volumes, e.g. sweat and blood serum, which causes a need to develop novel analytical devices and measurement protocols ensuring reliable analysis in small sample volumes. Furthermore, in remote locations there may be a need for portable and low-cost analytical instrumentation. These requirements can be fulfilled by microfluidic paper-based analytical devices (μ PADs), which offer portability and enable cost-effective point-of-care and on-site analysis [7-15]. Moreover, various detection methods, e.g. colorimetric, fluorometric, electrochemical, chemiluminescent and electrochemiluminescent have been coupled with μ PADs, which has increased their applicability in analytical chemistry [16-20]. Interestingly, the electrochemical techniques are not sensitive to ambient light and sample color, which makes them advantageous in various analytical devices [8-10]. Among others, Citterio et al. developed a paper-platform combining both, an all-solid-state ISE and a reference electrode, for detection of potassium and sodium, without the necessity to pre-condition the ISEs before measurements [21]. Also, Bühlmann et al. proposed disposable paper-based potentiometric sensors for Cl^- , K^+ , Na^+ , and Ca^{2+} , where wax-printed hydrophobic barriers were applied to contain the aqueous solution within the platform [22]. In fact, the application of paper as sample wicking substrate creates a possibility of conducting chemical reactions within the limited volume of the paper matrix, acting like a filter, separating solid particles from the sample and providing a relatively clear solution to the detection zone. Also simple modifications to the paper substrates, e.g. by applying hydrophobic barriers or by varying the shape of the paper allow directing the flow of the solution within the paper matrix driven only by capillary forces in a controlled way [9, 23-29].

In the potentiometric cell, except for the ISE, a reference electrode must be used. For that purpose, in conventional aqueous samples, a saturated calomel or a silver/silver chloride reference electrode is commonly applied [30]. It is well known that the Ag/AgCl reference element provides a constant and stable potential only when in contact with a solution of constant chloride activity. Usually the chloride activity in the sample is not constant, so then an equitransferent chloride solution of high concentration (e.g. $3 \text{ mol}\cdot\text{L}^{-1}$ KCl or saturated KCl) needs to be in place between the Ag/AgCl element and the sample solution. Thus integration of the reference electrode at/with the paper substrate is rather challenging. One of the options is stencil-printing of a reference Ag/AgCl electrode to achieve a potentiometric cell embedded into paper [31]. Another possibility involves screen-printing using conductive carbon ink on the patterned paper devices [32], which is usually used for both working and reference electrodes printed on the paper substrate surface [33-35]. The reference electrodes can be printed on the paper surface either on the same or opposite side of the paper substrate to the working electrode [36]. Another option is to fabricate paper-based solid-contact ISEs integrated with a solid-contact reference electrode integrated in the form of single strip [37].

In this work we present an alternative approach where the Ag/AgCl reference element is in contact with a paper substrate containing solid KCl that dissolves when the sample is transported into the paper via capillary forces. The novelty of this approach is that the KCl solution was not applied by directly drop-casting any KCl solution during the analysis step, but instead, KCl solid was deposited beforehand on the reference zone of the disposable paper substrate (DPS). Upon addition of the sample solution to the paper platform, the sample solution dissolved the KCl solids providing a saturated KCl electrolyte for the Ag/AgCl reference element. Ideally, this will result in a well-defined Ag/AgCl/KCl(sat.) reference electrode comparable to a conventional design of

the reference electrode. The concept is evaluated here using various paper-based microfluidic devices with ISEs.

2. Materials and methods

2.1 Materials and reagents

In the course of all experiments the quantitative filter paper Whatman 589/2 (Merck, product no. WHA10300114) was used. Potassium chloride (KCl, purity \geq 99%) was purchased from Merck KGaA (Germany). Sodium chloride (NaCl, purity \geq 99%) and a potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$, purity \geq 99%) were obtained from VWR International (USA). A 4-tert-butylcalix[4] arene-tetraacetic acid tetraethyl ester (sodium ionophore X) (product no. 71747), valinomycin (potassium ionophore I) (product no. 60403), tridodecylmethylammonium chloride (TDMACl) (product no. 91661), potassium tetrakis [3,5-bis(trifluoromethyl) phenyl] borate (KTFPB) (product no. 60588), sodium tetrakis (4-fluorophenyl) borate dihydrate (NaTFPB) (product no. 72017), 2-nitrophenyl octyl ether (o-NPOE) (product no. 73732), bis(2-ethylhexyl) sebacate (DOS) (product no. 84818), poly(vinyl chloride) of high molecular weight (PVC) (product no. 81387), tetrahydrofuran (THF) (product no. 401757), 3,4-ethylenedioxythiophene (EDOT) (product no. 483028) and sodium (polystyrene sulfonate) (NaPSS) (product no. 243051) were purchased from Sigma–Aldrich (Germany).

The inductively coupled plasma-optical emission spectrometry (ICP-OES) calibration standard 2 was obtained from PerkinElmer, Inc. (USA). Ion chromatography (IC) standard IV-STOCK-59 was purchased from Inorganic Ventures (USA). For all experiments ultra-pure water, 18.2 M Ω cm

resistivity, obtained with the ELGA purelab option DV25 ultra water system (High Wycombe, United Kingdom) was used.

2.2 Ion-selective electrodes

Solid-contact ion-selective electrodes sensitive to K^+ , Na^+ and Cl^- were prepared. For that, a glassy carbon (GC) electrode with a teflon body were firstly polished using mesh paper with $0.3\ \mu m$ Al_2O_3 , then washed with distilled water and 99.5% ethanol and left for air drying. In the next step, the conducting polymer PEDOT was electropolymerized on the GC disk electrode surface. The electropolymerization solutions consisted of $0.01\ mol\ L^{-1}$ EDOT and either $0.1\ mol\ L^{-1}$ NaPSS (in case of K^+ - and Na^+ -ISEs) or $0.1\ mol\ L^{-1}$ KCl (in case of Cl^- -ISE). The electropolymerization was done galvanostatically by applying a current corresponding to the current density of $0.2\ mA\ cm^{-2}$ to the working electrode for 714 s [38]. The electropolymerization was done in a three electrode cell with the GC disk electrode as the working electrode, the single junction silver/silver chloride electrode ($Ag/AgCl/3\ mol\ L^{-1}$ KCl) (6.0733.100, Herisau, Switzerland) as the reference electrode, and a glassy carbon rod as the counter electrode. After electropolymerization the GC/PEDOT electrodes were washed with deionized water and left to dry for 3 h. Later the membrane cocktail was cast on the top of each GC/PEDOT electrode (w/w%): for K^+ -ISEs (1% of potassium ionophore I, 0.4% of KTFPB, 65.7% of DOS, 32.9% of PVC); for Na^+ -ISEs (1% of sodium ionophore X, 0.3% of NaTFPB, 66.7% of o-NPOE, 32.7% of PVC); and for Cl^- -ISEs (15% of TDMACl, 51% of o-NPOE, 34% of PVC). The membrane cocktails were prepared from a total dry mass of 100 mg membrane components, followed by dissolution in 2 mL of THF. The membrane cocktails were cast in three portions of $10\ \mu L$ (up to the final volume of $30\ \mu L$) on top of each GC/PEDOT electrode. After each $10\ \mu L$ deposition, electrodes were left for THF

evaporation (ca 1 hour). All solid-contact ISEs were then left overnight for evaporation of the residual solvent, followed by conditioning (K^+ -ISEs in 10^{-3} mol L⁻¹ KCl, Na^+ -ISEs in 10^{-3} mol L⁻¹ NaCl and Cl^- -ISEs in 10^{-3} mol L⁻¹ KCl) for 24 hours. In-between measurements, all ISEs were stored in their respective conditioning solutions.

2.3 Reference electrodes

Except of a conventional single junction silver/silver chloride electrode (Ag/AgCl/3 mol L⁻¹ KCl) (cRE) (6.0733.100, Herisau, Switzerland), a new type of reference electrode (nRE) for potentiometric measurements utilizing paper-based sampling was prepared. In the new design, the electrode consisted of two elements, namely: (i) Ag/AgCl element and (ii) a disposable paper substrate (DPS) containing various concentrations of KCl(s). The experimental potentiometric setup with cRE, nRE and DPS is schematically shown in Figure 1. The Ag/AgCl element was prepared by electrodeposition of AgCl on a silver rod in a solution of 1 mol L⁻¹ KCl for three hours by applying 0.1 mA current to the electrochemical cell. After that the Ag/AgCl wires were conditioned in 3 mol L⁻¹ KCl overnight. Then the Ag/AgCl wire was rinsed with ultra-pure water and left to dry in air. The Ag/AgCl wire was kept dry in-between potentiometric measurements. The DPSs were prepared by cutting squares (1×1 cm²), which were then modified by addition of various solutions of KCl (1 mol L⁻¹, 3 mol L⁻¹ and saturated KCl in ultra-pure water). The KCl solutions were added in portions of 50 μ L (1-3 portions/DPS). After each portion the DPS was left to dry for ca 75 min in air. By registering the mass change of the DPSs before and after addition of KCl, the mass/concentration of KCl in each paper element was estimated (taking into account the maximum liquid holding capacity of the paper element). All prepared DPSs were connected to the Ag/AgCl reference elements and the potential was measured versus the conventional Ag/AgCl/3

mol L⁻¹ KCl electrode as shown in Fig. 1. The potential of the new reference electrode was measured in the following solutions: NaCl (10⁻², 10⁻¹ and 1.0 mol L⁻¹), NH₄Cl (10⁻⁶, 10⁻³ and 1.0 mol L⁻¹), MgCl₂ (5×10⁻⁷, 5×10⁻⁴, 0.5 mol L⁻¹), KNO₃ (10⁻⁶, 10⁻³, 1.0 mol L⁻¹) by drop casting (50 μL) of each solution onto the DPS that was in direct contact with the Ag/AgCl element and the conventional Ag/AgCl/3 mol L⁻¹ KCl electrode (Fig. 1).

2.4 Microfluidic paper-based platforms

The nRE reference electrode (Ag/AgCl reference element coupled with DPS) was combined with a microfluidic paper-based platform to obtain a potentiometric paper-based cell. Several shapes and sizes of microfluidic paper-based platforms for potentiometric detection were investigated (Fig. 2). Most of the proposed microfluidic paper-based platforms (Fig. 2. A to D) were tested in three variants based on the linking paper element (marked grey in Fig. 2) between the solution sampling zone and the DPS, namely: (i) without any modification (same paper substrate as the substrate in the solution sampling zone), (ii) top side and (iii) both sides of the linking paper element coated with wax, using ordinary wax crayons. The wax layer/layers were drawn only on the paper surface. The wax modification was implemented in order to retard the flow of solution between the sampling zone and the DPS. Then, the time for wicking the solution from the sample deposition zone to the entire microfluidic paper-based platform was measured. The wetting time of the paper platform was the main criteria for choosing the optimal microfluidic paper substrate for construction of the potentiometric cell. Furthermore, the best performing paper substrates (Fig. 2 E and F) were investigated without any wax coverage of the linking paper element. The choice of the best size and geometry of the paper was made by estimating the time

for the solution (i) to reach the detection zone, (ii) to reach the DPS zone and (iii) to fully cover the paper-based platform. The wetting of the substrate was observed by naked eye. Additionally, the temperature effect on the DPS before and after wicking of 30 μL of ultra-pure water was examined (90 s measurements with temperature readout every 10 s) by using a K-type thermocouple (purchased from Extech Instruments, USA).

2.5 Potentiometry with ISEs and nRE on paper-based platforms

The potentiometric response of the ISEs vs. nRE on the paper-based platform were measured using an EMF16 interface potentiometer (Lawson Labs Inc., USA). Before their use in the paper-based potentiometric cell, the potentiometric response of the ISEs was initially recorded in conventional beaker-based measurements and only electrodes that exhibited almost Nernstian response (slope = $56 \pm 2 \text{ mV dec}^{-1}$ for K^+ - and Na^+ -ISEs, $-56 \pm 2 \text{ mV dec}^{-1}$ for Cl^- -ISEs) were utilized further. In those measurements, the potentiometric response was investigated in the range of $10^{-7.0}$ to $10^{-1.1} \text{ mol L}^{-1}$ of the standard solutions, with tenfold increase in the primary ion concentrations. In beaker-based measurements, the single-junction Ag/AgCl (3 mol L^{-1} KCl) was used as the reference electrode. Both, the ISE and the reference electrode were immersed in the standard solutions during the potentiometric measurements for 60 s while recording the potential (EMF) of the potentiometric cell.

The potentiometric paper-based cell is schematically illustrated in Fig. 3 and a photo of the laboratory setup is shown in Fig. S1. In addition to the microfluidic paper substrates (μPS) that was combined with the DPS, two additional key regions can be identified, i.e. the sample deposition point and the detection zone (Fig. 3).

During the measurements the ISEs and the Ag/AgCl reference element were placed (using gravitational force) in direct contact with their respective paper elements. Further on, ca 80 μL of standard solution was pipetted in the middle part of the paper platform (sample introduction point). Then the solution was wicked into (absorbed by) the paper matrix, in approx. 10 s, and transported towards the detection zone and towards the DPS. When the sample reached the DPS a closed electrical circuit between the ISE and the nRE was established. Also, once the sample solution entered into the DPS, the dissolution of KCl accumulated in the paper occurred, providing the KCl electrolyte for the Ag/AgCl reference element. The EMF of the potentiometric paper cell was then measured for 60 or 120 s. A new piece of paper substrate and DPS were used for each ion concentration measurement. The K^+ -, Na^+ - and Cl^- -ISEs were investigated one by one in the potentiometric paper cell. Then, to investigate the influence of both paper substrates and the nRE on the potentiometric response, two additional measurement protocols were carried out. For both protocols, instead of nRE, the conventional single junction silver/silver chloride electrode (Ag/AgCl/3 mol L^{-1} KCl) was used. In the first one (protocol A), the cRE replaced Ag/AgCl reference element and was directly placed on the DPS. In the second (protocol B), the cRE was placed on the end part of the μPS that did not contain any KCl, thus eliminating any paper-based reference electrode element from the potentiometric cell, similarly to what has been done previously [39-42]. Two additional setups (based of the setup presented in Fig. 3) were investigated, namely: (i) when nRE was connected as indicator electrode while the conventional single junction silver/silver chloride electrode (Ag/AgCl/3 mol L^{-1} KCl) was connected as reference electrode and (ii) when nRE was connected as reference electrode while the conventional single junction silver/silver chloride electrode (Ag/AgCl/3 mol L^{-1} KCl) was connected as indicator electrode. All potentiometric results were corrected by activity coefficients (obtained by Debye-Hückel approximation) for each ion/concentration. For each measurement, three repetitions were

conducted and the uncertainty of the measurements were estimated. All experiments were carried out at room temperature.

Additionally, the impedance of the paper-based potentiometric cell was measured to assess whether the newly developed paper-based potentiometric cell could be used as an analytical device together with a simple voltmeter. Electrochemical impedance spectroscopy (EIS) was performed by using a Gamry interface 100 potentiostat, Metrohm (Switzerland). EIS was performed on a two electrode system (Fig. 3.), where the working electrode was either a Na⁺- or K⁺-ISE while the nRE was connected as counter and reference electrode. In such a design, the sum of all impedance contributions from the ISE, nRE and paper substrate were measured. For comparison, EIS measurements were also performed on the same electrode system in a beaker (keeping the same distance between electrodes). The measurements were performed in 0.1 mol·L⁻¹ NaCl and 0.1 mol·L⁻¹ KCl for Na⁺ and K⁺-ISEs, respectively, in the frequency range of 100 kHz to 0.01 Hz, with amplitude of 10 mV for the excitation signal. Three consecutive measurements were done (n=3). All tests were conducted at room temperature (23 ± 2 °C).

2.6 Real samples analysis

The proposed new potentiometric paper cell was validated by measuring three sludge samples collected from the Water Reclamation Plant (UPWRP) in Singapore, and two sweat samples obtained from a human subject exposed to physical stress (jogging). The sludge samples were collected in glass bottles, whereas, the sweat samples in 5-mL sterile plastic tubes. Both kinds of real samples were stored at 4°C (fridge) before analysis. No further pretreatment was done to the samples, except that the samples were allowed to warm up to room temperature before the actual ion analysis. Concerning reference analysis, the potassium and sodium concentrations in sludge

and sweat samples were determined by ICP-OES (PerkinElmer Optima 8300, USA), while the concentration of chloride was determined by ion chromatography (IC) (ThermoFisher ICS110, USA). For potentiometric measurements unfiltered samples were used, while all samples used for ICP-OES and IC were filtered prior to analysis by using an Acrodisc 25 mm Syringe Filter with 0.45 μm Supor membrane (Pall, USA). For potentiometric determinations, the calibrations of K^+ -, Na^+ - and Cl^- -ISEs were performed in the potentiometric paper cells in $10^{-4.0}$ - $10^{-1.0}$ mol L^{-1} KCl, NaCl and KCl ranges, respectively. Then samples were dropped onto the sample introduction zone of the paper (Fig. 3). While the liquid wicked in both directions in the paper substrates, the solid impurities from the sample solution were retained at the sample introduction point so that a relatively clear solution was delivered to both the detection zone and the DPS. The EMF was recorded for 60 s. Three measurements were carried out for each sample and the uncertainties of the measurements were estimated.

3. Results and discussion

3.1 Optimization of the disposable paper substrate (DPS)

The design of the nRE was optimized in order to be coupled with the μPS . During optimization, the Ag/AgCl reference element was always kept the same, while the DPSs with various KCl loadings were prepared. When the sample solution reaches the DPS, the KCl in the DPS undergo dissolution, resulting in various concentration of KCl (Table S1, Supplementary information) required for the stability of the potential readout of the Ag/AgCl reference element. The estimated concentration of KCl in the DPS was proportional to the number of additions of KCl solution to the DPS. Similarly, the higher the concentration of KCl used for modification of the DPS, the

higher the resulting concentration of the KCl in the DPS substrate. For conventional reference electrodes, a typical concentration of the filling solutions is 3 mol L^{-1} KCl. A high concentration of the reference KCl solution provides a stable potential of the electrode [42, 43]. From a storage perspective both the DPS containing KCl crystals and a conventional paper-based reference electrodes can be stored dry. While the DPS may lose some KCl crystals owing to mechanical friction between DPS and the packing material, the amount of KCl in the DPS is sufficient to assure a saturated KCl solution when in contact with the sample solution. In such a situation, both the DPS containing KCl crystals and conventional paper-based reference electrode provide sufficiently stable electrolyte for the reference electrode. Furthermore, dissolution of KCl during the process of sampling of the solution could decrease the temperature of the paper substrate. However, it was found that the temperature change during the dissolution process is very small: the temperature of dry DPSs was $21.2 \pm 0.3^\circ\text{C}$, whereas after addition of ultra-pure water, the temperature changed (after 90 s measurement time) to $20.0 \pm 0.2^\circ\text{C}$.

The potential stability of the paper-contact reference electrode was studied in comparison with the conventional $\text{Ag}/\text{AgCl}/3 \text{ mol L}^{-1}$ KCl reference electrode. In these measurements the conventional $\text{Ag}/\text{AgCl}/3 \text{ mol L}^{-1}$ KCl electrode was connected as the reference and the nRE was connected as the indicator electrode. Fig. 4 presents the potentiometric response of the Ag/AgCl element coupled with different DPSs modified with various amounts of saturated KCl, namely I ($1 \times 50 \mu\text{L}$), II ($2 \times 50 \mu\text{L}$) and III ($3 \times 50 \mu\text{L}$) (Table S1, Supplementary information). For each kind of DPS, measurements were conducted in triplicate, each time using a newly prepared DPS.

The concentration of the KCl resulting from the dissolution of the KCl salt deposited on/in the DPS is expected to stabilize gradually and thus influence the response time and repeatability of the

recorded potentiometric signal. As can be seen in Fig. 4, the EMF of the paper cells stabilized within ca 10 s, assuring a potential drift lower than 3 mV/min. The final EMF values of the potentiometric cell for DPS I, II and III (as taken at 60 s measurement time) were -1.2 ± 1.4 mV, -13.9 ± 3.3 and -12.7 ± 2.9 mV, respectively and for DPS I, II and III (as taken at 120 s measurement time) were -1.3 ± 1.7 mV, -14.1 ± 3.2 and -13.0 ± 2.1 mV, respectively. The measured cell potentials of the nRE vs. Ag/AgCl (3 mol L⁻¹ KCl) are close to the values expected for a KCl concentration of 3 mol L⁻¹ (DPS I) and saturated KCl (DPS II and III). Indeed, the use of all DPSs resulted in sufficiently reliable potential of the potentiometric cell, same for both shorter and longer measurement times. The potentiometric response of the Ag/AgCl element coupled with DPSs modified with saturated KCl (I, II and III) showed a shorter equilibration time and lower uncertainty of the potential readout when compared to DPSs modifications made with 3 mol L⁻¹ (IV, V and VI) and 1 mol L⁻¹ KCl (VII and VIII) (see Figs. S2, in the Supplementary information). For example, the final EMF value of the potentiometric cell, utilizing DPS IV, V, VI, VII and VIII (as taken at 60 s measurement time) were 3.4 ± 7.0 , -3.6 ± 6.8 , -7.1 ± 2.5 mV, 11.7 ± 1.2 and 4.7 ± 4.3 mV, respectively and for DPS IV, V, VI, VII and VIII (as taken at 120 s measurement time) were 2.8 ± 7.3 , -5.7 ± 5.6 , -8.9 ± 2.0 mV, 10.1 ± 2.6 and 2.4 ± 4.2 mV, respectively. More negative potentials were measured for nREs with higher amounts of KCl deposited (Fig. S3). The DPS I was chosen as the most suitable for the use in the new reference electrode based on its fast potential equilibration and its final potential close to that of Ag/AgCl/3 mol L⁻¹. Moreover, owing to the preparation/fabrication method of DPSs, the DPS I was preferential as the deposition of each KCl layer on the paper substrates was found laborious and time consuming (casting solution of KCl and leaving it to dry for 75 min), thus application of a single layer made the procedure much simpler and shorter. Finally, increasing the number of layers could possibly increase the risk of

crumbling KCl crystals, which would result in possible problems with the storage of a larger number of pieces of the prepared DPS.

Furthermore, to validate if the nRE as reference electrode consisting of Ag/AgCl reference element being in contact with 3 mol L⁻¹ KCl, the potentiometric cell setup presented in Fig. 3 was adapted to measure the potential of the nRE against the conventional Ag/AgCl (3 mol L⁻¹ KCl) reference electrode. Two setups were investigated, namely: (i) when nRE was connected as indicator electrode and (ii) when nRE was connected as reference electrode in this newly designed potentiometric cell. The measurements were performed in 0.1 mol L⁻¹ KCl, which should assure near zero potential of the potentiometric cell, taken the nRE was indeed equipped with 3 mol L⁻¹ KCl. As a result, both electrode arrangements were found to give near zero potentials, namely -2.69 ± 4.13 and 0.39 ± 1.84 mV for (i) and (ii), respectively. This confirms that the nRE acts as a Ag/AgCl (3 mol L⁻¹ KCl) reference electrode. Finally, the influence of the background electrolyte on the potentiometric stability of the Ag/AgCl element coupled with DPS I was examined. The DPS was tested in contact with different concentrations of KNO₃, NH₄Cl, NaCl and MgCl₂ (Fig. 5). The chosen salts represented ions with different charge and mobility and some of them are common ions present in body fluids [45]. The final potential values of the potentiometric cell in various solutions at two different measurement times are presented in Table 1. For DPS I, the equilibration time was found different for various background electrolytes. Nonetheless, the data collected after 60 and 120 s was found to be similar to the theoretically predicted cell potential of the Ag/AgCl electrode equipped with 3 mol L⁻¹ KCl. Since the cell potential was found relatively stable, the DPS I together with Ag/AgCl element was investigated as new reference system coupled with ion-selective electrodes. The 60 s measurement time was found sufficient for the cell equilibration.

3.2 Optimization of the microfluidic paper-based potentiometric cell

Various shapes and sizes of the microfluidic paper substrate were examined (Fig. 2). The sampling zone marked with a triangle was assumed to be placed in the middle of the paper strip to ensure solution transport in two directions simultaneously. The solution flow provided by capillary forces could be influenced in various ways. For example, by (i) changing the shape and size of the channel connecting the paper substrate with the DPS and (ii) covering one or two sides of that channel with wax to reach an optimal flow rate of the solution in the paper matrix. The used wax was placed by drawing (using crayons) only on the surface of the filter paper without heating. This was applied in order to slow down the transport of the solution and not to stop it completely (as it would be if the wax was heated). The time needed for the solution to reach the detection and DPS zones varied for various substrates and modifications made for the linking paper element (Table S2, Supplementary information). As expected, the introduction of the wax to slow down the transport of solution to the DPS and back transport of dissolved KCl salt from the DPS to the detection zone was the most effective when both sides of the paper-based platforms were modified, followed by one side modification. The fastest wetting was observed for paper-based platforms without modification of the paper linking element. Interestingly, by varying the shape of the paper linking element and thus the pathway of solution transport, the time needed for wetting the paper-based platforms could be regulated. For that purpose additional paper-based platforms, with no modifications to the paper linking elements (Fig. 2. E and F) were prepared and investigated, resulting in wetting times of 33 and 30 s, respectively. The longer wetting time was found beneficial for possible separation of the transport of solution to the DPS and back transport of dissolved KCl salt from the DPS to the detection zone, while lack of wax modification simplified the procedure

of paper-based platform preparation. Since the paper-based platform (F) assured better contact (2 mm overlap) between the solution sampling zone and the DPS, this platform was investigated in detail. It was found that the time needed for the solution applied at the sample deposition zone to reach the sample detection zone was 3.5 s while to reach the DPS it was 7 s, with full solution coverage after 30 s. In this case, the ISE was first in contact with the sample solution. The electric circuit of the potentiometric cell was then closed by solution entering to the DPS, while full dissolution of the KCl stored in the DPS was accomplished within 30 s, indicating minimal time required for analytical measurement.

3.3 ISE measurements utilizing the paper-based cell

The measurements were conducted using ion-selective electrodes sensitive to K^+ , Na^+ and Cl^- ions as indicator electrodes (as indicated in Fig. 3). The cell's EMF was measured against two reference electrodes, namely conventional single junction silver/silver chloride ($Ag/AgCl/3 \text{ mol L}^{-1} \text{ KCl}$) and paper-based silver/silver chloride electrodes. Potentiometric ISE measurements in the traditional beaker-based mode utilized a conventional $Ag/AgCl/3 \text{ mol L}^{-1} \text{ KCl}$ as reference electrode, while ISE measurements in the paper-based device used the paper-based silver/silver chloride electrode DPS I as reference electrode. The calibration curves and analytical parameters for both beaker- and paper-based measurement with ISEs are presented in Figure 6 and Table 2.

As can be seen in Table 2, the LOD for beaker-based sampling are tenfold and 100 times lower than paper-based sampling for K^+ - and Na^+ -ISEs, respectively. The loss of the detection limit for the paper-based potentiometric cell in comparison to the beaker-based measurement has been observed in various studies. Although this behaviour is not yet fully understood, a number of

possible causes have been identified. For example, paper has been identified to be slightly anionic in nature, possessing anionic carboxylate groups, which may interfere especially with cation-sensitive ISEs [13,21,46]. In addition, these ionic sites can take part in complexation and ion-exchange equilibria, that can influence the ion activity in the solution phase, especially at low ion concentrations. Finally, the physicochemical nature of the paper may allow physical and chemical adsorption of ions onto the paper substrate, which is especially valid for heavy metal ions. Some of these interactions are based on weak van der Waal's forces, resulting in sorption and desorption (if paper substrate already had particular ion in the matrix originating from paper making process) of ions [40,41,43,47]. In fact paper was found to be a good sorption material for a number of ions [48,49]. On the other hand, for Cl^- -ISEs, the beaker-based measurements and the paper-based measurements show comparable LODs. The potentiometric response utilizing two additional protocols, where the cRE replaced the Ag/AgCl reference element and was directly placed on the DPS and where the cRE was placed on the end part of the paper substrate are presented in Fig. 7a and 7b, respectively. For K^+ -ISEs, the slopes in protocol A (Fig. 7a) and B (Fig. 7b) were 60.0 ± 2.0 and $57.2 \pm 2.9 \text{ mV dec}^{-1}$, respectively. In both protocols, the LODs of K^+ -ISEs were similar ($10^{-4.1} \text{ mol L}^{-1}$) and comparable with those obtained when the paper substrate was combined with nRE (Fig. 6a). For Na^+ -ISEs, the slopes in protocol A and B were 62.2 ± 1.0 and $58.4 \pm 0.8 \text{ mV dec}^{-1}$, respectively. In protocol A, the LOD was ca $10^{-3.2} \text{ mol L}^{-1}$, and was similar to the one obtained when the paper substrate was combined with nRE (Fig. 6b). However, the LOD at ca $10^{-4.1} \text{ mol L}^{-1}$ level obtained in the case of protocol B was tenfold lower than the potentiometric response utilising nRE. From protocol A and B it can be concluded that the LOD is driven by the paper-ISE interactions rather than the application of the nRE or reference electrode arrangement onto the paper platform.

Additionally the impedance of the potentiometric cell applied in paper- and solution-based measurements was investigated. It was found that for both, Na⁺- and K⁺-ISEs, the sum of all impedance contributions from the ISE and the nRE in paper-based measurements was slightly higher than in the solution-based measurements. For Na⁺-ISEs the impedance of the potentiometric cell was 538 ± 8 and 515 ± 22 k Ω , for paper- and solution-based measurements, respectively. For K⁺-ISEs, the impedance of the potentiometric cell was 2.7 ± 0.2 and 2.5 ± 0.2 M Ω , for paper- and solution-based measurements, respectively. Thus, the proposed potentiometric paper-based cell was found compatible with affordable low-impedance voltmeters as a potential measuring device. Similar observations, using a conventional paper-based reference electrode were reported by Bühlmann et al. [12].

Summarizing, the new solid paper-contact reference electrode is compatible with paper-based microfluidics allowing the design of a completely dry potentiometric measurement system. The studied ISEs show satisfactory Nernstian responses when measured against the solid reference electrode to be considered for semi-quantitative determination of K⁺, Na⁺ and Cl⁻ in clinical and some of the environmental samples [50,51]. Thus, the newly-designed paper-based microfluidic sampler coupled with the paper-contact reference electrode was further used in the determination of ions in sludge and sweat samples.

3.4 Analysis of ions in real samples

The determination of K⁺, Na⁺ and Cl⁻ ions in environmental and clinical samples was carried out using the paper-based system coupled with the nRE. ICP-OES and IC were used to validate the results. The ion concentrations determined in all analyzed samples are listed in Table 3. The results

of potassium determination using K^+ -ISEs were found overestimated, e.g. by 23-47% in sludge and by 34-58% in sweat samples, in comparison to the ones measured by ICP-OES. The results of sodium and chloride determinations using Na^+ -ISEs and Cl^- -ISEs were found underestimated, e.g. for Na^+ -ISEs, by 9-41% in sludge and by 19-22% in sweat samples while for Cl^- -ISEs, by 19-41% in sludge and by 32-40% in sweat samples, in comparison to the ones measured by ICP-OES and IC, respectively. It is worth to mention, that contrary to analysis done with these methods, potentiometric measurements were performed without any pretreatment, using unfiltered sludge samples. Thus the solids in sludge (containing organic and inorganic residues of bio digestion) may have actively participated in equilibration of ions in the solution phase [52-54]. The differences in the measured ion concentrations might result from the complexity of the samples, in terms of possible ligands availability in the solution [55-57]. Finally, solvent evaporation from the paper substrate could cause pre-concentration of analytes in liquid samples [58]. Taken the complexity of both samples, the paper substrate coupled with new reference electrode seems to be a promising tool for analysis of environmental and clinical samples.

4. Conclusions

In this work a novel concept of a solid reference electrode was introduced. In this approach, the Ag/AgCl reference element was connected to a paper-based microfluidic device via a disposable paper substrate (DPS) containing solid KCl(s). Upon contact with the sample solution, KCl in the DPS dissolved and provided the KCl(aq) electrolyte that stabilized the potential of the Ag/AgCl reference element. It was found that the reference electrodes equipped with substrates offering higher KCl levels were characterized by shorter equilibration time and higher potential

reproducibility. In general, it was found that such reference electrodes required at least 30 s equilibration time to attain the near constant potential required for potentiometric measurement. Moreover, the potential was not affected by the presence of electrolytes commonly existing in real samples.

The new solid paper-contact reference electrode was partially successfully applied for ion determination in environmentally and clinically relevant samples, i.e. wastewater sludge and sweat. Ideally, the nRE should provide a solid reference electrode with comparable performance as a conventional Ag/AgCl/KCl(sat.). However, the experimental results suggest that the paper-based potentiometric cell works rather as a semi-quantitative analytical device. In the present design, the potentiometric cell utilizing the nRE would have to be subjected together with the indicator electrode to calibration before actual use. Nonetheless, after further optimization, the novel approach of the reference electrode may open new possibilities for conducting potentiometric analysis of samples containing high amounts of solid impurities and samples where no additional pre-treatment or solution collection is needed, for example moisture of polluted surfaces or perspiration.

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Table 1. The equilibrated potential of the Ag/AgCl element coupled with DPS I, measured versus Ag/AgCl (3 mol L⁻¹ KCl) on the paper-based platform.

Salt	Measurement time [s]	Potential [mV] when in contact with different concentrations of the electrolyte		
		10 ⁻⁶ mol L ⁻¹	10 ⁻³ mol L ⁻¹	10 ⁻¹ mol L ⁻¹
KNO ₃	60	6.8 ± 7.0	8.2 ± 2.2	3.4 ± 1.7
	120	2.0 ± 5.1	2.6 ± 2.1	-0.7 ± 1.6
NH ₄ Cl	60	14.1 ± 6.6	10.1 ± 6.5	0.1 ± 5.6
	120	6.9 ± 1.8	2.9 ± 3.1	-3.6 ± 3.7
NaCl	60	4.1 ± 2.6	-0.8 ± 2.6	-2.9 ± 3.8
	12	-4.0 ± 2.8	2.8 ± 2.0	6.1 ± 2.2
MgCl ₂	60	4.1 ± 3.6	-2.1 ± 4.0	4.8 ± 3.0
	120	4.8 ± 3.0	2.3 ± 4.3	6.0 ± 2.3

Table 2. Comparison of slope and limit of detection (LOD) of beaker- and paper-based measurements of K⁺-, Na⁺- and Cl⁻-ISEs in KCl, NaCl and KCl solutions, respectively.

	beaker-based			paper-based		
	K ⁺ -ISE	Na ⁺ -ISE	Cl ⁻ -ISE	K ⁺ -ISE	Na ⁺ -ISE	Cl ⁻ -ISE
Concentration ranges [mol L⁻¹]	10 ^{-5.0} -10 ^{-1.1}	10 ^{-5.0} -10 ^{-1.1}	10 ^{-4.0} -10 ^{-1.1}	10 ^{-4.0} -10 ^{-1.1}	10 ^{-3.0} -10 ^{-1.1}	10 ^{-4.0} -10 ^{-1.1}
Slope [mV dec⁻¹]	56.3 ± 1.2	53.4 ± 0.5	-56.9 ± 0.9	59.1 ± 1.5	57.5 ± 0.5	-56.4 ± 0.6
LOD [mol L⁻¹]	10 ^{-5.4 ± 0.1}	10 ^{-5.4 ± 0.1}	10 ^{-4.1 ± 0.1}	10 ^{-4.1 ± 0.1}	10 ^{-3.3 ± 0.1}	10 ^{-4.1 ± 0.1}

Table 3. Determination of potassium, sodium and chloride in sludge and sweat samples by ISEs, ICP-OES and IC.

Sample	log c_{K⁺} K⁺-ISEs	log c_K ICP-OES	log c_{Na⁺} Na⁺-ISE	log c_{Na} ICP-OES	log c_{Cl⁻} Cl⁻-ISE	log c_{Cl⁻} IC
Sludge A	-2.97 ± 0.08	-3.10 ± 0.01	-2.61 ± 0.08	-2.51 ± 0.01	-1.68 ± 0.07	-1.45 ± 0.01
Sludge B	-3.09 ± 0.10	-3.18 ± 0.01	-2.55 ± 0.06	-2.51 ± 0.01	-1.55 ± 0.07	-1.46 ± 0.01
Sludge C	-3.02 ± 0.08	-3.19 ± 0.01	-2.73 ± 0.05	-2.50 ± 0.01	-1.57 ± 0.07	-1.45 ± 0.01
Sweat A	-1.71 ± 0.09	-1.91 ± 0.01	-1.15 ± 0.02	-1.04 ± 0.01	-1.07 ± 0.03	-0.90 ± 0.01
Sweat B	-1.62 ± 0.11	-1.75 ± 0.01	-1.05 ± 0.05	-0.96 ± 0.01	-1.05 ± 0.10	-0.85 ± 0.01

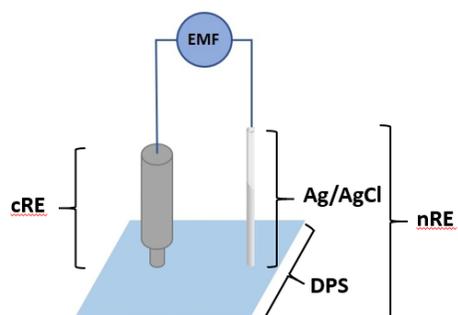


Fig. 1. Setup for testing the stability of nRE against the conventional single junction silver/silver chloride electrode ($\text{Ag}/\text{AgCl}/3 \text{ mol L}^{-1} \text{ KCl}$); nRE - new concept of the reference electrode, DPS – disposable paper substrate, cRE - conventional single junction silver/silver chloride reference electrode.

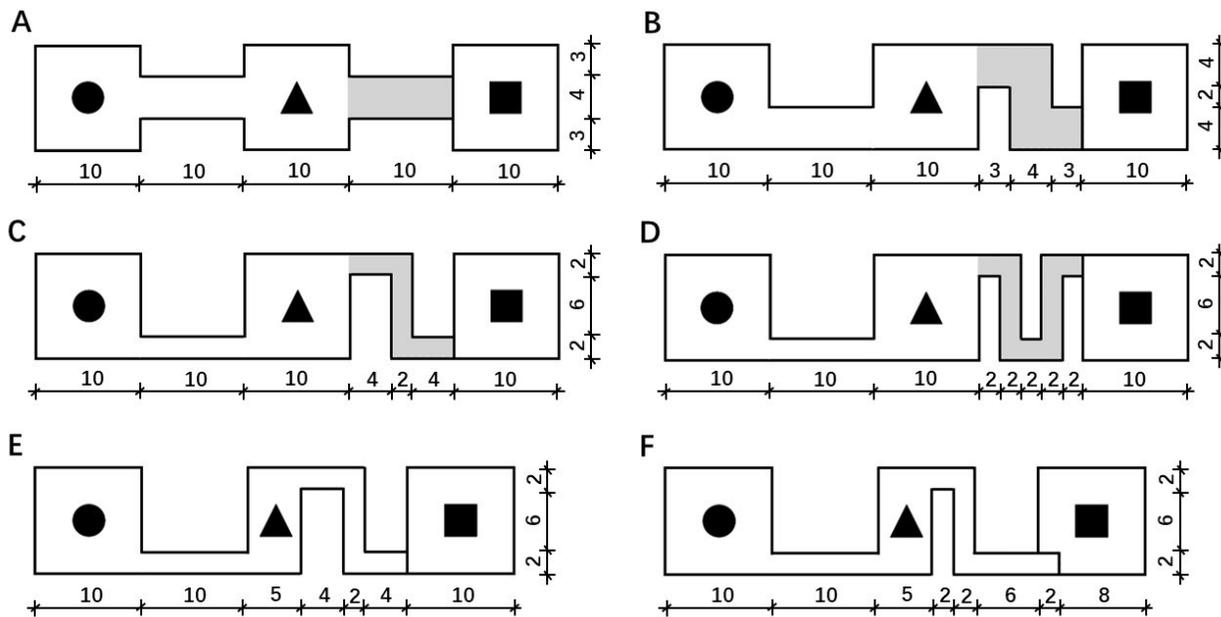


Fig. 2. Various shapes (A-D) of microfluidic paper-based platforms. ● - detection zone/ISE contact, ▲ - sample deposition zone, ■ - reference (nRE) zone; grey zone – area which was modified with wax layers on the paper surface. The dimensions are expressed in millimeters.

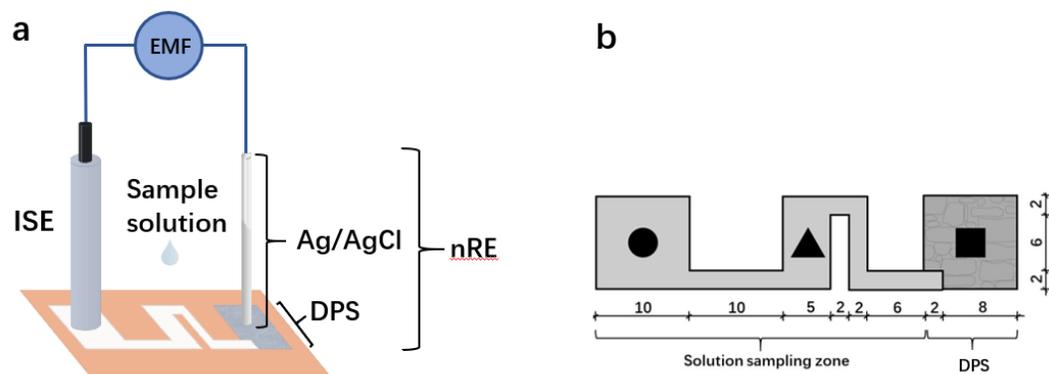


Fig. 3. Optimized potentiometric paper-based cell (a) and close up of the paper-based element of the chosen paper platform (b), where: ● is the detection zone, ▲ is the sample introduction point and ■ is the DPS with the Ag/AgCl reference element of the nRE. The dimensions are expressed in millimeters.

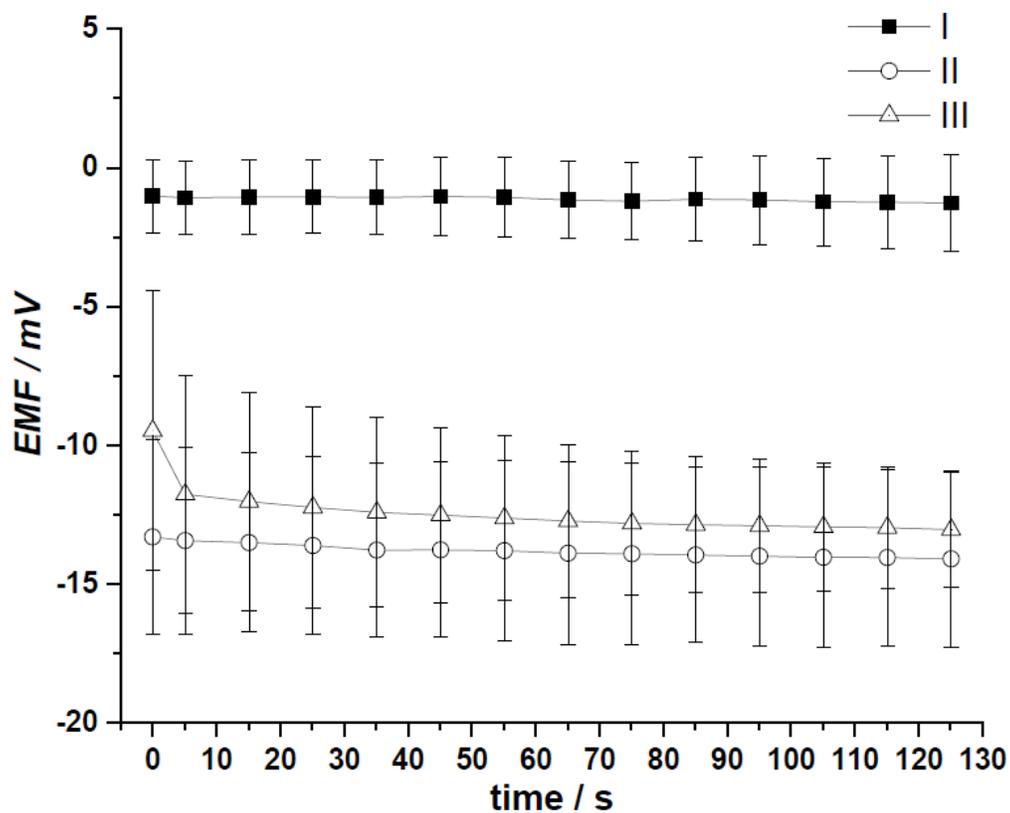


Fig. 4. Potentiometric response of the Ag/AgCl element coupled with DPSs modified with various amounts of saturated KCl (I, II and III in Table S1, Supplementary information) ($n= 3$). In this paper-based potentiometric cell, the the single-junction Ag/AgCl ($3 \text{ mol L}^{-1} \text{ KCl}$) was connected as reference electrode, while the Ag/AgCl element coupled with DPS was connected as indicator electrode.

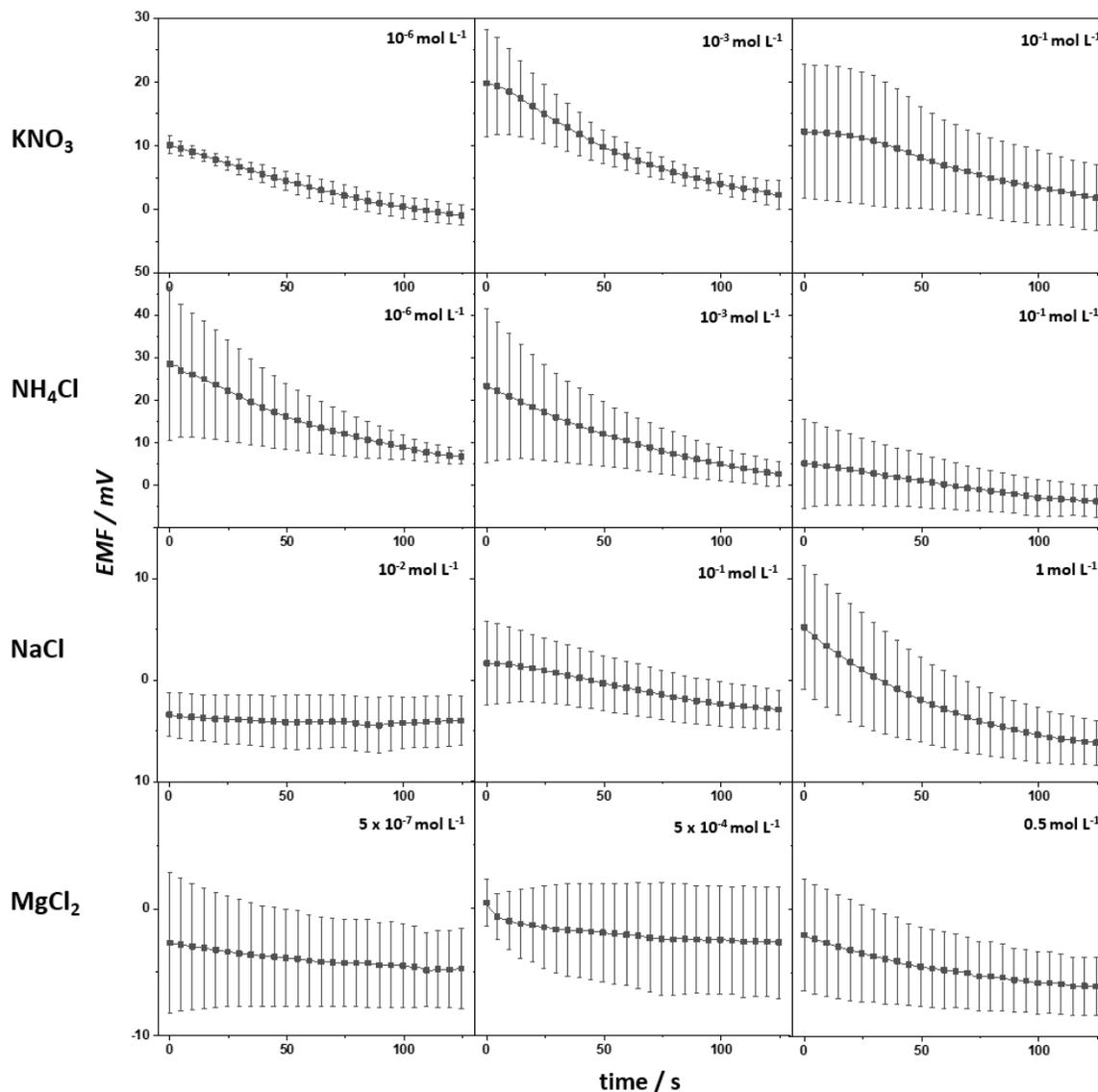


Fig. 5. The influence of the background electrolyte on the potentiometric response of the Ag/AgCl element coupled with DPS I. In this potentiometric cell, the the single-junction Ag/AgCl ($3 \text{ mol L}^{-1} \text{ KCl}$) was connected as reference electrode, while the Ag/AgCl element coupled with DPS was connected as indicator electrode.

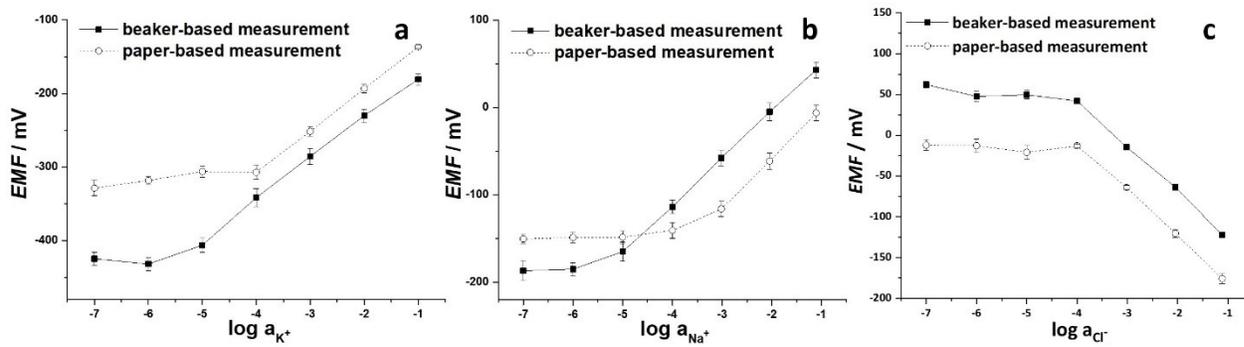


Fig. 6. Potentiometric response of potassium (a), sodium (b) and chloride (c) ISEs in $10^{-7.0}$ - $10^{-1.1}$ mol L⁻¹ standard solutions utilizing beaker-based and paper-based microfluidic sampling measuring setups.

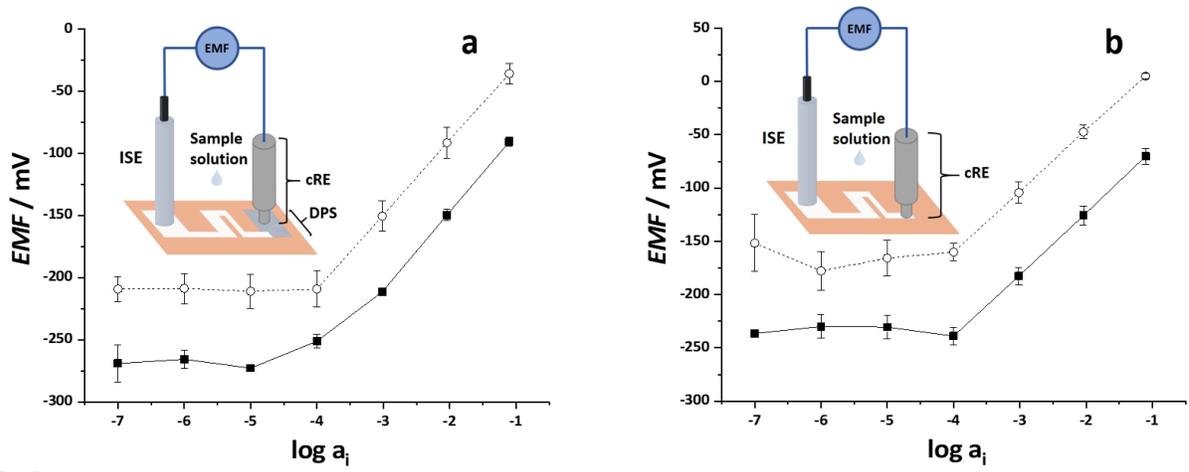


Fig. 7. Potentiometric response of both potassium (○) and sodium (■) ISEs in $10^{-7.0}$ - $10^{-1.1}$ mol L⁻¹ corresponding standard solutions utilizing protocol A (a) and protocol B (b).