Paper-based solution sampling coupled with potentiometric ion sensors

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**PAPER-BASED SOLUTION SAMPLING COUPLED
WITH POTENTIOMETRIC ION SENSORS**

DING RUIYU

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2021

**PAPER-BASED SOLUTION SAMPLING COUPLED
WITH POTENTIOMETRIC ION SENSORS**

DING RUIYU

School of Civil and Environmental Engineering

A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarized materials, and has not been submitted for a higher degree to any other University or Institution.

12th April, 2021

Date

Ding Ruiyu

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

12th April, 2021

Date

Grzegorz Lisak

Authorship Attribution Statement

This thesis contains material from 7 papers published/submitted in the following peer-reviewed journal in which I am listed as an author.

Chapter 2.2 and 9.2 contains material published as V. Krikstolaityte*, **R. Ding***, E.C. Hui, G. Lisak, Paper as sampling substrates and all-integrating platforms in potentiometric ion determination, TrAC Trends Anal. Chem. (2020) 116070. (**equally contributed*)

The contributions of the co-authors are as follows:

- Asst. Prof. Lisak provided the initial direction and supervision throughout duration of projects and edited the manuscript drafts.
- I, E.C. Hui and Dr Krikstolaityte prepared the manuscript drafts together. The manuscript was revised by Asst. Prof. Lisak.

Chapter 3 contains material published as **R. Ding***, Y.H. Cheong*, A. Ahamed, G. Lisak, Heavy Metals Detection with Paper-Based Electrochemical Sensors, Anal. Chem. (2021) 1880–1888. (**equally contributed*)

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Chapter 5.1 contains material published as **R. Ding**, V. Krikstolaityte, G. Lisak, Inorganic salt modified paper substrates utilised in paper based microfluidic sampling for potentiometric determination of heavy metals, Sens. Actuators B Chem. 290 (2019) 347–356.

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- Asst. Prof. Lisak provided the initial project direction, supervision and edited the manuscript drafts.
- I prepared the manuscript draft. The manuscript was revised by Dr Krikstolaityte.
- I co-designed the study with Asst. Prof. Lisak and performed all the laboratory work at the School of Civil and Environmental Engineering. I also analysed the data.

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The contributions of the co-authors are as follows:

- Asst. Prof. Lisak provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Asst. Prof. Lisak.
- I co-designed the study with Asst. Prof. Lisak and performed all the laboratory work at Nanyang Environmental and Water Research Institute. I also analysed the data.

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- Asst. Prof. Lisak provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Asst. Prof. Lisak.
- I co-designed the study with Asst. Prof. Lisak and performed all the laboratory work at the School of Civil and Environmental Engineering and Nanyang Environmental and Water Research Institute. I also analysed the data.

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- Asst. Prof. Lisak provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Asst. Prof. Lisak, Dr. Guzinski, Dr. Wagner, Dr. Ruzgas and Dr. Bobacka.
- I co-designed the study with Asst. Prof. Lisak and performed most of the laboratory work at Nanyang Environmental and Water Research Institute. I also analysed the data.
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The contributions of the co-authors are as follows:

- Asst. Prof. Lisak and Bobacka provided the initial project direction and edited the manuscript drafts.
- I and M. Fiedoruk-Pogrebniak prepared the manuscript drafts together. The manuscript was revised by Asst. Prof. Lisak and Dr. Bobacka.
- I co-designed the study with Asst. Prof. Lisak and performed most of the laboratory work at Nanyang Environmental and Water Research Institute. I also analysed the data.
- M. Fiedoruk-Pogrebniak assisted in some parts of the experiment.

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丁睿星 于新加坡

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY	I
SUPERVISOR DECLARATION STATEMENT	II
AUTHORSHIP ATTRIBUTION STATEMENT	III
ACKNOWLEDGEMENT	VI
TABLE OF CONTENTS	VII
ABSTRACT	X
LIST OF PUBLICATIONS	XI
LIST OF ABBREVIATIONS	XII
LIST OF SYMBOLS	XIII
LIST OF FIGURES	XIV
LIST OF TABLES	XVIII
CHAPTER 1 BACKGROUND	20
1.1 INTRODUCTION.	20
1.2 HEAVY METALS.	22
1.3 CLINICALLY RELEVANT IONS.	24
CHAPTER 2 THEORY AND LITERATURE REVIEW	27
2.1 POTENTIOMETRY.	27
2.1.1. Reference Electrodes (REs).	29
2.1.2. Ion-Selective Electrode (ISEs).	30
2.1.2.1 Types of ISEs.	30
2.1.2.2. Principle of ISEs response.	32
2.1.2.3 Detection limits and selectivity of ISEs.	34
2.2. PAPER USE IN POTENTIOMETRIC ION SENSING.	35
2.2.1. Paper properties.	35
2.2.2. General application of paper modification in paper-based solution sampling.	36
2.2.3. All-integrating paper-based sensors for potentiometric ion determination.	37
2.2.4. Paper as a microfluidic platform in paper-based potentiometric ion sensing.	38
CHAPTER 3 RESEARCH GAPS AND OBJECTIVES	44
3.1. PAPER PRE-TREATMENT FOR PAPER-BASED POTENTIOMETRIC HEAVY METAL ION SENSING.	44
3.2. SPONGE-BASED SAMPLING FOR POTENTIOMETRIC HEAVY METAL ION SENSING.	45
3.3. PAPER MODIFICATION TO REDUCE BIOFOULING FOR SAMPLING IN CLINICAL SAMPLES.	46
3.4. PAPER-BASED SOLID REFERENCE ELECTRODE.	46
CHAPTER 4 CHEMICALS, EQUIPMENTS AND METHODS	48
4.1. CHEMICALS AND MATERIALS.	48

4.2. METHODS.	49
4.2.1. Characterisation methods.	49
4.2.1.1. Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES).	49
4.2.1.2. Ion chromatography (IC).	50
4.2.1.3. Field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy analysis (EDXA).	50
4.2.1.4. Ellipsometry.	50
4.2.2. Preparation of electrodes and paper substrates.	51
4.2.2.1. Solid-state Cd^{2+} - selective electrodes.	51
4.2.2.2. Solid-contact ion-selective electrodes.	51
4.2.2.3. Paper substrates preparation.	52
4.2.3. The paper-based potentiometric measurements with ISEs.	52
4.2.4. Microwave digestion of paper substrates.	53
CHAPTER 5 PAPER MODIFICATIONS IN PAPER-BASAEED MICROFLUIDIC SAMPLING FOR HEAVY METAL DETECTION	54
5.1 INORGANIC SALT MODIFICATIONS OF PAPER SUBSTRATES FOR PAPER-BASED SOLUTION SAMPLING COUPLED WITH ISEs.	54
5.1.1. Experimental.	54
5.1.2. The paper-based potentiometric measurements with Cd^{2+} - and Pb^{2+} -ISEs.	56
5.1.3. Paper substrate pre-treated with different primary and interfering ion concentrations.	60
5.1.3 Interactions between paper substrates and the Cd^{2+} and Pb^{2+} ions.	67
5.2 ACIDIFICATION OF PAPER SUBSTRATES FOR PAPER-BASED SOLUTION SAMPLING COUPLED WITH ISEs.	70
5.2.1. Paper-based potentiometric sampling utilising acidified paper substrates.	70
5.2.2 Lead adsorption onto paper substrates after their acidification.	71
CHAPTER 6 SPONGE-BASED SAMPLING AS ALTERNATIVE TO PAPER-BASED SOLUTION SAMPLING IN HEAVY METAL ION DETECTION	74
6.1. EXPERIMENTAL.	74
6.2. OPTIMISATION OF TYPES OF SPONGE SUBSTRATES USED IN MICROFLUIDIC SPONGE-BASED SAMPLING.	75
6.3. SPONGE-BASED MICROFLUIDIC SOLUTION SAMPLING COUPLED WITH Cd^{2+} - AND Pb^{2+} -ISEs.	80
6.4. CONCLUSIONS.	81
CHAPTER 7 PAPER MODIFICATION IN PAPER-BASAEED MICROFLUIDIC SAMPLING FOR CLINICALLY RELEVANT IONS DETECTION	82
7.1. EXPERIMENTAL.	82
7.1.1. Au modification onto paper substrates.	82
7.1.2. Biofouling test.	83
7.1.2. BSA transportation through paper substrates.	84
7.1.4. Determination of K^+ , Na^+ and Cl^- concentrations in clinical samples.	86
7.2. CHARACTERISATION FOR AU-MODIFIED PAPER SUBSTRATES.	86
7.3. BIOFOULING OF ISEs DURING MICROFLUIDIC PAPER-BASED SAMPLING.	89
7.4. BSA TRANSPORTATION TIME ACROSS THE PAPER SUBSTRATES.	89
7.5. DETERMINATION OF K^+ , Na^+ AND Cl^- CONCENTRATIONS IN CLINICAL SAMPLES.	93
7.6. CONCLUSION.	95
CHAPTER 8 PAPER-BASED REFERENCE ELECTRODE COUPLED WITH ION-SELECTIVE ELECTRODE PAPER-BASED POTENTIOMETRIC SOLUTION SAMPLING SYSTEM	96
8.1. EXPERIMENTAL.	96
8.2. OPTIMISATION OF THE NEW REFERENCE ELECTRODE (NRE).	97

8.3. OPTIMISATION OF THE MICROFLUIDIC PAPER-BASED POTENTIOMETRIC CELL COUPLED WITH NRE.....	102
8.4. POTENTIOMETRY WITH ISEs AND NRE ON PAPER-BASED PLATFORMS.	104
8.5. REAL SAMPLES ION DETERMINATION.....	108
8.6. CONCLUSION.....	109
CHAPTER 9 CONCLUSIONS AND FUTURE PROSPECTS	111
9.1. CONCLUSIONS.....	111
9.2. FUTURE PROSPECTS.....	113

Abstract

The determination of heavy metals and clinically relevant ions is vital to maintain environmental quality and human health. In some situations, the samples do not contain a sufficient volume of the liquid to be collected for the analysis and to be measured using standard analytical methods. Potentiometric analysis based on ion-selective electrodes (ISEs) is performed by simple, portable and low-cost equipment, which can be used to monitor real-time ion activity in small-volume samples. To wick and deliver to the electrodes by capillarity action micro volumes of sample solutions with high solid-to-liquid ratios, as well as to avoid the electrodes being mechanically damaged by a rough surface of some samples/sampling surfaces, paper substrates were previously investigated as microfluidic sampling devices. However, the application of paper-based sampling coupled with potentiometric cell in heavy metal sensing was characterised with super-Nernstian response, thus was deemed as not suitable for reliable ion determination. Additionally, the ISEs and reference electrodes (REs) are prone to biofouling when used in the clinical samples, causing errors in ion determination. Lastly, the commonly used reference electrode Ag/AgCl/3M KCl is not the most suitable electrode for this particular application, namely: it is bulky and requires refilling the internal reference solution.

In this work, commercial crystalline membrane solid-state and solid-contact ISEs were used to investigate the possibility to facilitate paper-based microfluidic solution sampling coupled with potentiometric sensors for the ion determination in various environmentally (containing Cd^{2+} , Pb^{2+} and pH) and clinically relevant (containing K^+ , Na^+ , Cl^-) samples. To eliminate the unfavourable effects, different paper substrate pre-treatments were investigated, namely: (i) modifications of paper substrates with primary or interfering ions and acidification of paper substrates to chemically bind the active groups on the paper, thus avoid subsequent paper substrate interactions with metal ions in the sample solution; (ii) modifications of paper substrates by gold nanoparticles (AuNPs) to selectively remove/diminish transport of proteins from the clinical samples. Moreover, a new design of the solid-state paper-based reference electrode was investigated with the paper-based potentiometric sensors to provide constant and stable potential without refilling the internal reference solution. Lastly, the microfluidic sponge-based sampling was developed and studied as a novel sampling and sample handling method to serve as an alternative for microfluidic paper-based solution sampling, allowing measurements of heavy metals without prior modification of the sampling substrate.

List of publications

- [1] **R. Ding**, V. Krikstolaityte, G. Lisak, Inorganic salt modified paper substrates utilised in paper based microfluidic sampling for potentiometric determination of heavy metals, *Sens. Actuators B Chem.* 290 (2019) 347–356.
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- [3] **R. Ding**, M. Fiedoruk-Pogrebniak, M. Pokrzywnicka, R. Koncki, J. Bobacka, G. Lisak, Solid reference electrode integrated with paper-based microfluidics for potentiometric ion sensing, *Sens. Actuators B Chem.* 323 (2020) 128680.
- [4] V. Krikstolaityte*, **R. Ding***, E.C. Hui, G. Lisak, Paper as sampling substrates and all-integrating platforms in potentiometric ion determination, *TrAC Trends Anal. Chem.* 133 (2020) 116070. (**equally contributed*)
- [5] **R. Ding***, Y.H. Cheong*, A. Ahamed, G. Lisak, Heavy Metals Detection with Paper-Based Electrochemical Sensors, *Anal. Chem.* 93 (2021) 1880–1888. (**equally contributed*)
- [6] **R. Ding**, N. K Joon, A. Ahamed, A. Shafaat, M. Guzinski, M. Wagner, T. Ruzgas, J. Bobacka, G. Lisak, Gold-modified paper as microfluidic substrates with reduced biofouling in potentiometric ion sensing. *Sens. Actuators B Chem.* 344 (2021) 130200.
- [7] **R. Ding**, Y.H. Cheong, K. Zhao, G. Lisak, Acidified paper substrates for microfluidic solution sampling integrated with potentiometric sensors for determination of heavy metals, *Sens. Actuators B Chem.* 347 (2021) 130567.

List of abbreviations

AuNPs	gold nanoparticles
cRE	conventional single junction silver/silver chloride reference electrode (Ag/AgCl/3 M KCl)
DL	detection limit
DPS	disposable paper substrate
EDXA	energy dispersive X-ray spectroscopy analysis
EMF	electromotive force
FE-SEM	Field emission scanning electron microscopy
GC	Glassy carbon
IC	ion chromatography
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
ISE	ion-selective electrode
ISM	ion-selective membrane
LDL	lower detection limit
nRE	new concept of the reference electrode
PEDOT	poly(3,4-ethylenedioxythiophene)
PVC	poly(vinyl chloride)
PU	polyurethane
RE	reference electrodes
SC-ISEs	solid contact-ion selective electrodes
UDL	upper detection limit
μPADs	microfluidic paper-based analytical devices

List of symbols

a_i	activity of the primary ion
A and B	Debye-Hückel constants
c_i	the concentration of ion
d_i	the average effective ion radius
E_{cell}	potential of the potentiometric cell
E_i	electrode potential
E_j	electrode potential of the interfering ion
E_i^0	standard potential of the electrode
F	Faraday constant, $F = 9.649 \times 10^4 \text{ C mol}^{-1}$
I	ionic strength
$k_{i,j}$	selectivity coefficient
K_{sp}	solubility product constant
R	the universal gas constant, $R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$
T	temperature in Kelvin degrees
z_i	charge of the primary ion
γ_i	the activity coefficient
$\tilde{\mu}_i$	the electrochemical potential
μ_i^0	the standard chemical potential
μ_i^{s0}	the standard chemical potential in sample solution
μ_i^{m0}	the standard chemical potential in membrane
φ^i	the inner electric potential
φ^m	the inner electric potential of in membrane
φ^s	the inner electric potential in sample solution

List of figures

Figure 1. Schematic visualization of the standard potentiometric cell.

Figure 2. Illustration of a single junction silver/silver chloride reference electrode ($\text{Ag}/\text{AgCl}/3\text{M KCl}$).

Figure 3. Types of ISEs: (A) solid-state, (B) liquid-contact, (C) conducting polymer used as ISE and (D) solid-contact.

Figure 4. The charge propagation for liquid-contact (top) and solid-contact (bottom) K^+ -ISEs [72].

Figure 5. Detection limits of ISEs.

Figure 6. A simplified schematic depiction of potentiometric setup prototypes exploiting all-solid-state electrodes when micro-electrodes are integrated with a paper-based potentiometric sensor.

Figure 7. Illustration of (A) Vertical [9] and (B) horizontal [10] microfluidic paper-based sampling for potentiometric ion sensing.

Figure 8. (A) A vertical potentiometric setup utilising paper-based sampling; (B) Calibration curves of K^+ -ISE utilising filter paper types T1 (pore size 12-25 μm) and T2 (pore size 4-12 μm) in two different shapes [9].

Figure 9. Calibration curves for the Pb^{2+} -ISE using (---) paper substrates that were pre-conditioned for 1 h in $\text{Pb}(\text{NO}_3)_2$ and fresh paper substrates (—), while (···) is the dynamic response of the Pb^{2+} -ISE in a conventional, beaker-based measurement [10];

Figure 10. A schematic representation of a paper-based microfluidic sampling and separation system for potentiometric ion sensing. SEM images of the paper for separation zone (A1) and detection zone (A) are also accordingly shown [86].

Figure 11. Potentiometric response of the Cd^{2+} - and Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$ respectively when coupled with microfluidic paper-based sampling protocols 1 and 2 using (A) PS1 (B) PS2 (C) PS4 (D) PS5.

Figure 12. Potentiometric response of the Cd^{2+} - and Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$ respectively when coupled with microfluidic paper-based sampling by PS3 in the microfluidic paper-based sampling protocols 1 and 2.

Figure 13. Potentiometric response of the Cd^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS4 (D) PS5, utilising microfluidic paper-based sampling protocol 3 when the paper was pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}

M of primary ion concentrations. The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Figure 14. Potentiometric response of the Cd^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ using PS3, utilising microfluidic paper-based sampling protocol 3 when the paper was pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M of primary ion concentrations. The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Figure 15. Potentiometric response of the Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Pb}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5, utilising microfluidic paper-based sampling protocol 3 when the paper was pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M of primary ion concentrations. The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Figure 16. Potentiometric response of the Cd^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5, in microfluidic paper-based sampling protocol 4 when the paper substrates were pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M interfering ions, Pb^{2+} . The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Figure 17. Potentiometric response of the Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Pb}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5, in microfluidic paper-based sampling protocol 4 when the paper substrates were pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M interfering ions, Cd^{2+} . The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Figure 18. The concentration of cadmium and lead in (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5 as measured by ICP-MS after paper substrates pre-treatment in the concentration range of 10^{-6} - 10^{-3} M of $\text{Cd}(\text{NO}_3)_2$ (circles) and $\text{Pb}(\text{NO}_3)_2$ (triangles).

Figure 19. Potentiometric response of the Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Pb}(\text{NO}_3)_2$ in microfluidic paper-based sampling when the paper substrates were acidified by pH 1, 2, 3, 4 and 5 HCl solutions.

Figure 20. Lead concentration determined by ICP-MS after 10^{-4} , 10^{-5} and 10^{-6} M $\text{Pb}(\text{NO}_3)_2$ modification onto pH 1, 2, 3, 4 and 5 acidified paper substrates.

Figure 21. Schematic representation of potentiometric cell utilising sponge-based microfluidic sampling.

Figure 22. The sorption capacity of heavy metals (Cd and Pb) in PU sponge substrates determined using ICP-OES after sponge substrates pre-treatment in various concentration of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$.

Figure 23. The cross-section of a PU sponge substrate before (i) and after (ii, iii) microfluidic solution sampling of wastewater sludge.

Figure 24. Potentiometric cell response using the Cd^{2+} -ISEs (A) and Pb^{2+} -ISEs (B) in $10^{-7.0}$ - $10^{-1.0}$ M $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$, respectively utilising beaker-based and sponge-based microfluidic sampling measuring setups.

Figure 25. Sample mounting assembly (A) and substrate assembled device and gold substrate arrangement (B) used in protein transport studies null ellipsometry.

Figure 26. (A) The LSPR absorption spectrum and (B) TEM micrographs of prepared AuNPs.

Figure 27. FE-SEM of AuNPs distribution on unmodified paper (A), NPPS1 (B), NPPS2 (C), NPPS3 (D) and NPPS0 (E). Microscope photo of surfaces ($\times 20$) of unmodified paper (F), SPS1 (G), SPS2 (H), SPS3 (I) and SPS4 (J) with cross-sections (K, L, M, N, O, respectively).

Figure 28. The optical images captured in real-time contact angle measurements when the droplet contacted the unmodified paper (A), NPPS0 (B) and SPS1 (C).

Figure 29. Amount of BSA adsorbed onto the ellipsometric gold substrate after its transport through empty (blank), unmodified paper and AuNPs-modified paper substrates in the mounting assembly (Figure 28). The aliquot of $0.55 \mu\text{L}$ of 1 mg mL^{-1} BSA in 0.01 M PBS was added at 300 s.

Figure 30. EDXA mapping of carbon, gold, nitrogen and sulphur on the NPPS0 after conditioning in 1 mg mL^{-1} BSA in 0.01 M PBS.

Figure 31. Setup for testing the stability of nRE against the conventional single junction silver/silver chloride electrode ($\text{Ag}/\text{AgCl}/3 \text{ M KCl}$): nRE - new concept of the reference electrode, DPS - disposable paper substrate, cRE - conventional single junction silver/silver chloride reference electrode.

Figure 32. Potential stability for the Ag/AgCl element coupled with paper substrates (A) I, II, III (B) IV, V and VI (C) VII and VIII (Table 16) ($n=3$), measured versus the single-junction Ag/AgCl (3 M KCl) (cRE as the reference electrode, nRE as the indicator electrode).

Figure 33. Potential of the Ag/AgCl element at DPSs modified with various amounts of KCl (Table 16) measured versus Ag/AgCl (3 M KCl) after an equilibration time of 60 s (left) and 120 s (right).

Figure 34. The influence of the background electrolyte on the potentiometric response of the Ag/AgCl element coupled with DPS I. In this potentiometric cell, the single-junction Ag/AgCl (3 M KCl) was connected as reference electrode, while the Ag/AgCl element coupled with DPS was connected as indicator electrode.

Figure 35. 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 millimetres.

Figure 36. Optimised 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 millimetres.

Figure 37. Potentiometric response of potassium (A), sodium (B) and chloride (C) ISEs in $10^{-7.0}$ - $10^{-1.1}$ M standard solutions utilising beaker-based and paper-based microfluidic sampling measuring setups.

Figure 38. Potentiometric response of both potassium (○) and sodium (■) ISEs in $10^{-7.0}$ - $10^{-1.1}$ M corresponding standard solutions utilising protocol A (A) and protocol B (B).

List of tables

Table 1 K^+ , Na^+ and Cl^- concentration range (mM) and pH range in human serum, urine, sweat, tear and saliva.

Table 2 Summary of research on paper-based sampling and solution handling for *in situ* potentiometric ion determination using conventional ISEs.

Table 3 The list of chemicals used in this thesis.

Table 4 The list of equipment and tools used in this thesis.

Table 5 Lower detection limit (LDL) and slope of the Cd^{2+} - and Pb^{2+} - ISEs using microfluidic paper-based sampling protocols 1 and 2.

Table 6 Lower detection limit (LDL) and slope of the Cd^{2+} - ISEs applying microfluidic paper-based sampling protocol 3.

Table 7 Lower detection limit (LDL) and slope of the Pb^{2+} - ISEs applying microfluidic paper-based sampling protocol 3.

Table 8 The concentration of cadmium and lead in all paper substrates after paper substrate pre-treatment in the concentrations of 10^{-6} to 10^{-3} M of $Cd(NO_3)_2$ and $Pb(NO_3)_2$.

Table 9 Concentration function: slope Cd^{2+} and Pb^{2+} measured concentration= f(pre-treatment concentration).

Table 10 Physico-chemical characterisation of sponge substrates used for microfluidic sampling.

Table 11 The sorption capacity of heavy metals (Cd and Pb) in sponge substrates determined using ICP-OES after sponge substrates pre-treatment in various concentrations of $Cd(NO_3)_2$ and $Pb(NO_3)_2$.

Table 12 The potential change after the first and second contact with 1 mg mL^{-1} BSA in 0.01 M PBS in beaker- and microfluidic sampling-based measurement setups utilising unmodified and gold modified papers.

Table 13 The transportation times of the $0.55\text{ }\mu\text{L}$ of 10 mg L^{-1} BSA in 0.01 M PBS and 0.01 M PBS through unmodified and gold modified paper substrates.

Table 14 The concentrations (mM) of Na^+ , K^+ and Cl^- in artificial saliva, sweat, serum, artificial tear and artificial serum samples determined by ICP-OES/IC, Na^+ , K^+ and Cl^- -ISEs coupled with paper-based microfluidic sampling (applying NPPS0, NPPS1, NPPS2, NPPS3, SPS1, SPS2, SPS3 and SPS4). The concentrations fall into $\pm 10\%$ of the concentration determined by ICP-OES and IC are highlighted, fall into $\pm 20\%$ are in bold.

Table 15 The concentrations (mM) of Na^+ , K^+ and Cl^- in saliva, sweat, serum, artificial tear and artificial serum samples determined by ICP-OES/IC, Na^+ , K^+ and Cl^- -ISEs (before and after BSA modification).

Table 16 Concentration and volume of KCl solution and the corresponding mass of KCl at the disposable paper substrate (DPS).

Table 17 The equilibrated potential of the Ag/AgCl element coupled with DPS I, measured versus Ag/AgCl (3 M KCl) on the paper-based platform.

Table 18 The time for wicking the solution to the entire microfluidic paper-based platforms.

Table 19 Comparison of slope and lower detection limit (LDL) of beaker- and paper-based measurements of Na^+ , K^+ and Cl^- -ISEs in KCl, NaCl and KCl solutions, respectively.

Table 20 Determination of potassium, sodium and chloride in sludge and sweat samples by ISEs, ICP-OES and IC.

CHAPTER 1 BACKGROUND

1.1 Introduction.

In sustainable environmental development, ion sensing has been of great relevance as it brings widespread ionic descriptions of environmental systems and biological processes. The sensing of heavy metals and clinically relevant ions is crucial to maintain environmental quality and human health. General techniques used for the determination of heavy metals and clinically relevant ions are atomic absorption spectrometry (AAS) [1], inductively-coupled plasma mass spectrometry (ICP-MS) [2], inductively coupled plasma-optical emission spectrometry (ICP-OES) [3,4], colorimetry [4], fluorescence spectroscopy [5] and ion chromatography (IC) [6]. These, however, are non-portable, relatively costly and complicated devices that usually require complex laborious procedures for their operation and maintenance. Also, these require a certain minimal sample volume (no less than millilitre), which becomes limitations in their practical applications [4–7]. For both environmental and clinically relevant samples, the available volumes of different samples vary from virtually unlimited to less than millilitre. Solution sampling in lakes, rainwater and river, where the volume for analysis is sufficient can be simple and convenient. While sampling in samples containing a small amount of liquid or/and high solid content, e.g. sludge, wet soil and sweat on skin, the liquid fraction can be hard to collect for the analysis for the above mentioned techniques [8–10].

Therefore, new techniques should be developed to address the challenges of solution sampling and measurement in low sample volumes over their solid content. Potentiometric analysis using solid-contact ion-selective electrodes (SC-ISEs), where the electromotive force (EMF) (the electric potential produced by the electrochemical cell) is directly proportional to ion activity in the sample solution, is a promising alternative for measurements of heavy metals or clinically relevant ions in small sample volumes (as low as 10 to 30 μl sample solutions) when appropriate sampling methodology is provided [11]. Moreover, the time delay between the sampling and the

analysis occurs when performing the analysis using AAS, ICP-MS, ICP-OES, colorimetry, fluorescence spectroscopy and IC, contrary to potentiometry which is portable, relatively cheap and does not require sample preparation (e.g. filtration, buffering and dilution) that can be taken on-site for immediate measurement and can be handled in user-friendly manner [1,3,9,10,12]. However, the potentiometric response majorly depends on the physico-chemical state of the ion-selective membrane (ISM) and the analytical protocol of the electrode handling. To determine the ion concentration in small volume samples containing high solid-to-liquid ratios, various solution sampling substrates have been investigated when integrated with the potentiometric sensors, e.g. paper and textile substrates. The role of the use of the solution sampling substrates is: (i) to wick and deliver by capillarity action micro volumes of sample solutions to the detection place (electrodes), (ii) to filter out and retain the solid particles from reaching the fragile ion-sensitive membrane and (iii) to protect the ISM from mechanical damage from potentially rough measuring surface [9,10,13]. Nevertheless, the use of paper and textile substrates as microfluidic sampling substrates coupled with potentiometric cell in pH and heavy metals detection were found to be affected by side reactions and nonspecific adsorption of metal ions to the sampling substrates [9,10,13]. Utilising a paper/textile as a sample collecting substrates when performing heavy metal ions determination may cause hindrance during potentiometric analysis which can lead to the super-Nernstian response, thus not reliable ion determination. Additionally, ISEs and REs are prone to biofouling whenever biomolecules (e.g. proteins, fatty acids, enzymes, etc.) are present in the clinical samples, causing errors in ion determination. Moreover, the commonly used reference electrode Ag/AgCl/3M KCl is not the most suited electrode for this particular application, namely: it requires refilling the internal reference solution [14]. Otherwise, comparing to textile, paper substrates are lower cost and can be easier manufactured in different sizes, shapes, pore sizes, thicknesses and chemical structures, thus in this study paper substrates are utilized as the solution sampling substrates.

The aim of this study is to improve the applicability of paper-based solution sampling

integrated with potentiometric sensors in heavy metals and clinically relevant ions determination in samples with low liquids or/and high solids contents. In this thesis, protocols to modify the paper substrates to eliminate heavy metal interaction with paper substrates during potentiometric measurements, as well as to slow down the biofouling from clinical samples are investigated. Furthermore, a new design of the solid-state paper-based reference electrode is investigated with the paper-based potentiometric sensors. Finally, sponge-based microfluidic solution sampling was investigated as possible alternative to that of paper-based solution sampling for more reliable ion determination.

1.2 Heavy metals.

The determination of heavy metals is essential to monitor and maintain environmental quality, which is currently under pressure from the exponential increase of pollution originating from industrial, agricultural and domestic activities [15]. Heavy metals mainly enter the human body *via* inhalation of contaminated dust particles and ingestion of contaminated food and water. The most hazardous heavy metals are considered to be lead, cadmium, mercury and arsenic as they are characterised with high toxicity towards living organisms, including humans [16]. These metallic elements even at low concentrations of exposure might have a detrimental effect on human health and the environment, e.g. induce multiple organs and nervous system damage or when bioaccumulated cause cancer [15,17,18]. Thus, accurate monitoring of toxic heavy metals in the surrounding environment and foodstuff is crucial for human well-being.

Heavy metals are metals with a specific gravity of at least five times that of water [15]. They ultimately originate from the natural resource, e.g. metal ore. However, their increasing pollution is mainly caused by man-made activities, such as mining, agricultural, heavy industry and sewage irrigation. As a result, heavy metals are present in the atmosphere, water, soil and subsequently accumulate in living organisms [19]. In the environment, heavy metals are difficult to be naturally removed. They spread fast and widely, often move along water and further result in the contamination of soil and

plants. Also, heavy metals are hard to metabolise by the human, thus easily accumulating in the body [19]. In this thesis, sensors sensitive to cadmium and lead are mainly investigated, as they are one of the most toxic and commonly found heavy metals in the environment.

Cadmium is a heavy metal with world reserves estimated at 9 million tons [20]. Cadmium exists naturally mainly as greenockite (CdS). There are also small amounts of cadmium in the forms of cadmoselite (CdSe), otavite (CdCO_3), cadmoindite (CdIn_2S_4). Cadmium has strong corrosion resistance to alkaline media, thus it is used mostly to protect other metals from corrosion when electroplating steel, iron, copper, brass and various alloys. Cadmium is also used as yellow pigment in paints [17,21]. The main sources of cadmium pollution are wastewater coming from the electroplating process, mining, smelting, chemical industries and inadequately addressed wastes, e.g. dyes and batteries. The acidity of water can have an obvious effect on the solubility of cadmium, where the more acidic the water the more ionised cadmium it contains [21]. Plants easily uptake cadmium from polluted soil and water, similarly animals introduce cadmium *via* ingestion of polluted water and plant-based products [17,22]. It is reported that intake doses of 3 mg of cadmium have no acute influence on adults, however, more than 10 mg of cadmium per kg of diet or 10 mg L^{-1} in water will have a severe negative influence on the kidneys, liver, bones, hematopoietic and immune systems [21,23].

Lead occurs naturally in small deposits, where much of that is within minerals in Earth's crust. Lead is being extracted from lead-rich ores, thus the main source of lead is galena (PbS), while other forms of lead ore are anglesite (PbSO_4) and cerrusite (PbCO_3). Nowadays, lead is commonly used in the manufacturing of lead batteries, X-ray protective equipment, building materials, ammunition and projectiles, as well as in welding, trophies, and some alloys [24]. It is also used for casting type to make solder [25]. The industrial pollution of lead comes from the production of wastewater and waste from, e.g. mining, smelting, rubber production, dyestuff, printing, ceramics, lead glass, soldering tin, cables and lead pipes. Lead does not degrade and remains toxic

once it is released into the environment. Through its easy partitioning between air, soil and water, it can be easily absorbed by organisms, accumulating in live tissues [26]. Thus, lead and its compounds can also easily accumulate in the human body. After ingesting, it is mainly stored in bones, partially replacing calcium phosphate and is difficult to discharge. It also has a great effect on the neural system and further cause defects in behaviour and cognitive function. Children are much more likely to get lead poisoning than adults, as children continuously grow, thus can absorb more lead than adults. It is generally accepted that the excessive amount of lead in children's blood can affect their intelligence, including speech, memory and attention abilities [27–29]. The maximum acceptable blood lead (PbB) level is $400\ \mu\text{g L}^{-1}$, as established by the Surgeon General of the United States [30,31].

1.3 Clinically relevant ions.

Clinically relevant ions are considered as ions mainly present in human body fluids, animals and plants cells, which include cations and anions, such as H^+ , K^+ , Na^+ , Mg^{2+} , Ca^{2+} and Cl^- . They possess essential physiological functions for organisms, e.g. acting as the electrolyte to maintain osmotic pressure and pH balance of human body fluids [32–34], coordinate a huge number of physiological functions in the human body and adjust the metabolism processes of plants [35]. The detection of the concentration change of these ions in human body fluid, e.g. blood, urine and sweat, can evaluate the health conditions and provide reliable information for the diagnosis of potentially pathogenic diseases [32–34,36–39]. For plants, these ions are also significant nutrients assisting metabolism [35]. Although nowadays ISEs are applied in clinical analysers, the determination is only done in blood serum after the separation of red blood cells from the whole blood. Such time-consuming process may alter the ion concentration as the concentration can be easily influenced due to time delay between sampling and measurement and temperature change during the sample storage [40,41]. For this reason, it is important to develop on-site sampling and measurements of ions without time delay of clinically relevant ions in human fluids, plants and plant-related environment, e.g. stalk, root and planting soil. In this thesis, attention is paid mostly to the determination

of K^+ , Na^+ and Cl^- , using potentiometric sensors, which are extensive in human and animal bodily fluids and are key electrolytes related to human physiology. The K^+ , Na^+ and Cl^- concentration and pH in general body fluid, e.g. serum, urine, sweat, tear and saliva, has important clinical significance for the diagnosis of various diseases. The concentration range (mM) of K^+ , Na^+ and Cl^- and pH range in general body fluid, namely serum, urine, sweat, tear and saliva are shown in Table 1.

Table 1 K^+ , Na^+ and Cl^- concentration range (mM) and pH range in human serum, urine, sweat, tear and saliva [42].

	K^+	Na^+	Cl^-	pH
serum	3.5-5.3	130-155	100-106	7.35-7.45
urine	25-100	130-260	170-255	4.6-8.0
sweat	2-10	10-40	4-60	4.2-7.5
tear	13.8-24.4	108.3-142.5	24 ± 236	6.4-7.7
saliva	19-23	6.5-21.7	6.5-21.7	6.2-7.6

For humans and animals, K^+ is the main cation in the intracellular fluid, 98% of the potassium of the body is in the cell. K^+ can participate in transmitting nerve signals and promote muscle contraction, both myocardium and neuromuscular tissue need a relatively constant potassium ion concentration to maintain the stress [43]. When the serum potassium level is too high, it will have an inhibitory effect on the myocardium, which can stop the heartbeat during diastole. While too low serum potassium level can excite the myocardium and can stop the heartbeat during the systole [32,44]. Thus, controlling the K^+ concentration is crucial in surgery and intensive care [45]. In plants, K^+ is one of the most abundant cations in plant cells, as well as the leading cation balancing the negative charges, thus it is of great consequence for long-distance transport of anions [46]. Moreover, K^+ can promote the activity of enzymes in cells. There are more than 50 enzymes in the cell that are completely dependent on or activated by K^+ , e.g. pyruvate kinase and glutamine synthetase [47]. Also, K^+ can regulate a series of physiological functions of plants, namely enhancing plant photosynthesis, substance synthesis and transport, and improving energy metabolism [48].

About 30% of the sodium in the human body is on the surface of bones, the rest is mostly in extracellular tissue fluid, and a small amount in nerve and muscle cells. Na^+ is the main (93%) cation in body fluids and comparatively predominate for the electrolyte balance in body fluids. The extracellular sodium and intracellular potassium need to maintain a certain ratio in concentration, which is so-called the sodium-potassium balance [49]. Na^+ together with K^+ in body fluids help regulate the amount and pH of body fluids as well as participate in the nerve signals transmission and regulation of heart muscle [50].

The chloride level in the human body is 1.17g per kg on average, with a total amount of about 82-100 g, accounting for 0.15% of body weight, being widely distributed throughout the body [51]. Cl^- is also the major anion in human body fluids, together with Na^+ and K^+ to maintain electrolyte balance and acid-base balance [49,52]. Cl^- and Na^+ are main ions in the extracellular fluid to control the volume and sustain the osmotic pressure. Furthermore, Cl^- has crucial functions in metabolism, digestive system, immune system and circulatory system [53]. For instance, Cl^- participates in the transportation of divalent ion in blood, stimulates the liver and promotes the discharging of metabolic waste in the liver, as well as stabilises the potential of the nerve cell membrane [49,53].

CHAPTER 2 THEORY AND LITERATURE REVIEW

2.1 Potentiometry.

Potentiometry is a commonly used electrochemical analytical technique. It is sensitive, compact, portable, low energy powered and relatively low-cost. It dates back to the end of the 19th century [54,55]. It can be used widely to detect and study both inorganic and organics moieties [54,56–58]. It has been successfully applied for different sample analyses, e.g. in hospitals for blood electrolyte measurement, in environmental monitoring of pH and the food industry [59]. The potentiometric measurement setup is composed of two electrodes, namely a reference electrode and an indicator electrode. The potential of the reference electrode is ought to be constant and independent of the activity of ions in analyte solutions, while the indicator electrode potential is dependent on the activity of the target (primary) ion in analyte solutions. To close the electric circuit, the two electrodes must be immersed/in contact with standard or sample solutions (Figure 1).

Figure 1. Schematic visualization of the standard potentiometric cell.

Meanwhile, the use of a high impedance voltmeter ensures the measurement at close to “zero current” conditions, then no net electrochemical reactions occur and the potential reading related to the activity of the primary ion activity will not be affected by current flow in the system, thus the potential equilibrium is achieved [60]. The electromotive force (EMF) (potential of the electrode) is measured by the difference between the reference and the indicator electrodes. The potential of the cell can be written as

(Equation 1):

$$E_{cell} = E_{indicator} - E_{reference} \quad (\text{Equation 1})$$

The relationship between the activity of ions in the solution and the overall potential of the potentiometric cell is described by the Nernst equation, (Equation 2):

$$E_i = E_i^0 + \frac{RT}{z_i F} \ln a_i \quad (\text{Equation 2})$$

where E_i is the electrode potential (mV), E_i^0 is the standard potential of the electrode (mV), R is the universal gas constant, $R = 8.314 \text{ J K}^{-1} \text{ mol}^{-1}$, F is Faraday constant, $F = 9.649 \times 10^4 \text{ C mol}^{-1}$, T is temperature in Kelvin degrees, z_i is charge of the primary ion, a_i is activity of the primary ion. Activity is the effective concentration of a species in a mixture, as the interactions between ions at high concentrations cause deviation of effective concentration of the analyte. The relation between activity and concentration is described as (Equation 3):

$$a_i = c_i \cdot \gamma_i \quad (\text{Equation 3})$$

where c_i is the concentration of ion, γ_i is the activity coefficient (ranges from 1 to 0).

Similarly, the Nernst formulation can also be expressed as following when the temperature is 25 °C (Equation 4):

$$E_i = E_i^0 + \frac{0.059V}{z_i} \log a_i \quad (\text{Equation 4})$$

The activity coefficient depends on the ionic contribution in an analyte solution and the total ionic strength (I) of the solution, which can be defined by Debye-Hückel theory (Equation 5):

$$\log \gamma_i = - \frac{Az^2 \sqrt{I}}{1 + Bd_i \sqrt{I}} \quad (\text{Equation 5})$$

where A and B are constants for each sample solution which are dependent on the temperature and pressure, z is the charge number and d_i is the average effective ion

radius [61].

The ionic strength of the solution is estimated using (Equation 6):

$$I = \frac{1}{2} \sum c_i z_i^2 \quad (\text{Equation 6})$$

2.1.1. Reference Electrodes (REs).

Most of the currently used reference electrodes contain a metal electrode, which is in contact with a sparingly soluble salt of the same metal and immersed in the relatively concentrated solution of electrolyte containing the same anion as the sparingly soluble salt. For example, the commonly applied reference electrode is the silver/silver chloride reference electrode (Ag/AgCl/ 3M KCl). It consists of silver metal covered with a silver chloride salt immersed in a concentrated electrolyte containing chloride salt (Figure 2). According to its thermodynamic equilibrium $AgCl \rightleftharpoons Ag^+ + Cl^-$, the activity of the silver ion can be derived from the solubility product constant $K_{sp}(AgCl) = a(Ag^+)a(Cl^-)$, then the Equation 2 can be written as (Equation 7) [62,63]:

$$E_{Ag/AgCl} = E_{Ag/Ag^+}^0 + \frac{RT}{F} \ln(K_{sp}(AgCl)) - \frac{RT}{F} \ln(a_{Cl^-}) \quad (\text{Equation 7})$$

When the activity of chloride ion stays constant, the activity of the silver will also stay constant, thus assuring constant potential.

Figure 2. Illustration of a single junction silver/silver chloride reference electrode (Ag/AgCl/ 3M KCl).

A liquid junction is created with a thin film of the filling solution at the tip of the probe, which separates the reference electrode's internal filling solution from the test electrolyte and allows interdiffusion of ions between the reference filling solution and the sample [64]. The liquid junction potential forms due to different diffusion rates of cations and anions. Electrolytes containing ions of comparable diffusion rates (ion mobilities) for cation and anion are called equitransferent electrolytes, e.g. KCl, LiCH_3COO and NH_4NO_3 , they are commonly used to minimise the liquid junction potential [14,65]. A high concentration of KCl, namely 3 M or saturated, as the inner electrolyte is commonly applied to realise the predomination over other species in the diffusion layer, thus the liquid junction potential stays constant and stable [14,65].

2.1.2. Ion-Selective Electrode (ISEs).

2.1.2.1 Types of ISEs.

Based on the membrane types, there are mainly four different types of ISEs, namely solid-state, liquid-contact, conducting polymer-based and solid-contact (Figure 3).

Figure 3. Types of ISEs: (A) solid-state, (B) liquid-contact, (C) conducting polymer used as ISE and (D) solid-contact.

Solid-state ISEs have polycrystalline or single crystals-based membranes. For example, insoluble membrane materials are made of crystals of Ag_2S , CdS , PbS , LaF_3 , AgCl , AgBr , where the anion is the salt that the electrode is sensitive to [66]. For crystalline membrane solid-state ISEs, the lower detection limit (LDL) is dominated by the solubility of the ISM in relation to the experimental conditions. The membrane components are possible to oxidize resulting in the release of membrane components at the membrane | solution interface, thus influence the performance of the ISEs [66].

Liquid-contact ISEs have membranes based on organic liquid-immiscible phases e.g. polymer (commonly, poly(vinyl chloride) (PVC)). The ISE is equipped with Ag/AgCl electrode (called the internal reference electrode) and internal reference solution (usually containing a certain concentration of chlorides). The polymeric membranes are composed of a polymer matrix (as supporting material), a plasticizer (as the organic solvent of the membrane phase), ionophores (a chemical recognition element specific for the primary ion) and lipophilic ionic sites [67]. For example, the ionic sites for metal ion detection are composed of lipophilic anions and hydrophilic cations. The lipophilic anion is forming cation-exchange sites in the ISM. The concentration of the ionic sites in ISM counterbalances the charge of the primary ion [68]. Furthermore, the adding of ionophores to complex the primary ion is to obtain stronger interaction and increase the selectivity of the ISM towards the primary ion in the ISM [69]. During the equilibration of the ISE, the hydrophilic cations of the ionic sites are exchanged with the primary ion. Also, plasticizers are added to increase the elasticity to the ISM, as well as enhance the mobility of ionophores and ions in the ISM [70].

For solid-contact ISEs, the liquid internal filling solution together with the internal reference electrode is replaced by a solid contact layer between the electronic conductor and ion-selective polymeric membrane, called an ion-to-electron transducer [71]. Figure 4 compares the charge propagation for liquid-contact (top) and solid-contact (bottom) K^+ -ISEs. A high electrical capacity of the ion-to-electron transducer is required to reduce the impact of arbitrary charges on the electrode potential, thus

realizing the potential stabilisation [72,73]. Conducting polymers (CPs) are mostly used as the solid contacts with high stability of the potential, e.g. poly(3,4-ethylenedioxythiophene) (PEDOT), poly(pyrrole) (PPy), poly(3-octylthiophene) (POT) and polyaniline (PANI) [74]. Especially, PEDOT containing large redox capacity is commonly applied [75].

Moreover, CPs can be used directly as ion-selective films, however, such arrangement is characterised with poor ion selectivity and selectivity, thus not explored in this work.

Figure 4. The charge propagation for liquid-contact (top) and solid-contact (bottom) K⁺-ISEs [76]. R refers to ionic sites, L refers to ionophore, Q refers to conducting polymer.

2.1.2.2. Principle of ISEs response.

As the potential of the reference electrode stays constant, the overall potential of the whole potentiometric cell depends on the potential changes of the ion-selective electrode. When the ion-selective electrode is in contact with a solution containing primary ions, a primary ions activity related membrane potential is generated at the membrane | solution interface. The ion-selective membrane is optimally preferential for

the detection of only one primary ion. The phase-boundary model for the potential formation at the membrane | solution interface has an assumption that, the electrochemical equilibrium occurs at the interface. Thus, the electrochemical potential of the ion in the solution equals to the electrochemical potential of the ion in the membrane [77–79] (Equations 8 to 10):

$$\tilde{\mu}_i^s = \tilde{\mu}_i^m \quad (\text{Equation 8})$$

$$\tilde{\mu}_i = \mu_i^0 + RT \ln a_i + z_i F \varphi \quad (\text{Equation 9})$$

$$\mu_i^{s^0} + RT \ln a_i^s + z_i F \varphi^s = \mu_i^{m^0} + RT \ln a_i^m + z_i F \varphi^m \quad (\text{Equation 10})$$

where $\tilde{\mu}_i$ is the electrochemical potential, μ_i^0 is the standard chemical potential, φ^i is the inner electric potential. Also, *s-index* refers to a sample solution and *m-index* refers to an ion-selective membrane phases.

Then, the potential of ISEs is defined by the potential difference of ion in the sample solution and in the membrane (Equation 11):

$$E_i = \Delta\varphi = \varphi^m - \varphi^s = \frac{\mu_i^{s^0} - \mu_i^{m^0}}{z_i F} + \frac{RT}{z_i F} \ln \frac{a_i^s}{a_i^m} \quad (\text{Equation 11})$$

As $\mu_i^{s^0}$, $\mu_i^{m^0}$, F are constant and $a_i^m = 1$, $\frac{\mu_i^{s^0} - \mu_i^{m^0}}{z_i F}$ can be written as E_i^0 . Thus the potential of the indicator electrode can be finally described by the Nernst equation (Equation 12):

$$E_i = E_i^0 + \frac{RT}{z_i F} \ln a_i^s \quad (\text{Equation 12})$$

For practical applications, the formulation also can be described as (Equation 4):

$$E_i = E_i^0 + \frac{0.059V}{z_i} \log a_i \quad (\text{Equation 4})$$

The factor $\frac{0.059V}{z_i}$ is the slope of the calibration curve E_i vs. $\log a_i$. For example, for both Cd^{2+} - and Pb^{2+} -ISEs, the theoretical slope ought to be 29.6 mV dec⁻¹ (at 25°C), while

for K^+ - and Na^+ -ISEs, the theoretical slope ought to be 59.2 mV dec^{-1} (at 25°C), while for Cl^- -ISE the theoretical slope ought to be $-59.2 \text{ mV dec}^{-1}$.

2.1.2.3 Detection limits and selectivity of ISEs.

The potential response in the function of the logarithm of the primary ion activity ($E = f(\log a_i)$) provides the representation of the working range of the ISE. The detection limits of ISEs are defined by International Union of Pure and Applied Chemistry (IUPAC) as the cross-section of the two linear parts of the calibration curve (Nernstian and non-Nernstian), as shown in Figure 5. The lower detection limit (LDL) and the upper detection limit (UDL) reveal the activity range of the ISEs response to the primary ions being a function of proportional changes to the potential of the electrode, so-called Nernstian response [80]. Furthermore, more scientific attention is paid to the lower detection limit (LDL) as it is more vital when ISEs are considered for much-wanted trace detection of heavy metals [81]. The LDL is mainly affected by the zero-current fluxes of ions inside ISM, the primary ions leaching and contamination at membrane | sample interface [68]. The insufficient selectivity of the ISEs to the primary ion over interfering ions can also lead to the increase of LDL [82].

Figure 5. Detection limits of ISEs.

ISEs respond to the primary ion, however, the presence of other ions may cause interference to the potential formation. Selectivity is used to describe the ability of an ISE to discriminate potentially interfering ions in the presence of the primary ion in

sample solutions. The selectivity of ISEs is described through selectivity coefficient $k_{i,j}$ (i -index refers to the primary ions, j -index refers to the interfering ions). The potentiometric response of the ISEs including possible interference from other ions, e.g. as can be expected in complex environmental and clinical samples, is described by the Nikolsky-Eisenman equation (Equation 13) [83]:

$$E_i = E_i^0 + \frac{RT}{z_i F} \ln [a_i^S + \sum_j (k_{i,j} a_j^{z_i/z_j})] \quad (\text{Equation 13})$$

The selectivity of ISEs is mainly dependent on the physical and chemical properties of the electrode and its active components, e.g. ionophores. Thus, continuous studies are undertaken to research new materials, ionophores and ion exchangers to design ISEs with superior selectivity [84].

There are two methods for obtaining the selectivity coefficients according to IUPAC, namely separate solution method (SSM) and matched potential method (MPM). For SSM, it's to measure the potential of cell for two separate solutions, one contains the primary ion (E_i), one contains the interfering ion at the same concentration (E_j). The selectivity coefficient can be calculated as Equation 14:

$$E_{i,j} = a_i^{1-\frac{z_i}{z_j}} e^{(E_j - E_i) \frac{F}{RT}} \quad (\text{Equation 14})$$

The MPM is to measure potential of a solution of the primary ion i , and a mixed solution containing i and j . The selectivity coefficient can be calculated as Equation 15, where $\Delta E = E_{mix} - E_i$

$$E_{i,j} = a_i \frac{(e^{\frac{(\Delta E z_i F)}{RT}} - 1)}{a_i^{\frac{z_i}{z_j}}} \quad (\text{Equation 15})$$

2.2. Paper use in potentiometric ion sensing.

2.2.1. Paper properties.

Solution sampling in environmental, clinical and foodstuff samples sometimes faces

difficulties when samples containing high solid content with a limited amount of liquid [85–87]. This can be solved by applying adsorptive materials for sample solution collection to realise on-site potentiometric ion determination of various untreated samples. In certain situations, the adsorptive material is supposed to serve as a sample pre-handling substrate (e.g. filtration [88] and/or separation [89]) to carry out determination procedures with single manipulation. Furthermore, when the sampling substrate retains the solid impurities from the samples, at the same time it protects the ISM from mechanical damage by the solids. Thus, the substrate material should be hydrophilic, porous, flexible, lightweight and thin. Additionally, it should be low-cost and eco-friendly as it is used only once. Taking all the above requirements, paper is readily available at a very low cost and can be manufactured in different sizes, shapes, pore sizes, thicknesses and chemical structures, thus it has a great potential in developing affordable uses [90]. Paper also has high mechanical strength even when wet. Paper can be employed as a substrate for sample collection as well as swab/adsorb samples from different solid surfaces and solutions, because when testing solid-liquid mixtures, it enables to retain solid particles in the sample while the liquid part is delivered to the sensing zone. Thus, it can also be used as a separation substrate to filter the unwanted substrates [91–93].

The paper substrates are commonly applied as the microfluidics, as they have the abilities of passively transport mass/fluid through capillary action or wicking, without any requirement of external force [94]. The wicking abilities are relative to physical and geometrical properties of porous media such as , structure of the paper substrate, pore size, permeability, paper size and shape as well as it depends on the physical properties of the sample (liquid, high solid to liquid ratio, etc.

2.2.2. General application of paper modification in paper-based solution sampling.

Paper substrates can also be easily chemically and physically modified as well as customised for different applications. Different organic or inorganic materials can be grafted on the paper surface to serve as conducting layer, ion capture, network forming

and platforms for electrode printing, coating and integration [95–101]. A huge number of physical and chemical fabrication methods have been investigated for μ PADs, e.g. laser cutting, screen printing, wax printing, and plasma treatment [102–105].

Paper is widely used in chemistry, biology, biomedical engineering, electronics and agriculture. For example, paper can be used as a bioanalytical platform for optical detection, including colorimetric and fluorescence. Also, dipsticks and lateral flows are well-established assay formats for point-of-care testing [106,107]. Papers can also be used in electronics. Paper-based printed circuit boards are more environmentally friendly alternatives compared to traditional electronic components [108]. Devices such as biosensor for glucose, lactate and uric acid analysis in biological samples [109] and paper-based screen-printed voltammetric electrochemical sensor have been investigated [110]. For the detection of heavy metal ions, microfluidic paper-based analytical devices (μ PADs) have been developed [94,107,111]. The μ PADs are easy to manipulate, are low cost and disposable. They can also be combined with other detection technologies, such as colorimetric, fluorescent and electroanalytical, for the handling of low-volume aqueous heavy metal samples [112–114].

2.2.3. All-integrating paper-based sensors for potentiometric ion determination.

Paper substrates can be used coupled with potentiometry as miniaturised all-integrating paper-based sensors for on-site ion determination, conventional all-solid-state macro-electrodes were accordingly replaced by thin/thick-film micro electrodes, directly deposited on a paper substrate, thus designing all-integrating paper-based potentiometric sensors (Figure 6). In an all-integrating paper-based potentiometric setup, paper serves not only as a microfluidic sampling substrate, but also as a mechanical support for deposition of electrodes as well as the formation of sampling/detection and other functional zones depending on its design, thereby is further denoted as a paper platform. Paper-based micro electrodes are either designed as individual ISEs [115–119] and REs [120,121] or integrated in miniaturised potentiometric paper-based platforms [88,95,98,122–130]. The development of all-

integrating paper-based sensors are as portable and disposable tools for on-site ion determination of clinical/health [95,98,128–130], environmental [95,124,131] and foodstuff [124,126] samples.

Figure 6. A simplified schematic depiction of potentiometric setup prototypes exploiting all-solid-state electrodes when micro-electrodes are integrated with a paper-based potentiometric sensor.

2.2.4. Paper as a microfluidic platform in paper-based potentiometric ion sensing.

Paper substrates can also be served as microfluidic platforms in paper-based potentiometry when integrate the conventional potentiometric cell. It combines the advantages of paper and potentiometry. In potentiometry, similarly to the main advantages of the paper, it can be employed as a substrate for the sample collection from solid surfaces and solutions as well as avoid the damage of electrode surface when testing rough solid samples. Paper can retain bigger solid particles from a sample while the liquid fraction is sampled by wicking action into the paper matrix. The potentiometric cell can be simply pressed onto the paper substrate to achieve potentiometric measurements. Paper-based potentiometry is able to be realised through rapid microfluidic sampling and the paper can be disposed easily. It also achieves sampling and sample handling of low-volume aqueous samples [10]. Moreover, different sizes, structures, thicknesses and pore sizes of papers can be used to meet different requirements.

There have been several potentiometric studies for ion concentration determination that investigated microfluidic paper-based sampling for potentiometric ion sensing (Table 2). There are two approaches carried out for microfluidic paper-based potentiometric sampling (Figure 7), namely: (A) a paper substrate was placed vertically to a sample solution with the ISE and RE pressed against the paper from its opposite sides to hold the paper above the sample for vertical sampling [9]; (B) a paper substrate was positioned horizontally on a sample (or a sample was added on top of the paper) and the electrodes were placed on the paper for planar (horizontal) sampling [10,89,106].

Table 2 Summary of research on paper-based sampling and solution handling for *in situ* potentiometric ion determination using conventional ISEs.

Year	First Author & Ref.	Research Scope
2012	Gyurcsányi et al. [106]	Introduced the concept of paper-based sampling for <i>in situ</i> potentiometry using ISEs.
2014	Bobacka et al. [9]	Explored the impact of paper shape and porosity on liquid transportation within a paper substrate, thus possible impact on the potentiometric response in vertical paper-based sampling setup.
2015	Lisak et al. [10]	Explored the effects of paper sampling (horizontal) on potentiometric-based measurements of selected ions as a result of paper-analyte interactions.
2017	Ding et al. [89]	Utilised paper substrate as an ion separation platform to increase the accuracy of specific ion detection in the presence of interfering ions.

Figure 7. Illustration of (A) vertical [9] and (B) horizontal [10] microfluidic paper-based sampling for potentiometric ion sensing.

Gyuresányi et al [106] were the pioneers to perform proof-of-concept paper-based sampling in combination with ISEs for *in situ* potentiometry adapting this setup in dot-blot assay for protein analysis. Specifically, instead of employing optical detection for proteins adsorbed within a paper matrix as a regular dot-blot assay procedure, the authors exploited the possibility to improve the detection limit for the assay by combining *in situ* potentiometry with microfluidic paper-based sampling to detect Ag^+ ions dissolved within a paper substrate accordingly serving as a potentiometric tag in indirect highly selective protein analysis. In this measurement setup, the paper containing Ag^+ ions generated during bioassay was sandwiched between an Ag^+ -ISE and a Ca^{2+} -ISE acting as a pseudo RE (due to the highest selectivity against Ag^+). The calibration curve obtained during potentiometric measurements using the paper-based approach was compared with the results registered during conventional beaker-based measurements, and the detection limit for the paper-based measurement was found to be three orders of magnitude higher. The reasons behind this behaviour were subjected to possibly sluggish ion transport within the paper and the presence of Ag^+ ion complexing sites within the nitrocellulose paper [41].

Following the same principle, Bobacka et al. [9] used paper as a disposable sampling tool to transport sample solutions towards the electrode surface. In the potentiometric setup, solid-contact K^+ -ISE and RE were combined with vertical sampling in order to investigate how the difference in paper porosity and shape may affect the sample transportation towards the electrode surface, thus impact potentiometric signal (Figure 8A). It was found that the paper possessing smaller pores was more significantly affected by modifications in its shape which reflected in the super-Nernstian response of the potentiometric signal (Figure 8B). A super-Nernstian response is known as a phenomenon emerging due to a faster primary (target) ion uptake within an ion-selective membrane than the ion migration towards an ISE surface [132]. Thereby, the studies clearly showed that further investigation is essential to answer the questions regarding the optimal features of a paper substrate, such as porosity, shape, size, for its best performance regarding absorbing/transporting liquid.

Figure 8. (A) A vertical potentiometric setup utilising paper-based sampling; (B) Calibration curves of K^+ -ISE utilising filter paper types T1 (pore size 12-25 μm) and T2 (pore size 4-12 μm) in two different shapes [9].

In the following studies, Lisak et al. investigated the effect of a paper substrate on concentration determination of different ions (Cd^{2+} , Cl^- , Pb^{2+} and pH) in a potentiometric setup where paper substrates were positioned horizontally on the sample surface to minimise the transport distance of the liquid inside the paper, thus minimise the possible effect of paper physical properties (porosity, geometrical dimensions) as mentioned above (Figure 7B) [10]. This is in contrast to the previous measurement setup, where a paper substrate was positioned vertically so that only the lowest part of the paper was in direct contact with a sample [9]. When comparing potentiometric measurements in paper-based and conventional beaker-based setups, it was discovered that while the potentiometric response in Cl^- solutions was barely affected by paper-based sampling, the measurements in Pb^{2+} and Cd^{2+} solutions showed that in a lower concentration range paper-based sampling had a significant effect on a potentiometric signal resulting in undesired super-Nernstian response and higher detection limit. The observed impact was especially significant in Pb^{2+} -ISEs, thus, to better understand the occurring phenomenon, before potentiometric paper-based measurements the authors pre-conditioned paper substrates for 1 h in $Pb(NO_3)_2$ solution, and compared the obtained calibration curve with the one in the beaker-based measurements discovering both curves were closely comparable (Figure 9). This suggested a conclusion that when the concentration of Pb^{2+} was low, chemically active functional groups (carbonyl and

hydroxyl groups) inherently available in paper (cellulose) structure formed complexes with Pb^{2+} as previously reported [133] and accordingly affected Pb^{2+} concentration determination. The pH measurements were similarly affected by the paper substrate for samples in a neutral pH range ($\text{pH} \sim 7$). Regardless of the reported drawbacks, the pairing of potentiometry and a paper substrate has shown potential for future applications for environmental and food quality monitoring [10]. This also illustrated that more studies should be done to facilitate modifications of the paper substrate.

Figure 9. Calibration curves for the Pb^{2+} -ISE using (---) paper substrates that were pre-conditioned for 1 h in $\text{Pb}(\text{NO}_3)_2$ and fresh paper substrates (—), while (···) is the dynamic response of the Pb^{2+} -ISE in a conventional, beaker-based measurement [10].

Additionally, Ding et al. demonstrated the benefit of using a paper substrate for the purpose of ion sampling and separation which was essential to achieve accurate measurements in situations when an ISE was not selective enough to the primary ion [89]. Specifically, the authors made the use of the liquid chromatography concept and complexation chemistry to remove strongly interfering salicylate ions and conduct potentiometric detection of Cl^- . The constructed device consisted of two pieces of papers: a T-shaped filter paper of 12-25 μm pore diameter employed as a detection zone and a filter paper of 3.0 μm pore diameter complexed with Fe^{3+} to be used as a separation zone. The schematic diagram of this set up is shown in Figure 10. Thereafter, salicylate ions were effectively removed through complexation with Fe^{3+} .

Figure 10. A schematic representation of a paper-based microfluidic sampling and separation system for potentiometric ion sensing. SEM images of the paper for separation zone (A1) and detection zone (A) are also accordingly shown [89].

CHAPTER 3 RESEARCH GAPS AND OBJECTIVES

3.1. Paper pre-treatment for paper-based potentiometric heavy metal ion sensing.

Despite numerous advantages of using paper-based solution sampling in potentiometry, hindering effect of super-Nernstian ISE response when especially measuring heavy metals can occur. Cellulose-based paper substrates contain numerous hydroxyl and carboxyl groups, hence, the surface of commonly-used filter papers contains negatively charged adsorption sites [134]. These papers hence exhibit sorption potential for heavy metals [135], in fact the removal of heavy metals *via* adsorption onto modified cellulose-based substrates has been widely reported [136]. Engin et al. [137] reported the effect of the adsorptive property of filter papers on the determination of heavy metal ion concentrations. Running samples through filter paper caused systematic errors especially for the detection of lower concentrations in aqueous solution. Additionally, the potentiometric paper-based sampling exhibits super-Nernstian response in heavy metal determination. Usually, the super-Nernstian response occurs for polymeric membrane ISEs when the ion inward transport rate from the solution is not fast enough to supply the ion transport into the membrane at the sample | membrane interface [138]. In this situation of paper-based sampling, attention should be paid on paper | ISM interface. The super-Nernstian response is caused by the adsorption or binding of cations onto cellulose-based substrates, e.g. heavy metal ions *via* hydroxyl groups present in the cellulose [139]. Namely, ions may partially be bound to the functional groups in the paper, thus leading to the diminished concentration of the ions available at the ISM. Moreover, the inward rate of the solution from a paper towards an ISE surface may be constrained and not supply the steady ion transport at the sample | ISE interface in a lower concentration range [9,10]. The current solution proposed by Lisak et al in substrate conditioning for 30 min is unsustainable for fast throughput ion sensing [10]. Thus, research has to be performed in order to eliminate the hindering effect occurring when measuring heavy metals in paper-based sampling mode. At the same time, it was reported the adsorption of heavy metal ions was considerably reduced in the presence of competing electrolytes or at higher concentrations of acid, these ions

compete with metal ions for negatively charged sites onto the paper [137,140]. Thus the use of heavy metal ions and acid modification on paper substrates is investigated in this thesis to eliminate the super-Nernstian response of ISEs sensitive to heavy metals. To eliminate these, firstly method of pre-modification of paper substrates utilising inorganic salts was investigated. Potentiometric determination of heavy metals in environmental samples (Cd^{2+} and Pb^{2+}) utilising paper-based microfluidic sampling was studied with a commercial crystalline membrane solid-state Cd^{2+} - and a solid-contact Pb^{2+} -ISEs. Moreover, the sorption-desorption of heavy metal ions to/from paper substrates was studied. Then, considering the possible ion desorption from the inorganic modified paper substrates during paper-based microfluidic sampling, the acidification of the paper substrates was further studied. Different pH values were investigated for paper modification and the ability of the heavy metal binding onto paper substrates and the potentiometric response of Pb^{2+} -ISEs was evaluated.

3.2. Sponge-based sampling for potentiometric heavy metal ion sensing.

Although paper substrates have been widely applied in microfluidic sampling, hindering effects associated with heavy metals adsorption are still unsolved. A further search for a more suitable substrate that could be potentially applied directly as the microfluidic analyte sampling and sample handling substrates is necessary to assure reliable determination of ions in various environmental samples. For example, a sponge is a material that can be used for wicking, thus delivering the solution to the sensor surface for the detection of ions in small volume with high solid impurity content samples. Thereby different types of sponges were studied in this thesis to develop the microfluidic sponge-based sampling integrated with ISEs and evaluate their performance in the context of reliable measurements in environmental samples. Owing to the lowest heavy metal sorption capacity of polyurethane (PU) sponge, microfluidic sponge-based sampling was developed and studied as novel sampling and sample handling substrate to serve as an alternative for solution sampling for ion analysis. The microfluidic sponge-based sampling coupled with potentiometric cells was carried out in heavy metals (Cd^{2+} and Pb^{2+}) analyses.

3.3. Paper modification to reduce biofouling for sampling in clinical samples.

Furthermore, when monitoring ions in clinical samples, such as blood, sweat, tears and saliva, ISEs and REs undergo biofouling due to adsorption of biomolecules, e.g. proteins onto the electrode surface [141–143]. This in turn alters the potential at the electrodes | sample solution interface, affecting the analysis of ions in clinically relevant samples [141–143]. The paper substrates used for microfluidic sampling may cause physico- and chemo-sorption of ions and biomolecules onto their structures [10,144–147]. This property may be used to slow down the transport of biomolecules and prevent them from reaching the electrode surface where the ion determination takes place. Moreover, additional barriers to eliminate/diminish electrode biofouling in clinical samples should be investigated to further reduce biofouling and realise better potentiometric performance. Gold nanoparticles (AuNPs) have unique physicochemical properties with a high surface-to-volume ratio, excellent chemical stability and low cytotoxicity. AuNPs can be easily functionalised and produced in various sizes, and they have been widely studied in biological applications [148–151]. Moreover, gold has a high affinity to proteins, e.g. serum albumin making it a suitable material for selective removal of proteins from the sample *via* adsorption [146]. Thus in this thesis, the modification of paper substrates with AuNPs and sputtered gold was performed as additional barriers to eliminate/diminish electrode biofouling in clinical samples.

3.4. Paper-based solid reference electrode.

Finally, in the potentiometric cell except for the ISE, a reference electrode is used. For that purpose, in conventional aqueous samples, a saturated calomel or a silver/silver chloride reference electrode is commonly applied [152], but it is not the most suited electrode for the on-paper applications, as it is bulky and it requires refilling the internal reference solution [14]. Also 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 M 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 [153]. For that reason, a new design of the solid paper-contact reference electrode is developed in this thesis for ion determination in environmentally and clinically relevant samples with high solid-to-liquid content. The new type of solid reference electrode is based on Ag/AgCl element with salt containing paper compartment. After optimisation of concentration and layer on the salt containing paper, the solid reference electrode integrated with paper-based microfluidics for potentiometric ion sensing was applied in clinically relevant ion (K^+ , Na^+ and Cl^-) concentration determination.

Thus, the objective of the thesis is to realise the microfluidic solution sampling coupled with potentiometry in environmentally relevant and clinically relevant samples with high solid-to-liquid content.

CHAPTER 4 CHEMICALS, EQUIPMENTS AND METHODS

4.1. Chemicals and materials.

The list of chemicals and equipment used in the research work is shown in Table 3 and Table 4, respectively.

Table 3 The list of chemicals used in this thesis.

Chemicals	Purity	Vendor
Cadmium nitrate ($\text{Cd}(\text{NO}_3)_2$)	$\geq 98\%$	Sigma-Aldrich (Germany)
Lead nitrate ($\text{Pb}(\text{NO}_3)_2$)	$\geq 99\%$	Sigma-Aldrich (Germany)
70% nitric acid (HNO_3)	$\geq 99\%$, trace metal grade	Sigma-Aldrich (Germany)
Sodium polystyrene sulfonate (NaPSS)	$\geq 99\%$	Sigma-Aldrich (Germany)
3, 4-Ethylenedioxythiophene (EDOT)	$\geq 99\%$	Sigma-Aldrich (Germany)
Lead ionophore IV	$\geq 99\%$	Sigma-Aldrich (Germany)
Sodium ionophore X	$\geq 99\%$	Sigma-Aldrich (Germany)
Potassium ionophore I	$\geq 99\%$	Sigma-Aldrich (Germany)
Sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB)	$\geq 98\%$	Sigma-Aldrich (Germany)
Potassium tetrakis(4-chlorophenyl) borate (KTPCIPB)	$\geq 99\%$	Sigma-Aldrich (Germany)
2-Nitrophenyl octylether (<i>o</i> -NPOE)	$\geq 99\%$	Sigma-Aldrich (Germany)
Bis(2-ethylhexyl) sebacate (DOS)	$\geq 99\%$	Sigma-Aldrich (Germany)
Tridodecylmethylammonium chloride (TDMACl)	$\geq 99\%$	
Poly(vinyl chloride) (PVC)	$\geq 99\%$	Sigma-Aldrich (Germany)
Tetrahydrofuran (THF)	$\geq 97\%$	Sigma-Aldrich (Germany)
Gold(III) chloride trihydrate (HAuCl_4)	$\geq 99\%$	Sigma-Aldrich (Germany)
Trisodium citrate dihydrate	$\geq 99\%$	Sigma-Aldrich (Germany)
Phosphate-buffered saline tablet (PBS)	$\geq 99\%$	Sigma-Aldrich (Germany)
Bovine serum albumin (BSA, lyophilized powder)	$\geq 99\%$	Sigma-Aldrich (Germany)
Potassium chloride (KCl)	$\geq 99\%$	Merck KGaA (Germany)
Sodium chloride (NaCl)	$\geq 99\%$	VWR International (USA)
Potassium hexacyanoferrate (III) ($\text{K}_3[\text{Fe}(\text{CN})_6]$)	$\geq 99\%$	VWR International (USA)
30% hydrogen peroxide (H_2O_2)	$\geq 99\%$	VWR International (USA)
Instrument calibration standard 2	$\geq 99\%$	PerkinElmer (USA)
IV-ICPMS-71A standards	$\geq 99\%$	Inorganic Ventures (USA)
IV-STOCK-59 standards	$\geq 99\%$	Inorganic Ventures (USA)
Control Abtrol serum		Thermo Fisher (USA)
Artificial tear		Allergan, Inc. (USA)
Filter paper 388 particle retention, 12-15 μm	100 % cotton linters α -cellulose > 98 %	Sartorius (Germany)
Filter paper 389 particle retention, 8-12 μm	100 % cotton linters α -cellulose > 98 %	Sartorius (Germany)
Filter paper 390 particle retention, 3-5 μm	100 % cotton linters α -cellulose > 98 %	Sartorius (Germany)
Filter paper 391 particle retention, 2-3 μm	100 % cotton linters α -cellulose > 98 %	Sartorius (Germany)
Filter paper 392 particle retention, 5-8 μm	100 % cotton linters α -cellulose > 98 %	Sartorius (Germany)

White Ribbon Filter paper Whatman 589/2	Merck KGaA (Germany)
Acrodisc 25 mm Syringe Filter with 0.45 μ m supor membrane	Pall (USA)

Table 4 The list of equipments and tools used in this thesis.

Equipment and tools	Vendor
InLab Surface Pro ISM pH electrode	Mettler-Toledo (Switzerland)
Seven Compact pH/ion S220 meter	Mettler-Toledo (Switzerland)
Crystalline solid-state membrane Cd ²⁺ -selective electrode, Cd ²⁺ -ISE	Thermo Fisher (USA)
Single junction silver/silver chloride (3 M KCl) reference electrode	Thermo Fisher (USA)
Glassy carbon (GC) disk electrodes	Bioanalytical Systems (USA)
EMF16 Interface potentiometer	Lawson Labs (USA)
Integral Water Purification System	Millipore (USA)
CHI760E electrochemical workstation	CH Instruments (USA)
JSM-7600F Field Emission Scanning Electron Microscopy	Jeol (Japan)
Titan Microwave Sample Preparation System (MPS)	PerkinElmer (USA)
ELAN® DRC-e Inductively coupled plasma mass spectrometry (ICP-MS)	PerkinElmer (USA)
Optima 8300 Inductively coupled plasma Optical Emission Spectrometry (ICP-OES)	PerkinElmer (USA)
ICS110 ion chromatography (IC)	ThermoFisher (USA)
Gamry interface 100 potentiostat	Gamry Instruments Inc (USA)
43603-200E Automated Rudolph ellipsometer	Rudolph Research (USA)
OCA 15 goniometer	Dataphysics (Germany)

4.2. Methods.

4.2.1. Characterisation methods.

4.2.1.1. Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES).

Inductively coupled plasma mass spectrometry (detection limit can extend to ppb concentration level, element dependent) and inductively coupled plasma optical emission spectrometry (detection limit can extend to ppm concentration, level element dependent) are reliable analytical techniques used for trace element analysis. Both of them are based on sample pre-treatment using inductively coupled plasma (ICP). For ICP-MS, the mass spectrometry (MS) is coupled with ICP, where ions are detected by a mass analyser according to their mass-to-charge ratio [154]. For ICP-OES, the light emitted by moieties when de-excitation occurs is measured by optical emission spectrometry (OES). The dispersive system separates the wavelengths emitted by all the elements present in the sample to ensure the selectivity to different elements [155].

Both methods are calibration based, which means a series of calibration solutions containing a precise concentration of the elements in standards are used to generate calibration curve prior to the actual elemental analysis in the sample solution.

4.2.1.2. Ion chromatography (IC).

Ion chromatography is an analytical liquid chromatographic technique with wide applicability. It is able to determine nearly any kind of charged molecule, e.g. anions, large proteins and amino acids. The IC was used in this work to analyse major anions, e.g. chloride. The IC works on separating molecules based on their respective charged groups [156]. For anion analysis, the equilibrated cationic stationary phase is used, where the targeted anions of a mixture to be separated and quantified can bind while passing through the column. Then, the specific ionic concentrations are depended on their different retention times through the pressurised chromatographic column.

4.2.1.3. Field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy analysis (EDXA).

Field emission scanning electron microscopy is an advanced method for studying the morphology of solid materials. In this method, the electrons are first liberated by a field emission source, the secondary electrons then are caught and compared with the intensity of the scanning primary electron beam thus generating the image [157]. Moreover, the energy dispersive X-ray spectroscopy analysis (EDXA) is assisted with the FE-SEM to provide elements distribution in the investigated samples. It works on detecting x-rays emitted from a sample to characterise the difference in energy between the atomic structure of the emitting element, realising the measurement of the elemental composition [158].

4.2.1.4. Ellipsometry.

Ellipsometry is an optical technique to study the dielectric properties of thin films. It is a non-destructive and non-contact based technique. It analyses the state of polarisation

of the light upon reflection or transmission from the sample [159]. In this study, ellipsometry was used to quantify protein adsorption onto gold substrates after the use of unmodified and modified paper substrates used to stop or diminish transport of protein across a variety of solution sampling substrates.

4.2.2. Preparation of electrodes and paper substrates.

4.2.2.1. Solid-state Cd^{2+} -selective electrodes.

The solid-state Cd^{2+} -selective electrodes have the crystals-based membrane composed of cadmium sulfide. Before the use, the solid-state Cd^{2+} -ISE was gently polished using 0.05 μm alumina slurry (Al_2O_3) on a polishing soft pad to renew the surface of the electrode and then vigorously rinsed with ultra-pure water. The Cd^{2+} -ISE was stored in 10^{-3} M $\text{Cd}(\text{NO}_3)_2$ solution between the measurements and polished after each measurement.

4.2.2.2. Solid-contact ion-selective electrodes.

Glassy carbon (GC) disk electrodes (working surface area of 0.07 cm^2) were used for the construction of solid-contact Pb^{2+} -, K^+ -, Na^+ - and Cl^- -ISEs. Before use, GC electrodes were polished using 0.05 μm polishing alumina slurry (Al_2O_3) on a polishing soft pad to remove the unwanted impurities sticking to the surface, followed by vigorous rinsing with ultra-pure water. Then electropolymerization of PEDOT doped either by PSS^- for Pb^{2+} -, K^+ -, Na^+ -ISEs or Cl^- for Cl^- -ISEs on GC electrodes (working electrodes) was conducted in a three-electrode cell, where a Pt mesh and an Ag/AgCl (3 M KCl) served as counter and reference electrodes, respectively. The electropolymerization was performed using CHI760E electrochemical workstation, CH Instruments, Inc. (USA) by applying a constant current of 0.014 mA (0.2 mA cm^{-2}) for 714 s to obtain a polymer layer of approx. 790 nm on the electrode surface [75,160]. The electropolymerization was performed in the aqueous solution containing 0.01 M EDOT and 0.1 M NaPSS or KCl (for cation and anion sensitive ISEs, respectively). After the electropolymerization, the electrodes were rinsed with ultra-pure water and

dried overnight in the open air at room temperature (23-25°C). Then, ISMs were drop cast on top of the dried PEDOT (PSS⁻ or Cl⁻) by applying 60 µL of ISM cocktail in portions of 20 µL with a one-hour interval between each portion of ISM cocktail. The composition of the ISM cocktails were (w/w %): 1% lead ionophore (IV), 0.5% KTpClPB, 65.2% *o*-NPOE and 33.3% PVC for Pb²⁺-ISEs, 1% sodium ionophore X, 0.3% KTpClPB, 66% *o*-NPOE and 32.7% PVC for Na⁺-ISEs, 1% potassium ionophore I, 0.3% KTpClPB, 65.2% DOS and 33% PVC for K⁺-ISEs and 15% TDMACl, 51% *o*-NPOE and 34% PVC for Cl⁻-ISEs. Each ISM cocktail was prepared by dissolving membrane components in 2 mL THF. After ISM membrane cocktail casting, the ISEs were left overnight in the open air at room temperature (23 ± 2 °C) to evaporate the residual solvent. Before use, all ISEs were conditioned for 24 h in 10⁻³ M Pb(NO₃)₂ for Pb²⁺-ISEs, 10⁻³ M NaCl for Na⁺-ISEs, 10⁻³ M KCl for K⁺-and Cl⁻-ISEs and between measurements, ISEs were stored in 10⁻³ M Pb(NO₃)₂ for Pb²⁺-ISEs, 10⁻³ M NaCl for Na⁺-ISEs, 10⁻³ M KCl for K⁺-and Cl⁻-ISEs.

4.2.2.3. Paper substrates preparation.

The paper substrates of different grades were cut into pieces of different sizes and immersed in ultra-pure water for 30 min while gently stirring in order to remove any water-soluble components. Then, wet paper substrates were oven-dried at 70°C for 30 min before their subsequent use in physiochemical characterisation and in paper-based sampling.

4.2.3. The paper-based potentiometric measurements with ISEs.

The potentiometric measurements were carried out using an EMF16 Interface potentiometer, Lawson Labs Inc. assisted with L-EMF DAQ 3.0 software (USA). Potentiometric response in each standard solution was registered for 60 s at room temperature (23-25°C). The measurements were done with the approximately decade changes in concentration over the range 10⁻⁷ to 10⁻¹ M for all ISEs, respectively, in the order of increasing ion activity in the analyte. Standard deviation was calculated using three consecutive measurements of the same type. Before paper-based sampling

assisted potentiometric measurements, the ISEs were tested for their Nernstian response in a conventional beaker-based potentiometric cell. The electrodes that exhibited Nernstian response in beaker-based measurements were further used and coupled with paper-based microfluidic sampling. In the paper-based/sponge-based assisted potentiometric measurements, the potentiometric cell was pressed by gravitational force (if not stated otherwise) against paper/sponge substrates that contained standard solutions ($\text{Cd}(\text{NO}_3)_2$ for Cd^{2+} -ISEs, $\text{Pb}(\text{NO}_3)_2$ for Pb^{2+} -ISEs, NaCl for Na^+ -ISEs, KCl for K^+ - and Cl^- -ISEs). Once electrodes were positioned, the potentiometric response was then recorded for 60 s in each standard solution by registering EMF every 0.2 s. For each paper-based sampling, a new paper substrate was used.

4.2.4. Microwave digestion of paper substrates.

Paper substrates were weighed and cut into smaller pieces (approx. $2 \times 2 \text{ mm}^2$) and transferred into a 100 mL Teflon vessel to run microwave digestion in a solution of 7 mL 70% trace metal grade HNO_3 . The microwave digestion was performed using the Titan Microwave Sample Preparation System (MPS) (USA), where the sample was kept for 5 min in 120°C and 30 bar, then while keeping the pressure constant the temperature was further increased to 170°C and kept for another 5 min, finally the temperature was decreased to 50°C and kept for 10 min. After the digestion, the sample was left to cool down at a room temperature ($23\text{-}25^\circ\text{C}$). Then digested sample was transferred into 25 mL volumetric flasks and topped up with ultra-pure water. Acrodisc 25 mm Syringe Filter with $0.45 \text{ }\mu\text{m}$ supor membrane (Pall, USA) was used to filter out the possible impurities before the sample analysis by ELAN® DRC-e ICP-MS (USA).

CHAPTER 5 PAPER MODIFICATIONS IN PAPER-BASED MICROFLUIDIC SAMPLING FOR HEAVY METAL DETECTION

5.1 Inorganic salt modifications of paper substrates for paper-based solution sampling coupled with ISEs.

Recently, paper-based sampling coupled with potentiometry were developed to handle low-volume aqueous samples [9,10]. The measurements of clinically relevant ions, e.g. K^+ , Na^+ or Cl^- were not affected by the paper-based sampling. To the contrary, measurements of Cd^{2+} and Pb^{2+} were heavily interfered by the paper-based sampling. It was observed that heavy metals undergo either physical adsorption or chemical bonding to the cellulose material [11], thus the Super-Nernstian response was observed. Modifications of the paper substrates are intended to reduce the interaction between the paper substrates and the heavy metals in sample solutions, thus eliminating the Super-Nernstian response and providing more accurate ion analysis.

5.1.1. Experimental.

5.1.1.1. Paper pre-treatment protocols.

Cellulose filter papers 388, 389, 390, 391 and 392 (further named PS1, PS2, PS3, PS4 and PS5) were used in this study. The paper-based sampling were performed in two various protocols of the samplings, which further on are denoted as protocols 1 and 2 that differ in the time that the paper substrate was in contact with the standard solutions. Thus in protocol 1 sampling was performed when pipetting a standard solution of $Cd(NO_3)_2$ or $Pb(NO_3)_2$ on the clean oven-dried paper substrates, while in protocol 2 the clean oven-dried paper substrates were soaked in standard solutions for 30 min. After that paper substrates were taken out, the excess of the standard solution was removed through gentle shaking off the paper substrate, placed on the clean Teflon plate with the subsequent potentiometric cell placement, followed by immediate potentiometric measurement.

Subsequently, for paper based sampling two more paper substrates pre-treatments protocols were investigated, further one denoted as protocols 3 and 4. In both cases, the

paper substrates was pre-treated in the inorganic salt solutions ($\text{Cd}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$) at concentrations 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M for 30 min while gently stirring. Then, the soaked paper substrates were vigorously washed with ultra-pure water and oven-dried at 70°C for 30 min. Such paper substrates were used in protocols 3 and 4. In protocol 3, the ion used for pre-treatment of the paper based substrates was same as the one of primary ion in the standard solutions used in potentiometric measurement, e.g. paper based substrates were pre-treated with different concentrated solutions of $\text{Pb}(\text{NO}_3)_2$ or $\text{Cd}(\text{NO}_3)_2$, with subsequent measurement of Pb^{2+} and Cd^{2+} , respectively. In protocol 4, the ion (interfering ion) used for pre-treatment of the paper based substrates was different as the one of primary ion in the standard solutions used in potentiometric measurement, e.g. paper based substrates were pre-treated with different concentrated solutions of $\text{Pb}(\text{NO}_3)_2$ or $\text{Cd}(\text{NO}_3)_2$, with subsequent measurement of Cd^{2+} and Pb^{2+} , respectively. Then a standard solution of $\text{Cd}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$ was pipetted on the paper substrates, after that the excess of the standard solution was removed through gentle shaking off the paper substrate, placed on the clean Teflon plate with the subsequent potentiometric cell placement, followed by immediate potentiometric measurement.

In both cases, the paper substrates were pre-treated in the inorganic salt solutions ($\text{Cd}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$) at concentrations 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M for 30 min while gently stirring. Then, the soaked paper substrates were vigorously washed with ultra-pure water and oven-dried at 70°C for 30 min. Such paper substrates were used in protocols 3 and 4. In protocol 3, the ion used for pre-treatment of the paper-based substrates was same as the one of the primary ion in the standard solutions used in potentiometric measurement, e.g. paper-based substrates were pre-treated with differently concentrated solutions of $\text{Pb}(\text{NO}_3)_2$ or $\text{Cd}(\text{NO}_3)_2$, with subsequent measurement of Pb^{2+} and Cd^{2+} , respectively. In protocol 4, the ion (interfering ion) used for pre-treatment of the paper-based substrates was different as the one of primary ion in the standard solutions used in potentiometric measurement, e.g. paper-based substrates were pre-treated with different concentrated solutions of $\text{Pb}(\text{NO}_3)_2$ or

$\text{Cd}(\text{NO}_3)_2$, with subsequent measurement of Cd^{2+} and Pb^{2+} , respectively. Then a standard solution of $\text{Cd}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$ was pipetted on the paper substrates, after that the excess of the standard solution was removed through gentle shaking off the paper substrate, placed on the clean Teflon plate with the subsequent potentiometric cell placement, followed by immediate potentiometric measurement.

5.1.1.2. Cd^{2+} and Pb^{2+} ions retention in the paper substrates.

The retention of Cd^{2+} or Pb^{2+} within the paper substrates was evaluated by ELAN® DRC-e inductively coupled plasma mass spectrometry (ICP-MS) (USA) after paper substrates ($3 \times 3 \text{ cm}^2$) pre-treatment with various concentrations of $\text{Cd}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$ at concentrations 10^{-6} M , 10^{-5} M , 10^{-4} M and 10^{-3} M for 30 min while gently stirring. Then, the soaked paper substrates were vigorously washed with ultra-pure water and oven-dried at 70°C for 30 min. After that, paper substrates were sent to microwave digestion before going to ICPMS.

5.1.2. The paper-based potentiometric measurements with Cd^{2+} - and Pb^{2+} -ISEs.

Figure 11 (A, B, C and D) presents the paper-based potentiometric measurements utilising microfluidic paper sampling protocols 1 and 2 using PS1, PS2, PS4 and PS5, respectively, with a solid-state Cd^{2+} -ISEs and a solid-contact Pb^{2+} -ISEs. For both electrodes, namely Cd^{2+} - and Pb^{2+} -ISEs, the application of the microfluidic paper sampling protocol 1, resulted in a super-Nernstian potential response between $10^{-4.0}$ - $10^{-3.1} \text{ M}$ Cd^{2+} and Pb^{2+} . The super-Nernstian effect is a disturbance caused by the application of the paper substrates at the sample solution | ISM interfaces. Typically, such effects are associated with unconditioned membranes, where the flux of ion into ISM (enhanced by ionophore-ion binding) results in depletion of the ion at the sample solution | ISM interface [132,161–164]. Also, it was observed before that microfluidic paper-based sampling influences heavy metal measurements [10]. In this situation, the super-Nernstian response was caused by the binding of metal ions onto cellulose-based paper substrates, e.g. Cd^{2+} and Pb^{2+} via hydroxyl groups present in the cellulose [139].

In any case, the metal ions interacting with paper substrates at lower analyte concentration depleted the sample solution | ISM interface from heavy metals and the super-Nernstian response occurred [10].

Figure 11. Potentiometric response of the Cd²⁺- and Pb²⁺-ISEs in 10^{-7.0}-10^{-1.3} M and 10^{-7.0}-10^{-1.4} M standard solutions of Cd(NO₃)₂ and Pb(NO₃)₂ respectively when coupled with microfluidic paper-based sampling protocols 1 and 2 using (A) PS1 (B) PS2 (C) PS4 (D) PS5.

Obviously, this is a serious limitation considering the usefulness of the microfluidic paper-based sampling for the determination of heavy metals in various samples. Thus, the research directions in this work were to eliminate the Super-Nernstian effect and extend the Nernstian response of the heavy metal sensitivity of ISEs. One of the ways to eliminate the super-Nernstian response for the determination of heavy metals using microfluidic paper-based sampling was to condition the paper substrates in each of the standards and sample solution before actual potentiometric measurement, similarly as it was done before [10]. As a result, for both Cd²⁺- and Pb²⁺-ISEs, the super-Nernstian

response was eliminated after the application of microfluidic sampling protocol 2. For Cd^{2+} -ISEs the slope of the calibration curve in the concentration range $10^{-5.0}$ - $10^{-1.3}$ M Cd^{2+} and the slope of the calibration curve in the concentration range $10^{-5.0}$ - $10^{-2.2}$ M Pb^{2+} for Pb^{2+} -ISEs are presented in Table 5. For both types of ISEs the lower detection limit was $10^{-5.0}$ M, tenfold higher than that observed for solution-based potentiometric measurements (Table 5). All paper substrates, except for PS3, coupled with Cd^{2+} - and Pb^{2+} -ISEs in microfluidic sampling protocol 1 and 2 exhibited analogous behaviours (Figure 11 (A, B, C and D) and Table 5).

The same approach was investigated in previous work and was similarly found that conditioning of paper substrates for 30 min in each of the standard solutions (as in protocol 2) resulted in liner Nernstian response compared to measurements done without paper substrate conditioning. In fact, the performance of Cd^{2+} -ISE was found to be better than previously reported [10]. It is clear that the paper ion binding during the measurements is mitigated by preconditioning with cations. In this protocol, however, the paper substrate is simply equilibrated with the sample which has its disadvantages, namely: (i) time for equilibration (30 min) is too long for any practical applications, (ii) substances released from the paper matrix during the conditioning process may contaminate the standards and sample solution and (iii) the conditioning partially consumes cations from the standards and sample solution, thus altering the concentration of the analyte. For that reason, there is a clear need of another paper substrate modification protocol that would allow more reliable applications of paper-based microfluidic sampling in heavy metal sensing.

Table 5 Lower detection limit (LDL) and slope of the Cd^{2+} - and Pb^{2+} - ISEs using microfluidic paper-based sampling protocols 1 and 2.

ISEs/paper substrates	LDL/log a_i	Slope /mV dec ⁻¹
Cd^{2+}-ISE response utilising protocol 1		
In solution	-6.0	27.8 ± 1.3
PS1*	-3.1	33.3 ± 0.7
PS2*	-3.1	33.2 ± 3.2
PS3	-5.0	30.8 ± 0.5
PS4	-5.0	27.6 ± 1.3

PS5*	-3.1	34.3 ± 3.1
Cd²⁺-ISE response utilising protocol 2		
PS1	-5.0	36.0 ± 5.7
PS2	-5.0	34.3 ± 1.1
PS3	-5.0	30.7 ± 2.1
PS4	-5.0	38.8 ± 1.6
PS5	-5.0	33.8 ± 1.0
Pb²⁺-ISE response utilising protocol 1		
In solution	-5.0	35.7 ± 0.6
PS1*	-4.0	69.4 ± 1.3
PS2*	-4.0	61.4 ± 8.4
PS3*	-4.0	55.6 ± 7.8
PS4*	-4.0	53.5 ± 5.7
PS5*	-4.0	50.0 ± 0.5
Pb²⁺-ISE response utilising protocol 2		
PS1	-5.0	32.1 ± 0.8
PS2	-5.0	29.3 ± 1.5
PS3	-5.0	28.0 ± 0.7
PS4	-5.0	29.6 ± 0.5
PS5	-5.0	30.7 ± 1.1

*Non-Nernstian response.

Moreover, in microfluidic sampling protocol 1, the super-Nernstian potential jump was higher for Pb²⁺-ISEs comparing to Cd²⁺-ISEs utilising the same type of paper substrate (Figure 11 (A, B, C and D) and Figure 12). For example, when using Pb²⁺-ISEs with PS1, PS2, PS3, PS4 and PS5 the super-Nernstian jumps were 94.4 ± 5.3, 82.5 ± 32.1, 60.3 ± 17.8, 62.0 ± 25.0 and 62.1 ± 6.3 mV, respectively. On the other hand, when using Cd²⁺-ISEs with PS1, PS2, PS3, PS4 and PS5 the super-Nernstian jumps were 53.8 ± 3.1, 47.9 ± 1.0, 27.8 ± 0.1 (Nernstian response), 38.1 ± 5.1 and 46.6 ± 3.5 mV, respectively. The higher super-Nernstian jump for Pb²⁺-ISEs may be attributed to the fact that Pb²⁺ binds more strongly to cellulose than Cd²⁺ [165]. Uniquely, Cd²⁺-ISEs using microfluidic sampling protocol 1 with PS3 did not exhibit super-Nernstian response (Figure 12). The potentiometric measurements performed using PS3 for microfluidic sampling, in protocol 1 and 2, resulted in a similar and linear response between 10^{-5.0}-10^{-1.3} M Cd²⁺. In both cases, the slopes were close to Nernstian 30.8 ± 0.5 and 30.7 ± 2.1 mV dec⁻¹, respectively. The extended linear range, when performing immediate potentiometric measurements after sample collection, showed a great perspective of this paper to be directly used in potentiometric measurement for Cd²⁺

concentration determination. On the other hand, the measurements of Pb^{2+} using the same PS3 paper substrate still resulted with occurrence of super-Nernstian response (Figure 12). Thus it is clear that the paper substrate interacts weaker with Cd^{2+} than Pb^{2+} and modifications to paper substrates to block metal active groups in the structure of the paper are necessary to extend the applicability of paper-based sampling to measurements of all heavy metals.

Figure 12. Potentiometric response of the Cd^{2+} - and Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$ respectively when coupled with microfluidic paper-based sampling by PS3 in the microfluidic paper-based sampling protocols 1 and 2.

5.1.3. Paper substrate pre-treated with different primary and interfering ion concentrations.

In microfluidic paper-based sampling protocol 2 each of the paper substrates was allowed to stay in contact with the corresponding standard solution for 30 min. In this process, all metal active sites at the paper substrates were consumed by the inorganic salt/ions and the measurement could be performed. This protocol, however, has its serious disadvantages as mentioned in the previous chapter. To investigate the applicability of the inorganic salt modification to the paper substrate and to eliminate the disadvantages of microfluidic paper-based sampling protocol 2, the microfluidic paper-based sampling protocol 3 and 4 were developed. In these protocols, the paper substrate was treated with different inorganic salt solutions before the microfluidic

sampling of the analyte. Thus, the modification/blocking of the active sites on the paper substrates was a separate process and was performed before the calibration/sample concentration determination measurements.

Figure 13 (A, B, C and D) presents the potentiometric response of Cd^{2+} -ISE in $\text{Cd}(\text{NO}_3)_2$ solutions using PS1, PS2, PS4 and PS5 with prior pre-treatment of paper substrates in the concentration range of $10^{-7.0}$ - $10^{-1.3}$ M of $\text{Cd}(\text{NO}_3)_2$. The results were compared to the ones obtained using microfluidic paper-based sampling protocols 1 and 2. The super-Nernstian potential jump between $10^{-3.1}$ - $10^{-4.0}$ M Cd^{2+} as obtained applying the microfluidic paper-based sampling protocol 1 was eliminated after the pre-treatment of paper substrates with all concentrations of $\text{Cd}(\text{NO}_3)_2$ solution (microfluidic paper-based sampling protocol 3).

Figure 13. Potentiometric response of the Cd^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS4 (D) PS5, utilising microfluidic paper-based sampling protocol 3 when the paper was pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M of primary ion concentrations. The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

For all the paper substrates except of PS3 (the optimal paper substrate pre-treatment concentration was 10^{-5} M $\text{Cd}(\text{NO}_3)_2$) (Figure 14), the paper pre-treatment with 10^{-4} M $\text{Cd}(\text{NO}_3)_2$ solution resulted in the best potentiometric response (Table 6), which was comparable to the microfluidic paper-based sampling protocol 2 (Figure 13, Table 6).

Figure 14. Potentiometric response of the Cd^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ using PS3, utilising microfluidic paper-based sampling protocol 3 when the paper was pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M of primary ion concentrations. The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Table 6 Lower detection limit (LDL) and slope of the Cd^{2+} -ISEs applying microfluidic paper-based sampling protocol 3.

Cd^{2+} -ISE	LDL/ $\log a_i$	Slope / mV dec^{-1}
10^{-3}M		
PS1	-4	26.6 ± 0.8
PS2*	-5	28.0 ± 0.7
PS3	-4	23.7 ± 1.5
PS4	-4	25.0 ± 0.5
PS5	-4	28.3 ± 0.5
10^{-4}M		
PS1	-5	26.6 ± 0.8
PS2	-5	28.0 ± 0.7
PS3	-5	23.7 ± 1.5
PS4	-4	25.0 ± 0.5
PS5	-5	28.3 ± 0.5
10^{-5}M		
PS1	-4	33.4 ± 2.1
PS2	-4	34.9 ± 3.7
PS3	-4	30.6 ± 1.9

PS4	-4	29.8 ± 2.3
PS5	-5	22.2 ± 1.4
10^{-6} M		
PS1	-4	54.6 ± 6.8
PS2*	-5	26.0 ± 2.1
PS3	-5	30.9 ± 1.7
PS4*	-4	35.0 ± 14.4
PS5	-4	26.5 ± 3.3

* Non-Nernstian response.

Figure 15 (A, B, C, D and E) presents the potentiometric response of Pb^{2+} -ISE in $\text{Pb}(\text{NO}_3)_2$ solutions using PS1, PS2, PS3, PS4 and PS5 with prior pre-treatment of paper substrates in concentration range of $10^{-7.0}$ - $10^{-1.3}$ M of $\text{Pb}(\text{NO}_3)_2$. The super-Nernstian potential jump between $10^{-3.1}$ - $10^{-4.0}$ M Pb^{2+} as obtained applying the microfluidic paper-based sampling protocol 1 was eliminated after the pre-treatment of the paper substrates only with certain solutions of $\text{Pb}(\text{NO}_3)_2$ (microfluidic paper-based sampling protocol 3). The paper pre-treatment with 10^{-4} M $\text{Pb}(\text{NO}_3)_2$ solution resulted in the best potentiometric response (Table 7), which was comparable to the microfluidic paper-based sampling protocol 2. The pre-treatment of paper substrates with only 10^{-3} and 10^{-4} M $\text{Pb}(\text{NO}_3)_2$ resulted in close to Nernstian response of Pb^{2+} -ISEs utilising the microfluidic paper-based sampling protocol 3. In both cases, the pre-treatments of paper substrates with more concentrated inorganic salts (10^{-3} and 10^{-4} M) were more beneficial for overall potentiometric response when such paper substrates were further on utilised for solution sampling. The concentrations of inorganic salts at 10^{-5} and 10^{-6} M during pre-treatment of paper substrates were not effective for Pb^{2+} -ISEs that suggested the insufficient concentration of the ion to block all metal active sites on the paper substrates.

Figure 15. Potentiometric response of the Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M and $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Pb}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5, utilising microfluidic paper-based sampling protocol 3 when the paper was pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M of primary ion concentrations. The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Table 7 Lower detection limit (LDL) and slope of the Pb^{2+} -ISEs applying microfluidic paper-based sampling protocol 3.

Pb^{2+} -ISE	LDL/log a_i	Slope /mV dec^{-1}
10^{-3} M		
PS1	-5	21.0 ± 0.8
PS2	-5	21.2 ± 0.8
PS3	-5	30.0 ± 1.9
PS4*	-4	26.4 ± 0.9
PS5	-4	21.2 ± 2.4
10^{-4} M		
PS1	-5	24.8 ± 1.3
PS2	-4	29.4 ± 0.9
PS3*	-4	27.3 ± 1.9
PS4	-5	23.8 ± 0.3
PS5	-4	31.0 ± 1.9
10^{-5} M		
PS1*	-5	31.7 ± 2.5
PS2	-4	48.7 ± 4.8
PS3*	-4	55.5 ± 1.3
PS4	-4	32.6 ± 1.7
PS5	-4	32.6 ± 3.4

10⁻⁶ M		
PS1	-5	32.4 ± 1.4
PS2	-5	34.2 ± 2.1
PS3*	-4	60.3 ± 2.8
PS4*	-4	40.2 ± 1.0
PS5*	-4	36.1 ± 1.7

* Non-Nernstian response.

In protocol 3, the cation in inorganic salt used for modification of paper-based substrates was the same as the primary ion for ISEs. To investigate the applicability of the different inorganic salt modifications to the paper substrates than the primary ion for ISEs, the microfluidic paper-based sampling protocol 4 was introduced. For that paper-based substrates were pre-treated with differently concentrated solutions of Cd(NO₃)₂ or Pb(NO₃)₂ with subsequent measurement of Pb²⁺ and Cd²⁺, by Cd²⁺- and Pb²⁺-ISEs, respectively.

Figure 16 (A, B, C, D and E) presents the response Cd²⁺-ISEs in 10^{-7.0}-10^{-1.3} M using PS1, PS2, PS3, PS4 and PS5 utilising microfluidic paper-based sampling protocol 4 when paper substrates were pre-treated with 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶ M interfering ions, Pb²⁺. For the Cd²⁺-ISEs, the super-Nernstian potential jump between 10^{-3.1}-10^{-4.0} M Cd²⁺ as obtained applying the microfluidic paper-based sampling protocol 1 was eliminated after the pre-treatment of paper substrates with application of only 10⁻⁶ M Pb(NO₃)₂ solution (microfluidic paper-based sampling protocol 4), except of PS3 for which no Nernstian response was attained. In all other cases, the paper substrate pre-treatment in 10⁻³, 10⁻⁴ and 10⁻⁵ M interfering ions, except of PS5 pre-treatment in 10⁻⁵ M Pb(NO₃)₂ solution, resulted in sub-Nernstian potentiometric response. Thus, contrary to the microfluidic paper-based sampling protocol 3 where for paper substrate pre-treatment the high concentrations of the inorganic salt were needed, here the lowest concentration of interfering ion (10⁻⁶ M Pb(NO₃)₂) was needed to attain Nernstian response. In general, the selectivity of solid-state crystalline membranes suffers from the presence of interfering ions [54,166,167]. Thus the results indicated that Pb²⁺ undergoes partial desorption from the paper substrate to the standard solutions or ion-exchange at the ISM | paper substrate interface, negatively affecting the response of

Cd^{2+} -ISEs towards sub-Nernstian. Moreover, the fact that only 10^{-6} M $\text{Pb}(\text{NO}_3)_2$ was found to be the suitable pre-treatment solution indicates that paper substrate metal ion interactions are concentration-dependent (further explored in the next chapter).

Figure 16. Potentiometric response of the Cd^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.3}$ M standard solutions of $\text{Cd}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5, in microfluidic paper-based sampling protocol 4 when the paper substrates were pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M interfering ions, Pb^{2+} . The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

Figure 17 (A, B, C, D and E) presents the response Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.4}$ M using PS1, PS2, PS3, PS4 and PS5 utilising microfluidic paper-based sampling protocol 4 when the paper substrates were pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M interfering ions, respectively. None of the pre-treatment interfering ion concentrations, namely 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M Cd^{2+} were sufficient to attain Nernstian response of Pb^{2+} -ISEs. Contrary to the previous case, all Pb^{2+} -ISEs responses were super-Nernstian, similar to the ones obtained using the microfluidic paper-based sampling protocol 1. It has been documented before that Pb^{2+} bound more strongly to wood fibres than Cd^{2+} and alkali metals [138]. Thus, the pre-treatment of paper substrates with weaker bound Cd^{2+} resulted in its insufficient adsorption capacity onto the paper substrate that promoted further adsorption of Pb^{2+} onto the paper substrates when in contact with the Pb^{2+}

solutions. This indicates that paper substrates have a high adsorption capacity for metal ions (explored in the next chapter).

Figure 17. Potentiometric response of the Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Pb}(\text{NO}_3)_2$ using (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5, in microfluidic paper-based sampling protocol 4 when the paper substrates were pre-treated with 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} M interfering ions, Cd^{2+} . The potentiometric response was compared to the ones obtained using the microfluidic paper-based sampling protocols 1 and 2.

5.1.3 Interactions between paper substrates and the Cd^{2+} and Pb^{2+} ions.

Paper substrates pre-treated with different concentrations of primary and interfering ions as described in microfluidic sampling protocols 3 and 4 showed that the concentration of the inorganic salt used for conditioning of paper substrates influences the overall potentiometric performance of the ISEs coupled with paper-based microfluidic sampling.

Figure 18 (A, B, C, D and E) shows the concentration of cadmium and lead in PS1, PS2, PS3, PS4 and PS5 as measured by ICP-MS after paper substrates pre-treatment in the concentration range of 10^{-6} - 10^{-3} M of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$. After the conditioning, the excess of unbound and loosely bound metal ions were removed by vigorous washing the paper substrates with ultra-pure water. Thereby, the ICP-MS of digested paper

substrates represented only bound metal ions to the paper substrates. It was found that the sorption capacity of the paper substrate was directly proportional to the concentration of the ion in the solution during the paper substrate conditioning.

Figure 18. The concentration of cadmium and lead in (A) PS1 (B) PS2 (C) PS3 (D) PS4 (E) PS5 as measured by ICP-MS after paper substrates pre-treatment in the concentration range of 10^{-6} - 10^{-3} M of $\text{Cd}(\text{NO}_3)_2$ (circles) and $\text{Pb}(\text{NO}_3)_2$ (triangles).

The slope of the concentration measured vs. concentration used for conditioning was higher for Pb^{2+} sorption than Cd^{2+} (Table 8 and Table 9). Thus, the Pb^{2+} ions bounded to the paper substrate with higher affinity than Cd^{2+} ions which was in agreement as reported by other authors [138]. Moreover, the paper substrates, e.g. for PS4 were able to adsorb significant heavy metal amount, approx. 450 ppm of Pb^{2+} and 125 ppm of Cd^{2+} for pre-conditioning in 10^{-3} M solution. These results suggested that if untreated, the paper substrate used for microfluidic sampling or simply being a part of electrode construction may easily alter the concentration of the analyte in the sample solution, which is very valid for all the research that implements paper for the construction of paper devices, e.g. electrodes. Especially if those devices are developed for the intention to measure low analyte concentrations or/and to measure heavy metal concentrations. Cellulose based materials, e.g. sesame husk was found to be an efficient adsorbent for Cd^{2+} [135]. Both physical and chemical adsorption of heavy metals were

recognised to drive sorbent-metal interactions. Since paper substrates used in this study were cellulose-based, similar interactions properties between the metal ion and paper were expected. For that, as it was previously identified some of the ions are bound to the paper *via* physical sorption that is governed by weak Vander Waal's forces and they can be a subject of desorption from the paper to the solution [135]. Thus, if the paper is modified with inorganic salt/primary ion, the cation can be desorbed at low analyte concentration and pollute the sample solution, and at the same time negatively influence the lower detection limit of the sensor. That possibly is the reason why even after paper substrate modifications, the ISEs coupled with microfluidic paper-based sampling tend to have higher lower detection limit (at least tenfold higher) than the ones used in solution-based measurements. This may be a limitation for possible measurements of heavy metals at low analyte concentrations.

Table 8 The concentration of cadmium and lead in all paper substrates after paper substrate pre-treatment in the concentrations of 10^{-6} to 10^{-3} M of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$.

Grade of paper Pre-treatment ion concent.	PS1	PS2	PS3	PS4	PS5
Cd^{2+} concentration (ppm) bound to paper substrate					
10^{-3}	157.80 ± 0.71	183.18 ± 1.53	127.88 ± 1.10	145.98 ± 1.14	151.80 ± 1.62
10^{-4}	111.67 ± 0.12	102.67 ± 0.39	83.67 ± 0.64	99.00 ± 0.74	96.33 ± 0.61
10^{-5}	93.49 ± 0.72	73.10 ± 0.18	32.92 ± 0.27	48.68 ± 0.16	57.08 ± 0.28
10^{-6}	2.88 ± 0.05	7.95 ± 0.11	4.02 ± 0.04	3.79 ± 0.02	2.82 ± 0.03
blank	0.29 ± 0.01	0.93 ± 0.02	0.39 ± 0.03	0.95 ± 0.03	0.17 ± 0.01
Pb^{2+} concentration (ppm) bound to paper substrate					
10^{-3}	402.08 ± 1.85	510.00 ± 4.02	338.89 ± 3.97	430.77 ± 6.21	317.83 ± 6.18
10^{-4}	626.32 ± 14.61	425.00 ± 9.31	279.17 ± 3.42	315.68 ± 1.61	296.88 ± 3.15
10^{-5}	119.31 ± 0.52	187.50 ± 4.84	44.79 ± 0.48	123.92 ± 0.37	93.85 ± 1.75
10^{-6}	34.89 ± 0.37	18.79 ± 0.78	35.3 ± 0.61	15.10 ± 0.20	24.31 ± 0.20
blank	0.61 ± 0.005	1.03 ± 0.01	0.82 ± 0.04	0.60 ± 0.01	0.65 ± 0.09

Table 9 The slope of the concentration measured vs. concentration used for conditioning.

Grade of paper Concentration function	PS1	PS2	PS3	PS4	PS5
Cd²⁺ pre-treat.	48.29 ± 9.99	55.53 ± 6.58	42.2 ± 3.15	47.69 ± 0.70	48.62 ± 2.22
Pb²⁺ pre-treat.	175.23 ± 53.68	176.11 ± 10.10	115.88 ± 7.21	143.88 ± 11.33	110.20 ± 34.80

5.2 Acidification of paper substrates for paper-based solution sampling coupled with ISEs.

5.2.1. Paper-based potentiometric sampling utilising acidified paper substrates.

As PS3 paper substrate performed the best in potentiometric paper-based heavy metal ion sensing, this type of paper was further studied in terms of influence of its acidification on the potentiometric response of ISEs. Also, Pb²⁺ showed much greater sorption to paper substrates than cadmium, thus only lead was studied in the acidification of paper substrates for paper-based solution sampling coupled with ISEs.

A variety of HCl concentrations (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} M, referring to pH= 1, 2, 3, 4 and 5) were applied to acidify paper substrates. To do that, the clean paper substrates were soaked in different HCl solutions for 30 mins, and then dried in open air. Subsequently the pH of the acidified paper substrates was measured by positioning the flat-surface pH electrode against the paper after the addition of ultra-pure water to reach its full adsorption capacity. The measured pH of the acidified paper substrates were 2.2 ± 0.1 , 3.2 ± 0.1 , 4.3 ± 0.1 , 5.0 ± 0.1 and 5.3 ± 0.1 after pH 1, 2, 3, 4 and 5 of HCl solution modifications, respectively. Such paper substrates were utilised in paper-based solution sampling coupled with potentiometric sensors sensitive towards Pb²⁺. The paper-based potentiometric responses utilising pH 1, 2, 3, 4 and 5 acidified paper substrates are shown in Figure 19.

Figure 19. Potentiometric response of the Pb^{2+} -ISEs in $10^{-7.0}$ - $10^{-1.4}$ M standard solutions of $\text{Pb}(\text{NO}_3)_2$ in microfluidic paper-based sampling when the paper substrates were acidified by pH 1, 2, 3, 4 and 5 HCl solutions.

Previously, it was proven that the adsorption of heavy metals may be stopped at low pH [168]. Thus, the pH 2 acidified paper substrate showed elimination of the unwanted Super-Nernstian response, with the slope of $27.0 \pm 1.4 \text{ mV dec}^{-1}$ in the range of $10^{-5.0}$ to $10^{-2.2}$ M $\text{Pb}(\text{NO}_3)_2$ as the paper substrate did not participate in the sorption of Pb^{2+} from the solution. However, pH 1, 3, 4 and 5 acidified paper still resulted in a super Nernstian potential response of ISEs between $10^{-4.0}$ - $10^{-3.1}$ M Pb^{2+} . The unsuccessful elimination of Super-Nernstian response for pH 1 acidified paper substrates may be attributed to interference of hydronium ions at very low pH [169]. It was found most of the ISE response is independent of pH within the range of 3-6 [170–172], while the surface pH of the pH 1 acidified paper substrates was lower than the working range.

5.2.2 Lead adsorption onto paper substrates after their acidification.

To validate potentiometrically obtained results, the lead ion adsorption onto differently acidified paper substrates was investigated by immersing unmodified and pH modified paper substrates in 10^{-4} , 10^{-5} and 10^{-6} M $\text{Pb}(\text{NO}_3)_2$ solutions for 30 mins, subsequently rinsed by ultra-pure water, dried in open air and finally performing microwave digestion. The elemental composition of the lead in all paper substrates was investigated using

ICP-MS. The elemental lead concentration on differently acidified and pure paper substrates is compared in Figure 20.

Figure 20. Lead concentration determined by ICP-MS after 10^{-4} , 10^{-5} and 10^{-6} M $Pb(NO_3)_2$ modification onto pH 1, 2, 3, 4 and 5 acidified paper substrates.

The adsorption of lead onto paper substrates modified with pH 1 and pH 2 showed comparable and the lowest lead adsorption (8.0 ± 3.7 and 5.1 ± 1.7 ppm after soaking in 10^{-6} M $Pb(NO_3)_2$ for pH 1 and pH 2 acidified paper, 21.5 ± 3.3 and 13.1 ± 2.3 ppm after soaking in 10^{-5} M $Pb(NO_3)_2$ for pH 1 and pH 2 acidified paper, 48.5 ± 19.5 and 38.9 ± 7.9 ppm after soaking in 10^{-4} M $Pb(NO_3)_2$ for pH 1 and pH 2 acidified paper). On the other hand, the unmodified paper substrate showed the highest lead adsorption capacity (Figure 20). The results clearly indicate that lower pH on the paper substrates can significantly reduce the heavy metal adsorption, as higher concentration of H^+ will effectively compete with metal ions for negatively charged sites on the paper substrate, reducing the heavy metal interaction with paper substrates [137,140]. These result correlate well with the ones presented in Figure 19, where the super-Nernstian response of the Pb^{2+} -ISEs can be eliminated at lower pH. However, pH 1 paper modification, although resulting in low lead sorption capacity onto the paper substrate, it did not result in the improved potentiometric response of the Pb^{2+} -ISEs. This may be attributed to interference of hydronium ions at very low pH for the response of the potentiometric

sensor and generally stability of the ion-selective membrane at low pH [169,171,173]. Additionally, possible protonation of the ionophore in the ISM may occur under the effect of low pH [174]. As expected, higher pH (3 to 5) did not result in the elimination of the super Nernstian response between $10^{-4.0}$ - $10^{-3.1}$ M and Pb^{2+} , which is confirmed that at that pH high sorption of Pb^{2+} is observed at the paper substrates.

5.3 Conclusion.

In this chapter, paper pre-treatment by inorganic salts and acid was investigated to eliminate unfavourable super Nernstian response of the ISEs during heavy metal ion sensing. The paper substrates were found to be very good sorbents of metal ions, with stronger binding force given to Pb^{2+} over Cd^{2+} . Thus, the metal-paper interactions can lead to super Nernstian response. During inorganic salts pre-treatment, the Cd^{2+} - and Pb^{2+} -ISEs were used to investigate paper pre-treatment utilising primary and interfering ions. When the paper substrates were pre-treated with primary ions, relatively high concentration of inorganic salt (10^{-3} M) solution was required. While paper pre-treatment with interfering ions, only low concentration of 10^{-6} M Pb^{2+} solution was applicable when coupled with Cd^{2+} -ISEs. Further on, the acidification of paper substrates (pH 2 acidified) was found successfully eliminate the super Nernstian response when coupled with Pb^{2+} -ISEs is potentiometric cell.

CHAPTER 6 SPONGE-BASED SAMPLING AS ALTERNATIVE TO PAPER-BASED SOLUTION SAMPLING IN HEAVY METAL ION DETECTION

To address the unfavourable Super-Nernstian response occurring when paper-based sampling integrated with potentiometry in heavy metal analysis, pre-treatment of paper substrates in various concentrated solutions of primary ion and acidification of paper substrates, prior to the actual potentiometric measurements were applied. Nonetheless, a further search for a more suitable substrate that could be possibly applied directly (without any modification) for the microfluidic analyte sampling and sample handling is necessary to assure reliable determination of ions in environmental samples. A sponge is a material that can be used for wicking, thus delivering the solution to the sensor surface for the detection of ions in small volume with high impurity content samples. In this work, the use of sponges was tested in sponge-based sampling coupled with potentiometry in heavy metal detection. The illustration of the sponge-based sampling coupled with a potentiometric cell is shown in Figure 21.

Figure 21. Schematic representation of potentiometric cell utilising sponge-based microfluidic sampling.

6.1. Experimental.

The sponge substrates were prepared by cutting from the original material into pieces of $3 \times 2 \text{ cm}^2$ and soaked in ultra-pure water for 30 min while stirring to remove any water-soluble impurities. Subsequently, sponge substrates were subjected to drying in oven at 70°C for 30 min and were deemed to be ready for microfluidic sampling of

analytes.

The maximum liquid wicking ability of each sponge was tested by the mass change before and after soaking a $3 \times 2 \times 0.5 \text{ cm}^3$ piece of sponge in ultra-pure water for adsorbing until its full capacity. An evaporation rate of liquid registered for each sponge was obtained by calculating the change of mass between the sponge reaching its maximum wicking ability and 60 s after being exposed to evaporation in the open air at room temperature (23-25°C). The pH of sponges was carried out by placing the flat surface pH electrode on the top of a sponge substrate after reaching its full wicking capability with ultra-pure water.

To study the heavy metals sorption onto the sponge substrates, the sponge substrates were soaked in 10^{-6} , 10^{-5} , 10^{-4} and 10^{-3} M solutions of $\text{Cd}(\text{NO}_3)_2$ or $\text{Pb}(\text{NO}_3)_2$ for 30 min. After soaking, sponge substrates were taken out and vigorously rinsed with ultra-pure water and subjected to drying in the oven at 70°C for 30 min. Then, each sponge substrate was digested in microwave [144] and filtered before sent to ICP-OES to detect the concentrations of lead and cadmium in the obtained samples.

6.2. Optimisation of types of sponge substrates used in microfluidic sponge-based sampling.

To use sponge substrates as a novel sampling and sample handling substrates and to serve as an alternative for microfluidic paper-based sampling for application in ion sensing, three sponge substrates were evaluated, namely PU, natural and cellulose sponges. These sponges are three commonly used types in everyday life. PU sponges have good air permeability, elasticity, recovery ability under pressure and can absorb sound and shock. Natural sponges have neutral pH, super soft natural of fibers when wet and high liquid absorption capacity. Cellulose sponges have also high liquid absorption capacity. Various sponges can present different metal ion adsorption comparing to cellulose-based paper substrates.

The results of the physico-chemical characterisation of each type of sponge are shown

in Table 10.

Table 10 Physico-chemical characterisation of sponge substrates used for microfluidic sampling.

Sponges	PU sponge	Cellulose sponge	Natural sponge
Liquid absorption capacity /$\mu\text{L g}^{-1}$	70.2 ± 8.4	20.2 ± 1.7	20.1 ± 0.4
Evaporation rate /$\mu\text{L g}^{-1} \text{ min}^{-1}$	1.2 ± 0.3	1.7 ± 0.9	2.9 ± 0.8
pH	6.4 ± 0.1	7.3 ± 0.3	7.0 ± 0.2

The PU sponge had the highest liquid absorption capacity and had more porous structure and lower density (11 kg m^{-3}) than other cellulose (134 kg m^{-3}) and natural (59 kg m^{-3}) sponge substrates, thus it was able to retain a larger volume of liquid in its structure. Furthermore, the evaporation rate was the lowest for PU sponge substrates, owing to the evaporation induced concentration changes, subsequent error in concentration were found to be 1.8, 8.6 and 14.4% for PU, cellulose and natural sponge substrates, respectively. The highly porous nature of the PU sponge substrates and its ability to wick much more liquid directly translated into better retention of the liquid within the structure and lower influence of concentration change after undesired solution evaporation. Thus it was concluded that in the context of short solution sampling integrated with 60 s potentiometric measurement time the sponge-based microfluidic sampling poses no significant change in the concentration of the analyte that is wicked into the sponge matrix. Additionally, all sponges were characterised with close to neutral pH. Taken that the highest absorption liquid capacity of the PU sponge, while the evaporation rate was the lowest, PU-based sponge substrates were further considered for solid-liquid separation that would benefit the potentiometric measurements done by ISEs.

In previous studies, when microfluidic sampling utilising paper- and textile-based substrates integrated with potentiometric sensors were used, the Super-Nernstian response of ISEs occurred as a result of heavy metal ions depletion at the ISM surface. Such effect was ascribed to the binding of metal ions to the functional groups present in the paper substrates and/or to concurrent adsorption of metals onto the substrate

[9,10,144]. Table 11 presents the sorption capacity of heavy metals (Cd and Pb) in all sponge substrates (measured by ICP-OES) after sponge substrates pre-treatment in various concentrations of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$ (10^{-6} to 10^{-3} M).

Table 11 The sorption capacity of heavy metals (Cd and Pb) in sponge substrates determined using ICP-OES after sponge substrates pre-treatment in various concentrations of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$.

Heavy metal ion	Pre-treatment concentration	Total element concentration after pre-treatment /ppm		
		PU sponge	Cellulose Sponge	Natural sponge
Cadmium	10^{-3} M	40.9 ± 1.1	1205.9 ± 8.7	25710.5 ± 168.2
	10^{-4} M	34.4 ± 0.1	484.4 ± 5.2	21047.1 ± 82.0
	10^{-5} M	16.8 ± 0.2	35.7 ± 0.5	3010.4 ± 4.2
	10^{-6} M	3.0 ± 0.2	4.1 ± 0.0	356.3 ± 1.1
Lead	10^{-3} M	186.5 ± 1.4	3531.0 ± 50.0	46762.5 ± 151.3
	10^{-4} M	115.9 ± 1.3	1180.6 ± 6.2	34109.8 ± 146.3
	10^{-5} M	98.5 ± 1.0	224.1 ± 0.4	5336.5 ± 13.5
	10^{-6} M	34.9 ± 1.4	26.7 ± 0.5	300.9 ± 3.3

Furthermore, PU sponge substrate to visualise the concentration dependent pre-conditioning of the sponge substrate and its effect on the sorption of heavy metals (Cd and Pb) was shown in Figure 22. Similar to paper-based microfluidic sampling, heavy metals undergo sorption to sponge substrates. For all sponge substrates, much higher sorption rates of lead than cadmium were observed. Previously, the Pb^{2+} also showed much stronger binding affinity to paper substrates than Cd^{2+} [144]. The ion sorption of heavy metal ions onto sponge substrate may have either chemical or physical origin. For example, the adsorption of the metal ions is affected by the electronegativity and ionic radii of ions [175,176], thus the Pb^{2+} with greater electronegativity than Cd^{2+} can easier be adsorbed at the sampling substrate. Moreover, cellulose and natural sponge substrates were found to uptake significantly more cadmium and lead during the pre-treatment conditioning than PU-based sponge substrates. These in fact acted as heavy metal accumulators, and could be considered as suitable substrates for removal of heavy metals in the environmental samples, e.g. natural sponge directly or after modification can be used for purification of contaminated water [177,178]. Thus when applying

cellulose and natural sponge substrates in microfluidic sampling of analytes, the concentration of the sample may be easily altered. On the other hand, PU sponge substrates were characterised with the lowest heavy metal sorption capacity, even up to 2-4 times lower than that measured previously for paper-based substrates [144]. Since PU sponge substrate exhibited the lowest heavy metal sorption capacity, only the PU-based sponge substrates were further considered for microfluidic analyte sampling.

Figure 22. The sorption capacity of heavy metals (Cd and Pb) in PU sponge substrates determined using ICP-OES after sponge substrates pre-treatment in various concentrations of $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$.

The morphology of PU sponge before and after microfluidic sampling done on wastewater sludge taken from Water Reclamation Plant (UPWRP) Singapore was investigated by JSM-7200F field emission scanning electron microscopy (FE-SEM) (Japan). After PU sponge was applied for microfluidic solution sampling in wastewater sludge, the substrate was left to dry overnight in the open air at room temperature (23-25°C). Then, the sponge substrate was cut in half to expose the clear cross-section and two positions on that cross sections were investigated by FE-SEM. The cross-section of a PU sponge substrate before (i) and after (ii, iii) microfluidic solution sampling of wastewater sludge is presented in Figure 23. FE-SEM was performed at two different zones on the PU sponge substrate, namely (ii) the upper part where the wicked solution from the samples meet the potentiometric cell for ion determination and (iii) the

separation part where the PU sponge was in direct contact with the wastewater sludge. For comparison, a clean PU sponge was also presented (i). Already by naked eye inspection, there was a clear boundary between the part (ii) and (iii), indicating the separation of bigger solids from the solution. The PU sponge effectively stops the transport of major solids through its volume, which is assured by the highly porous nature of the sponge. The FE-SEM figure (Figure 23 (iii)) clearly presents full coverage of sponge walls with wastewater sludge solid matter. Naturally, it is not expected that the sponge would stop fine particles to go through the sponge to the electrode surface. From the FE-SEM figures, as compared to Figure 23 (i), it is clear that fine particles go through the volume of the sponge to the electrode surface (Figure 23 (ii)). Such particulates, however, should not pose a thread of damaging the ISM surface, as reported previously ISEs can tolerate solid fine particulates in the solution [179]. Thus the sponge substrate not only allows the transport of ions from the sample to the electrode surface for ion analysis but also protects the electrode from mechanical damages caused by the often rough nature of mixed solid-liquid samples.

Figure 23. The cross-section of a PU sponge substrate before (i) and after (ii, iii) microfluidic solution sampling of wastewater sludge.

6.3. Sponge-based microfluidic solution sampling coupled with Cd^{2+} - and Pb^{2+} -ISEs.

The potentiometric cell response in beaker- and sponge-based microfluidic solution sampling integrated with Cd^{2+} -ISEs and Pb^{2+} -ISEs is shown in Figure 24. The slopes of the calibration curves for Cd^{2+} -ISEs and Pb^{2+} -ISEs considering the concentration range of $10^{-5.0}$ - $10^{-1.3}$ M Cd^{2+} and $10^{-5.0}$ - $10^{-2.2}$ M Pb^{2+} were 27.2 and 30.0 mV dec⁻¹, respectively. Subsequently, for beaker-based potentiometric measurements, the slopes were 27.7 and 28.8 mV dec⁻¹, respectively.

The Cd^{2+} -ISEs presented comparable potentiometric responses for beaker- and sponge-based sampling (LDL= $10^{-5.0}$ M Cd^{2+}), while for Pb^{2+} -ISEs, the sponge-based had a tenfold higher lower detection limit than that of beaker-based measurement ($10^{-6.0}$ M Pb^{2+}).

Figure 24. Potentiometric cell response using the Cd^{2+} -ISEs (A) and Pb^{2+} -ISEs (B) in $10^{-7.0}$ - $10^{-1.0}$ M $\text{Cd}(\text{NO}_3)_2$ and $\text{Pb}(\text{NO}_3)_2$, respectively utilising beaker-based and sponge-based microfluidic sampling measuring setups.

Lead has higher sorption capacity than cadmium onto sponge-based substrates (as investigated in **Table 11**), thus for Pb^{2+} -ISEs the difference of the LDL between beaker- and sponge-based sampling potentiometric can be possibly attributed to concentration-dependent sorption of metals onto the sampling substrate. Furthermore, Cd^{2+} - and Pb^{2+} -ISEs showed comparable (none super-Nernstian behaviour) potentiometric responses between sponge-based and modified paper-based microfluidic sampling [144]. Contrary to sponge-based microfluidic sampling where no pre-treatment of the substrate is done, in order to eliminate unfavourable super-Nernstian effect observed when utilising paper-based microfluidic solution sampling, pre-treatment of paper in

the primary ion conditioning solution must be performed [144]. To conclude, the direct use of sponge-based substrates in microfluidic sampling in the determination of heavy metals, up to the sub-micromolar concentration range was found feasible. This also simplifies the protocol of measurement when microfluidic sampling is used, as previously the measurements of heavy metals were only possible with prior pre-treatment of paper substrates [144].

6.4. Conclusions.

This chapter developed sponge substrates as alternative microfluidic solution sampling materials for paper-based potentiometric heavy metal sensing. Sponge substrates can be used to realise sampling in samples with low volume of liquids and high content of solids with ability to transport liquid and protect the ISM from mechanical damages. The PU sponge substrates were found with the lowest heavy metal adsorption capacity out of three investigated sponge types, even lower than paper substrates. Then, the sponge-based microfluidic solution sampling integrated with ISEs was found compatible with ion-selective electrodes sensitive to Cd^{2+} and Pb^{2+} .

CHAPTER 7 PAPER MODIFICATION IN PAPER-BASAED MICROFLUIDIC SAMPLING FOR CLINICALLY RELEVANT IONS DETECTION

In clinical samples, ISEs and REs undergo biofouling due to the adsorption of biomolecules, e.g. proteins, onto the electrode surface. This in turn alters the potential at the electrodes | sample solution interface, affecting the analysis of ions in clinically relevant samples [141–143]. The paper substrates used for microfluidic sampling may cause physico- and chemo-sorption of ions and biomolecules into their structure [10,144–147]. This property may be used to slow down the transport of biomolecules and prevent them from reaching the electrode surface where the ion detection takes place. Furthermore, AuNPs have unique physicochemical properties with a high surface-to-volume ratio, excellent chemical stability and low cytotoxicity. AuNPs can be easily functionalised and produced in various sizes, and they have been widely studied in biological applications [148–151]. Moreover, gold has a high affinity to BSA making it a suitable material for selective removal of BSA from the sample *via* adsorption [146]. Thus, the modification of paper substrates with AuNPs and sputtered gold can act as additional barriers to eliminate/diminish electrode biofouling in clinical samples. Thus, in this work two gold materials were used to realise the Au-modification onto paper substrates, (i) AuNPs and (ii) sputtered gold.

7.1. Experimental.

7.1.1. Au modification onto paper substrates.

The AuNPs were prepared by first adding 10 mL 0.038 M trisodium citrate into 50 mL 10^{-3} M HAuCl₄ which was heated to 80°C while stirring. Then the mixture was further heated to 100 °C under stirring, until the colour turned into deep purple. Afterwards, the solution was left at room temperature to cool down with stirring. Subsequently, the cooled down AuNPs were stored at 4 °C in the fridge (further called AuNPs suspension) [180].

For AuNP-modified paper substrates, the cellulose filter paper 390 was used, either

conditioned for 4 hours in the prepared AuNPs suspension and then dried in the open air until completely dry (further named as NPPS0) or through pipetting different volumes of the AuNPs suspension, namely 30, 60 and 90 μL (further named as NPPS1, NPPS2 and NPPS3, respectively) and then dried in the open air until completely dry. For the sputtered gold modified paper substrates, different amounts/thicknesses of Au were sputtered using JEC-300FC Sputter Coater, JEOL (Japan) on only one side of the paper substrates. The amount of Au sputtered on untreated paper substrates was controlled *via* the sputtering time, namely 50, 100, 200 and 300 s (further named SPS1, SPS2, SPS3 and SPS4, respectively).

The changes in the hydrophobicity/hydrophilicity of the paper surface were measured by the contact angle of a water droplet (5 μL /droplet) on one side of the paper. The contact angle was measured by a Dataphysics OCA 15 goniometer (Hamburg, Germany) with inbuilt software (SCA20).

7.1.2. Biofouling test.

ISEs are prone to biofouling whenever biomolecules (e.g. proteins, fatty acids, enzymes, etc.) are present in the analyte solution. The BSA is considered as a model biomolecule to study the possible biofouling of sensors in clinical samples. In this protocol, freshly prepared and conditioned ISEs are brought into initial contact with the BSA [146]. Since the BSA adsorption at the ISM is considered irreversible, a second BSA adsorption is performed to observe if there are further changes to the potential of the ISEs. The ISEs were tested first when coupled with all paper substrates (reaching their full solution holding capacity) containing 0.01 M PBS. After that, a new microfluidic substrate was taken and the same measurement was done using 1 mg mL^{-1} BSA in 0.01 M PBS. The potentiometric response of the ISEs was measured for 60 s after each change in the solution composition. Between measurements electrodes were rinsed with ultra-pure water. The measurements were done on freshly prepared K^+ -ISEs and for K^+ -ISEs conditioned in 10^{-3} M KCl. The protocol was repeated twice to study the initial and subsequent potential response of the ISE to the BSA present in the solution. For

each electrode configuration, three consecutive measurements were performed, and the uncertainties of the measurements were reported ($n=3$).

7.1.2. BSA transportation through paper substrates.

Solution transport across the unmodified and Au-modified substrates were also investigated. For that purpose, substrate samples ($1 \times 1 \text{ cm}^2$) were cut from each Au-modified paper substrate prepared as mentioned in the previous chapter. After that, each sample substrate was fixed individually in a mounting assembly as shown in **错误!未找到引用源。** A. Each individual microfluidic sampling substrate was placed between two polypropylene pipette tips and gently tightened by pressing the pipette tips together while keeping the sampling substrate in-between (without tearing it apart). In this assembly, the active substrate area was approx. 7 mm^2 . The lower part of the assembly (lower tip) was then filled with 0.15 ml of 0.01 M PBS and carefully fixed in a cuvette (filled with 0.01 M PBS) in the vertical position, so that the lower part of the tip was in continuous contact with 0.01 M PBS (pH 7.4 at 25°C) (**错误!未找到引用源。** B).

Figure 25. Sample mounting assembly (A) and substrate assembled device and gold substrate arrangement (B) used in protein transport studies null ellipsometry.

Then, freshly prepared 550 μL PBS-BSA was pipetted carefully into the upper part of the mounting assembly. After passing through the substrate, the final concentration of

the BSA in the cuvette would be 1 mg mL^{-1} in 0.01 M PBS, if no BSA was absorbed by the substrate. The time needed for the solution to pass the substrate mounted in the mounting assembly was then measured. For each paper substrate (each measurement a new substrate was used), three replicates were recorded ($n=3$).

The ability of the microfluidic sampling substrates to retain or retard protein transport across the substrates was further studied by using an automated Rudolph ellipsometer (Rudolph Research, Fairfield, USA, type 43603-200E) that could detect the amount of BSA that passed through the sampling substrate. For each experiment, a fresh ellipsometric gold substrate was first carefully washed with ethanol (99.5%) and deionised water (washing step repeated 3 times) followed by drying with nitrogen and cleaning with plasma for 5 minutes. A glass trapezoid cuvette was repeatedly washed three times with ethanol (99.5%) and water, and then dried with nitrogen before each measurement. The cuvette was then placed in the ellipsometer setup and the gold substrate was vertically mounted. Then 5 ml of 0.01 M PBS was transferred to the cuvette. The ellipsometric cuvette was prepared in the same way as indicated in **错误!** **未找到引用源。** The mounting assembly was carefully positioned (vertically), retaining the PBS in the lower pipette tip so that the tip of the assembly was in continuous liquid contact (no air bubbles detected) with PBS in the cuvette and the fixed substrate.

Then the null ellipsometer was calibrated according to the procedures reported elsewhere [146]. The actual measurement was only attempted when the calibration procedure was deemed successful. The ellipsometric measurement was started and a baseline was recorded for the first 300 s. Then, an aliquot of the concentrated BSA in 0.01 M PBS solution, resulting in the 1 mg mL^{-1} total BSA concentration in the total volume in the cuvette, was added to the upper part of the substrate assembled device. The measurement of ellipsometric parameters was continued for 1800 s and the adsorption of BSA onto the gold substrate was recorded. The measurements were conducted without stirring to imitate no stirring conditions of microfluidic-based

sampling and potentiometric measurements. To investigate the possible enhancement of the BSA retention, the microfluidic sampling substrates were (i) modified with AuNPs, (ii) unmodified and (iii) with empty mounting assembly. The preparation of the substrates was done in the same way as indicated above for the solution transport studies.

7.1.4. Determination of K^+ , Na^+ and Cl^- concentrations in clinical samples.

The unmodified and Au-modified substrates were utilised in the potentiometric determination of K^+ , Na^+ and Cl^- in artificial tears, saliva, serum and sweat (collected from a human subject after physical activity). The calibration of each ISE was performed in $10^{-4.0}$ - $10^{-1.1}$ KCl for K^+ (slope = 59.1 mV dec⁻¹) and Cl^- -ISEs (slope = -57.6 mV dec⁻¹), $10^{-4.0}$ - $10^{-0.82}$ mol L⁻¹ NaCl for Na^+ -ISEs (slope = 59.4 mV dec⁻¹). The Ag/AgCl (3 M KCl) was used as a reference electrode. The potentiometric measurements were performed by placing the electrodes onto the microfluidic substrate containing standard and/or sample solutions. For comparison, beaker-based ion determination using ISEs was also performed. In both cases, the potential of the cell was registered for 60 s. All measurements were performed at room temperature (23 ± 2 °C).

Moreover, it is believed that an initial adsorption of BSA onto the ISM may be beneficial for the subsequent measurements [146]. For that reason, freshly prepared and conditioned ISEs and ISEs soaked in in 1mg mL⁻¹ BSA in 0.01 M PBS for 30 mins (fully adsorbed layer of BSA at the surface of the ISM) were used for the detection of ions in the same clinical samples, as above.

7.2. Characterisation for Au-modified paper substrates.

The concentration of the prepared AuNPs was approx. 0.04 mM, estimated from UV-Vis measurements (Figure 26A) and the size of the synthesised AuNPs as shown in TEM micrographs (Figure 26B) was 10-15 nm.

Figure 26. (A) The LSPR absorption spectrum and (B) TEM micrographs of prepared AuNPs.

The FE-SEM of all modified paper substrates are shown Figure 27. The AuNPs modification of the paper substrates was performed successfully, offering visible clusters of the AuNPs on the paper fibres. To quantify the amount of gold deposited on each paper substrate, the ICP-OES analysis (after microwave digestion) was performed and the total concentrations of gold in NPPS0, NPPS1, NPPS2 and NPPS3 were 4900 ± 407 , 1114 ± 232 , 1613 ± 214 and 2787 ± 330 ppm, respectively. Thus the amount of AuNPs was the highest in the substrate that was given time to equilibrate with the Au suspension over 4 h. Furthermore, optical microscopy (Figure 27) was carried out to examine the surface and cross-section of the Au-sputtered paper substrates. A coating on top of the fibres was observed using optical microscopy where the longer gold sputtering time resulted in a thicker gold layer on top of the paper substrates (Figure 27). From the cross-section of the gold modified paper substrates it is clear that the sputtered gold form a layer only on one side of the substrate, with limited penetration of the gold between the wood fibres.

Figure 27. FE-SEM of AuNPs distribution on unmodified paper (A), NPPS1 (B), NPPS2 (C), NPPS3 (D) and NPPS0 (E). Microscope photo of surfaces ($\times 20$) of unmodified paper (F), SPS1 (G), SPS2 (H), SPS3 (I) and SPS4 (J) with cross-sections (K, L, M, N, O, respectively).

The possible change of surface properties of the paper before and after Au-modification was investigated by comparing water droplet angle immediately after contact with the unmodified paper, NPPS0 and SPS1 (Figure 28). The NPPS0 had a noticeable increase

of hydrophobicity, as compared to unmodified paper. On the other hand, sputtering of Au uniform layer had significantly increased the hydrophobicity of the substrate. The increase of the hydrophobicity can be associated with the increase of elemental gold presence at the surface of the paper substrates, as naturally more uniform and thicker layer of gold is produced by sputtering than AuNPs modifications.

Figure 28. The optical images captured in real-time contact angle measurements when the droplet contacted the unmodified paper (A), NPPS0 (B) and SPS1 (C).

7.3. Biofouling of ISEs during microfluidic paper-based sampling.

The potentiometric responses of ISEs (potential change) due to biofouling caused by the first and second contact with 1 mg mL⁻¹ BSA in 0.01 M PBS in beaker- and microfluidic sampling-based measurement setups utilising NPPS0, NPPS1, NPPS2, NPPS3, SPS1, SPS2, SPS3 and SPS4 are grouped in Table 12. The lowest potential difference after the first and second contact was observed using NPPS0 microfluidic substrate.

Table 12 The potential change after the first and second contact with 1 mg mL⁻¹ BSA in 0.01 M PBS in beaker- and microfluidic sampling-based measurement setups utilising unmodified and gold modified papers.

Potential change /mV	Unmodified paper	NPPS0	NPPS1	NPPS2	NPPS3	SPS1	SPS2	SPS3	SPS4
After the first contact	3.9 ± 1.3	0.9 ± 0.4	2.2 ± 1.2	1.9 ± 1.1	1.1 ± 1.0	1.8 ± 1.6	2.3 ± 0.8	1.3 ± 0.2	3.2 ± 4.0
After the second contact	0.9 ± 1.1	1.4 ± 1.1	1.7 ± 1.0	2.3 ± 1.7	2.7 ± 2.2	3.4 ± 2.2	1.8 ± 1.5	1.4 ± 0.9	5.0 ± 2.8

7.4. BSA transportation time across the paper substrates.

The time needed to transport the entire volume of the 0.55 µL of 10 mg L⁻¹ BSA in 0.01 M PBS through gold modified papers is presented in Table 13. The higher the concentration of the AuNPs in the paper substrate, the slower the transport of the

solution was observed. Furthermore, unmodified paper, NPSP0, NPPS1, NPPS2 and NPPS3 showed comparable transportation time (approx. 300 s) for 0.01 M PBS (Table 13), indicating the AuNPs are unable to slow down the transportation of 0.01 M PBS. On the other hand, the transportation time increased with thicker Au sputtered layers substrates (Table 13), thus indicating the Au sputter created a physical barrier to the solution of 0.01 M PBS. The introduction of AuNPs to the paper substrate slowed down the transport of the solution through the substrate, as compared to the unmodified paper substrate (629 ± 46 s). This can be attributed to interactions between the AuNPs and BSA, creating a BSA sieve at the paper substrate, stopping and agglomerating the protein within the paper substrates. For comparison, the sputtered gold on the paper substrates was characterised with even slower solution transport, however, layered gold except for being available for BSA to adsorb it created a physical barrier for the solution to pass, contrary to AuNPs modification. Moreover, the much more hydrophobic surface of sputtered Au paper comparing to unmodified paper can also possibly slower down liquid transport.

It is expected, however, that AuNPs (NPPS0 substrate) have the highest surface area available for the BSA to adsorb and thus was found the most suitable for further investigation.

Table 13 The transportation times of the $0.55 \mu\text{L}$ of 10 mg L^{-1} BSA in 0.01 M PBS and 0.01 M PBS through unmodified and gold modified paper substrates.

Solution/ Time /s	Pure paper	NPPS0	NPPS1	NPPS2	NPPS3	SPS1	SPS2	SPS3	SPS4
PBS	319 ± 40	305 ± 19	300 ± 17	281 ± 11	306 ± 34	656 ± 56	727 ± 32	794 ± 65	856 ± 49
BSA in PBS	629 ± 46	921 ± 47	760 ± 36	742 ± 33	812 ± 33	917 ± 87	1132 ± 36	1199 ± 29	1232 ± 28

Figure 29 shows the amount of BSA adsorbed onto the ellipsometric gold substrate after its transport through empty (blank), unmodified paper and AuNPs-modified paper (NPPS0) substrates in the mounting assembly. In all cases, a nearly stable baseline (adsorption ca. zero) was recorded, prior to the addition of the aliquot of BSA in 0.01 M PBS to the top of the mounting assembly. The measured adsorption of BSA on the

ellipsometric gold substrate varied significantly between unmodified and AuNPs-modified paper substrates. For example, when no substrate in mounting assembly was used, the first noticeable adsorption of BSA was observed after ca. 51 s (from the addition of BSA-PBS solution to the mounting assembly). This represents the delay of BSA reaching the ellipsometric substrate in the diffusion-controlled transport of BSA, rather than convection. Additionally, the initial BSA adsorption rate was found to be $0.0012 \text{ mg m}^{-2} \text{ s}^{-1}$ and the maximum adsorbed amount of BSA was found to be 1.41 mg m^{-2} . The absence of stirring in the ellipsometric cuvette resulted in a slower transport of BSA in the solution, thus even after 3700 s no stable adsorption profile was observed. Typically, a full monolayer of BSA on the gold substrate is reached within 300 s and the amount of the adsorbed BSA is approx. 2.2 mg m^{-2} . The time delay caused by the presence of the unmodified paper substrate was 540 s. The initial BSA adsorption rate at the gold substrate was found to be $0.0005 \text{ mg m}^{-2} \text{ s}^{-1}$, reaching a maximum of 0.93 mg m^{-2} BSA adsorption at 3700 s BSA adsorption time. Those findings indicate that unmodified paper substrates pose as a physical barrier for the solution to pass through. Furthermore, paper substrates modified with AuNPs showed further delay in the first noticeable adsorption of BSA, namely after 1260 s. Additionally, the initial BSA adsorption rate was found to be $0.0003 \text{ mg m}^{-2} \text{ s}^{-1}$ and the maximum adsorbed amount of BSA was found to be 0.73 mg m^{-2} , which was significantly less (22%) compared to unmodified paper substrates. A similar observation was made earlier, i.e. that transport of BSA solution through paper modified with AuNPs was slowed down ($921 \pm 47 \text{ s}$ compared to unmodified paper $629 \pm 46 \text{ s}$). Thus, physico-chemical properties of the paper substrate modified with AuNPs improved the substrate's capabilities to slow down the BSA transport through the microfluidic sampling substrate, which may be very useful for practical applications of paper-based sensing systems in clinical samples.

Figure 29. Amount of BSA adsorbed onto the ellipsometric gold substrate after its transport through empty (blank), unmodified paper and AuNPs-modified paper substrates in the mounting assembly (错误!未找到引用源。). The aliquot of 0.55 μL of 1 mg mL^{-1} BSA in 0.01 M PBS was added at 300 s.

The EDXA mapping of NPPS0 after conditioning in 1 mg L^{-1} BSA in 0.01M PBS was carried out to detect the BSA adsorption onto AuNPs (Figure 30). Generally, BSA contains 31.65% C, 49.83% H, 8.42% N, 9.69% O and 0.42% S [181,182], thus through mapping of nitrogen and sulphur the BSA can be identified on the surface of gold modified paper substrates. Region of concentrated AuNPs on the NPPS0 was chosen to clearly observe between the paper and AuNPs BSA adsorption. The EDXA mapping of carbon and gold clearly differentiated between the paper substrate and AuNPs. Also, the mapping of nitrogen and sulphur, showed evidence of BSA adsorption onto the whole paper substrate, with a clear concentration of the BSA in AuNPs regions, indicating the AuNPs modified paper substrates are more efficient in adsorption of BSA from the sample solution.

Figure 30. EDXA mapping of carbon, gold, nitrogen and sulphur on the NPPS0 after

conditioning in 1 mg mL⁻¹ BSA in 0.01M PBS.

7.5. Determination of K⁺, Na⁺ and Cl⁻ concentrations in clinical samples.

The determination accuracies of Na⁺, K⁺ and Cl⁻ concentrations in most of the clinical samples by AuNPs modified papers greatly improved as compared to unmodified paper substrates, the error reduced from $\pm 20\%$ to $\pm 10\%$. This is especially valid for the NPPS0 substrate, with the most AuNPs modified, being applicable with sufficient accuracy (with error less than $\pm 10\%$ of the concentrations determined by ICP-OES/IC) in most of the samples and for all ions studied (Table 14).

Table 14 The concentrations (mM) of Na⁺, K⁺ and Cl⁻ in artificial saliva, sweat, serum, artificial tear and artificial serum samples determined by ICP-OES/IC, Na⁺, K⁺, and Cl⁻-ISEs coupled with paper-based microfluidic sampling (applying NPPS0, NPPS1, NPPS2, NPPS3, SPS1, SPS2, SPS3 and SPS4). The concentrations fall into $\pm 10\%$ of the concentration determined by ICP-OES and IC are highlighted, fall into $\pm 20\%$ are in bold.

			Saliva	Sweat	Artificial tears	Artificial serum
Na	ICP	beaker	1.58 $\pm$ 0.0002	35.09 $\pm$ 0.0003	52.17 $\pm$ 0.001	130.19 $\pm$ 0.001
	ISE	unmodified	2.41 $\pm$ 0.14	16.70 $\pm$ 0.30	31.98 $\pm$ 0.19	143.91 $\pm$ 9.66
		NPPS0	1.73 $\pm$ 0.57	35.69 $\pm$ 3.89	51.40 $\pm$ 0.29	135.12 $\pm$ 5.67
		NPPS1	2.33 $\pm$ 0.82	36.41 $\pm$ 1.62	47.97 $\pm$ 1.69	157.26 $\pm$ 5.11
		NPPS2	3.20 $\pm$ 1.94	56.60 $\pm$ 3.57	86.47 $\pm$ 5.58	145.18 $\pm$ 7.12
		NPPS3	1.77 $\pm$ 0.56	26.56 $\pm$ 1.30	40.92 $\pm$ 1.45	149.67 $\pm$ 4.81
		SPS1	3.27 $\pm$ 0.79	28.18 $\pm$ 4.19	38.02 $\pm$ 5.14	153.40 $\pm$ 12.82
		SPS2	3.01 $\pm$ 0.59	30.29 $\pm$ 0.31	44.75 $\pm$ 0.84	153.95 $\pm$ 7.82
		SPS3	2.38 $\pm$ 0.67	29.39 $\pm$ 4.16	10.45 $\pm$ 0.35	158.41 $\pm$ 5.14
		SPS4	3.51 $\pm$ 0.86	42.62 $\pm$ 5.60	60.81 $\pm$ 4.99	159.74 $\pm$ 13.09
K	ICP	beaker	17.31 $\pm$ 0.0001	8.62 $\pm$ 0.00003	14.86 $\pm$ 0.00005	8.69 $\pm$ 0.0001
	ISE	unmodified	20.39 $\pm$ 5.81	10.73 $\pm$ 0.05	20.44 $\pm$ 0.21	6.32 $\pm$ 2.41
		NPPS0	16.11 $\pm$ 2.10	6.60 $\pm$ 4.18	18.29 $\pm$ 4.11	7.95 $\pm$ 0.88
		NPPS1	13.77 $\pm$ 3.23	6.93 $\pm$ 13.21	20.76 $\pm$ 2.32	7.64 $\pm$ 2.09
		NPPS2	14.82 $\pm$ 4.64	6.12 $\pm$ 3.30	24.77 $\pm$ 2.49	8.03 $\pm$ 1.30
		NPPS3	15.83 $\pm$ 1.83	5.67 $\pm$ 4.74	17.46 $\pm$ 4.30	10.02 $\pm$ 1.12
		SPS1	21.77 $\pm$ 2.92	6.13 $\pm$ 1.32	20.34 $\pm$ 2.31	6.08 $\pm$ 2.11
		SPS2	22.51 $\pm$ 3.35	5.04 $\pm$ 0.96	15.03 $\pm$ 1.81	7.91 $\pm$ 1.89
		SPS3	19.88 $\pm$ 5.53	4.82 $\pm$ 1.96	16.46 $\pm$ 0.59	6.79 $\pm$ 2.70
		SPS4	20.42 $\pm$ 5.66	6.68 $\pm$ 0.78	19.38 $\pm$ 1.56	6.23 $\pm$ 2.73
Cl	IC	beaker	8.97 $\pm$ 0.0004	45.92 $\pm$ 0.0003	89.25 $\pm$ 0.0003	95.59 $\pm$ 0.0001
	ISE	unmodified	10.55 $\pm$ 2.23	41.17 $\pm$ 0.71	73.63 $\pm$ 0.16	112.41 $\pm$ 5.12
		NPPS0	9.85 $\pm$ 2.38	105.40 $\pm$ 25.39	76.51 $\pm$ 1.99	111.72 $\pm$ 3.35
		NPPS1	11.99 $\pm$ 1.97	142.78 $\pm$ 23.73	63.85 $\pm$ 3.59	120.15 $\pm$ 7.23

NPPS2	12.27 ± 4.67	145.89 ± 22.43	55.61 ± 12.83	113.11 ± 5.39
NPPS3	9.91 ± 5.12	93.22 ± 11.10	56.66 ± 0.99	118.82 ± 4.02
SPS1	11.42 ± 4.32	164.28 ± 3.14	155.12 ± 10.34	127.16 ± 2.61
SPS2	12.15 ± 2.45	88.64 ± 4.39	73.41 ± 2.51	130.21 ± 5.92
SPS3	12.46 ± 4.63	89.82 ± 11.74	67.65 ± 8.40	120.65 ± 4.12
SPS4	13.01 ± 2.50	136.88 ± 7.96	7.83 ± 0.33	125.29 ± 10.48

The results for using unmodified ISEs and ISEs adsorbed with BSA were compared to the ICP-OES/IC measurements (

Table 15). The determination of ions done with ISEs after full adsorption of the BSA showed poorer performance as compared to freshly prepared ISEs. One of the reasons for that may be the fact that slopes for freshly prepared ISEs in most cases was closer to the Nernstian (K^+ -ISE: 59.1 mV dec⁻¹, Na^+ -ISE: 59.4 mV dec⁻¹ and Cl^- -ISE: -57.6 mV dec⁻¹) than that for BSA modified ISEs (K^+ -ISE: 59.8 mV dec⁻¹, Na^+ -ISE: 56.9 mV dec⁻¹ and Cl^- -ISE: -50.7 mV dec⁻¹). It was previously reported that BSA adsorption lowers the Nernstian slope of the ISEs [146]. Although BSA adsorption at the surface of the ISEs was found beneficial to decrease the variations of the potential caused by the introduction of the adsorbing moiety in the sample solution, it may be detrimental to the accuracy of the ion determination because of the lower Nernstian slope of the sensor. For that reason, eliminating or diminishing BSA adsorption at the surface of the electrode had a positive effect in accuracy of the determination of ions in various samples.

Table 15 The concentrations (mM) of Na^+ , K^+ and Cl^- in saliva, sweat, serum, artificial tear and artificial serum samples determined by ICP-OES/IC, Na^+ , K^+ , and Cl^- -ISEs (before and after BSA modification).

Concentration		Saliva	Sweat	Artificial tear	Artificial serum
Na	ICP	1.58 ± 0.0002	92.26 ± 0.0003	76.65 ± 0.0005	130.19 ± 0.001
	unmodified ISE	1.46 ± 0.03	89.81 ± 7.58	61.48 ± 1.39	119.48 ± 1.70
	ISE after BSA modification	1.34 ± 0.02	80.87 ± 1.27	56.09 ± 1.48	114.73 ± 0.52
K	ICP	17.31 ± 0.0001	17.07 ± 0.0002	16.49 ± 0.0001	8.69 ± 0.0001
	unmodified ISE	16.76 ± 0.73	10.62 ± 0.17	13.72 ± 0.89	3.5 ± 0.19
	ISE after BSA modification	12.24 ± 0.74	9.87 ± 0.50	13.73 ± 0.89	3.33 ± 0.29
Cl	ICP	8.97 ± 0.0004	81.85 ± 0.0002	75.18 ± 0.0001	95.59 ± 0.0001
	unmodified ISE	8.04 ± 4.47	76.30 ± 3.38	73.70 ± 3.70	90.47 ± 1.17

ISE after BSA modification	7.32 ± 6.21	71.34 ± 9.83	65.38 ± 8.40	123.58 ± 14.05
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7.6. Conclusion.

This Chapter applied the modification of paper substrates by AuNPs and sputter Au. According to the transportation time of BSA and PBS through unmodified and modified papers, the Au sputter paper substrates were found providing only a physical barrier to BSA solution, while paper substrates modified by AuNPs can hinder/lower the transport of these biofouling moieties. The EDXA mapping also presented the BSA adsorption onto AuNPs. Furthermore, the Au modified paper substrates were compared with unmodified paper as sampling matrix for the potentiometric determination of Na⁺, K⁺ and Cl⁻ in sweat, saliva, artificial tears and artificial serum. Unmodified paper tended to show better precision (lower standard deviation between measurements) but lower accuracy, while the gold-modified paper tended to show better accuracy (values closer to those of ICP-OES and IC) but lower precision of the analytical results. The improved analytical accuracy obtained with gold-modified paper can be related to less biofouling as a result of BSA adsorption on AuNPs. Therefore, the obtained results can be a useful foundation for further design of antibiofouling microfluidic paper-based analytical devices utilizing electrochemical detection.

CHAPTER 8 PAPER-BASED REFERENCE ELECTRODE COUPLED WITH ION-SELECTIVE ELECTRODE PAPER-BASED POTENTIOMETRIC SOLUTION SAMPLING SYSTEM

In the potentiometric cell, except for the ISE, a reference electrode is used. For that purpose, in conventional aqueous samples, a saturated calomel or a silver/silver chloride reference electrode is commonly applied [152], but it is not the most suited electrode for the on-paper applications, as it is bulky and it requires refilling the internal reference solution [14]. For that reason, a new design of the solid paper-contact reference electrode should be developed for ion determination in environmentally and clinically relevant samples with high solid-to-liquid content.

8.1. Experimental.

A new type of reference electrode (nRE) for potentiometric measurements utilising 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). During all experiments, the quantitative filter paper Whatman 589/2 (Merck, Germany) was used. The experimental potentiometric setup with conventional single-junction silver/silver chloride electrode (Ag/AgCl/3 M KCl) (cRE), nRE and DPS is schematically shown in Figure 31. The Ag/AgCl element was prepared by electrodeposition of AgCl on a silver rod in a solution of 1 M KCl for three hours by applying 0.1 mA current to the electrochemical cell. After that, the Ag/AgCl wires were conditioned in 3 M 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 ($1 \times 1 \text{ cm}^2$) from the filter paper, which were modified and then optimised by addition of various solutions of KCl (1 M, 3 M and saturated KCl in ultra-pure water). The KCl solutions were added in portions of 50 μL (1-3 portions/DPS). After each portion, the DPS was left to dry for approx. 75 min in air. By registering the mass change of the DPSs before and after the addition of KCl, the mass/concentration of KCl in each paper element was estimated (considering 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 M KCl electrode as shown in Figure 31.

Figure 31. Setup for testing the stability of nRE against the conventional single-junction silver/silver chloride electrode (Ag/AgCl/3 M KCl): nRE - new concept of the reference electrode, DPS - disposable paper substrate, cRE - conventional single-junction silver/silver chloride reference electrode.

Then, the influence of the background electrolyte on the potentiometric stability of the Ag/AgCl element coupled with DPS I was examined (Figure 34). The DPS was tested in the following solutions: NaCl (10^{-2} , 10^{-1} and 1.0 M), NH_4Cl (10^{-6} , 10^{-3} and 1.0 M), MgCl_2 (5×10^{-7} , 5×10^{-4} , 0.5 M), KNO_3 (10^{-6} , 10^{-3} , 1.0 M) 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 M KCl electrode (Figure 31).

8.2. Optimisation of the new reference electrode (nRE).

When the sample solution reached the DPS, the KCl in the DPS undergo dissolution, resulting in various concentrations of KCl (Table 16) 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 M KCl. A high

concentration of the reference KCl solution provides a stable potential of the electrode [64,144]. From a storage perspective, both the DPS containing KCl crystals and a conventional paper-based reference electrode 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 electrolytes for the reference electrode. Furthermore, the dissolution of KCl during the process of sampling 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 the addition of ultra-pure water, the temperature changed (after 90 s measurement time) to $20.0 \pm 0.2^{\circ}\text{C}$.

Table 16 Concentration and volume of KCl solution and the corresponding mass of KCl at the disposable paper substrate (DPS).

DPS	Modification steps	KCl deposited at DPS /mg
I	sat. KCl ($50\ \mu\text{L} \times 1$ time)	13.89 ± 0.14
II	sat. KCl ($50\ \mu\text{L} \times 2$ times)	26.43 ± 1.60
III	sat. KCl ($50\ \mu\text{L} \times 3$ times)	36.39 ± 0.53
IV	3 M KCl ($50\ \mu\text{L} \times 1$ time)	9.47 ± 0.03
V	3 M KCl ($50\ \mu\text{L} \times 2$ times)	19.11 ± 1.40
VI	3 M KCl ($50\ \mu\text{L} \times 3$ times)	27.31 ± 0.41
VII	1 M KCl ($50\ \mu\text{L} \times 1$ time)	3.61 ± 0.14
VIII	1 M KCl ($50\ \mu\text{L} \times 2$ times)	7.07 ± 0.10

The concentration of the KCl resulting from the dissolution of the KCl salt deposited on/in the DPS is expected to stabilise gradually and thus influence the response time and repeatability of the recorded potentiometric signal. As can be seen in Figure 32 (A), the EMF of the paper cells stabilised within approx. 10 s, assuring a potential drift lower than $3\ \text{mV min}^{-1}$. 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 , -13.9 ± 3.3 and $-12.7 \pm 2.9\ \text{mV}$, respectively and for DPS I, II and III (as taken at 120 s measurement time) were -1.3 ± 1.7 , -14.1 ± 3.2 and $-13.0 \pm 2.1\ \text{mV}$, respectively. The measured cell potentials of the

nRE vs. Ag/AgCl (3 M KCl) are close to the values expected for a KCl concentration of 3 M (DPS I) and saturated KCl (DPS II and III). Indeed, the use of all DPSs resulted in the 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 M (IV, V and VI) and 1 M KCl (VII and VIII) (Figure 32 (B) and (C)). For example, the final EMF value of the potentiometric cell, utilising 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.

Figure 32. Potential stability for the Ag/AgCl element coupled with paper substrates (A) I, II, III (B) IV, V and VI (C) VII and VIII (Table 16) ($n=3$), measured versus the single-junction Ag/AgCl (3 M KCl) (cRE as the reference electrode, nRE as the indicator electrode).

More negative potentials were measured for nREs with higher amounts of KCl deposited (Figure 33). The DPS I was chosen as the most suitable for use in the new reference electrode based on its fast potential equilibration and its final potential close to that of Ag/AgCl/3 M. 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 the 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.

Figure 33. Potential of the Ag/AgCl element at DPSs modified with various amounts of KCl (Table 16) measured versus Ag/AgCl (3 M KCl) after an equilibration time of 60 s (left) and 120 s (right).

Furthermore, to validate if the nRE as reference electrode consisting of Ag/AgCl reference element being in contact with 3 M KCl, the potentiometric cell setup presented in Figure 31 was adapted to measure the potential of the nRE against the conventional Ag/AgCl (3 M KCl) reference electrode. Two setups were investigated, namely: (i) when nRE was connected as the indicator electrode and (ii) when nRE was connected as the reference electrode in this newly designed potentiometric cell. The measurements were performed in 0.1 M KCl, which should assure near zero potential of the potentiometric cell, taken the nRE was indeed equipped with 3 M 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 an Ag/AgCl (3 M 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 (Figure 34).

Figure 34. The influence of the background electrolyte on the potentiometric response of the Ag/AgCl element coupled with DPS I. In this potentiometric cell, the single-junction Ag/AgCl (3 M KCl) was connected as the reference electrode, while the Ag/AgCl element coupled with DPS was connected as the indicator electrode.

The chosen salts represented ions with different charges and mobility and some of them are common ions present in body fluids [183]. The final potential values of the potentiometric cell in various solutions at two different measurement times are presented in Table 17. 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 M KCl. Since the cell potential was found relatively stable, the DPS I together with Ag/AgCl element was investigated as a new reference system coupled

with ion-selective electrodes. The 60 s measurement time was found sufficient for the cell equilibration.

Table 17 The equilibrated potential of the Ag/AgCl element coupled with DPS I, measured versus Ag/AgCl (3 M KCl) on the paper-based platform.

Salt	Measurement time /s	Potential /mV when in contact with different concentrations of the electrolyte		
		10^{-6} M	10^{-3} M	10^{-1} M
KNO₃	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
NH₄Cl	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
MgCl₂	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

8.3. Optimisation of the microfluidic paper-based potentiometric cell coupled with nRE.

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 (Figure 35). 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.

Figure 35. 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 millimetres.

Most of the proposed microfluidic paper-based platforms (Figure 35 A to D) were tested in three variants based on the linking paper element (marked grey in Figure 35) 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 the construction of the potentiometric cell, it varied for various substrates and modifications made for the linking paper element (Table 18). 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.

Table 18 The time for wicking the solution to the entire microfluidic paper-based platforms.

Paper-based platform	Time /s		
	No modification	Top side wax modification	Both sides wax modification
A	4	6	23
B	6	12	46
C	20	43	180
D	21	47	191

For that purpose, additional paper-based platforms, with no modifications to the paper linking elements (Figure 35 E and F) were prepared and investigated, resulting in wetting time 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 the full dissolution of the KCl stored in the DPS was accomplished within 30 s, indicating the minimal time required for analytical measurement.

8.4. Potentiometry with ISEs and nRE on paper-based platforms.

The potentiometric measurement of the ISEs vs. nRE on the paper-based platform were investigated using ion-selective electrodes sensitive to K^+ , Na^+ and Cl^- ions as indicator electrodes. 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 (slopes; $56 \pm 2 \text{ mV dec}^{-1}$

for K^+ - and Na^+ -ISEs, $-56 \pm 2 \text{ mV dec}^{-1}$ for Cl^- -ISEs) were utilised further. In those measurements, the potentiometric response was investigated in the range of $10^{-7.0}$ to $10^{-1.1} \text{ M}$ of the standard solutions, with a tenfold increase in the primary ion concentrations. In beaker-based measurements, the single-junction $Ag/AgCl$ (3 M 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 Figure 36. In addition to the microfluidic paper substrates that were combined with the DPS, two additional key regions can be identified, i.e. the sample deposition point and the detection zone (Figure 36).

Figure 36. Optimised 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 millimetres.

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, approx. $80 \mu\text{L}$ of the 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 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 s. A new piece of the 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. The calibration curves and analytical parameters for both beaker- and paper-based measurement with ISEs are presented in Figure 37 and Table 19.

Figure 37. Potentiometric response of potassium (A), sodium (B) and chloride (C) ISEs in $10^{-7.0}$ - $10^{-1.1}$ M standard solutions utilising beaker-based and paper-based microfluidic sampling measuring setups.

As can be seen in Table 19, the LDLs 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, several possible causes have been identified. For example, the paper has been identified to be slightly anionic in nature, possessing anionic carboxylate groups, which may interfere especially with cation-sensitive ISEs [122,184,185]. In addition, these ionic sites can take part in complexation and ion-exchange equilibria, which 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 [9,64,89,106]. In fact, paper was found to be a good sorption material for a number of ions [135,165]. On the other hand, for Cl^- -ISEs, the beaker-based measurements and the paper-based measurements show

comparable LDLs.

Table 19 Comparison of slope and lower detection limit (LDL) 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 /M	$10^{-5.0}$ - $10^{-1.1}$	$10^{-5.0}$ - $10^{-1.1}$	$10^{-4.0}$ - $10^{-1.1}$	$10^{-4.0}$ - $10^{-1.1}$	$10^{-3.0}$ - $10^{-1.1}$	$10^{-4.0}$ - $10^{-1.1}$
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
LDL /M	$10^{-5.4 \pm 0.1}$	$10^{-5.4 \pm 0.1}$	$10^{-4.1 \pm 0.1}$	$10^{-4.1 \pm 0.1}$	$10^{-3.3 \pm 0.1}$	$10^{-4.1 \pm 0.1}$

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 M KCl) was used. In the first one (protocol A), the cRE replaced Ag/AgCl reference element and was directly placed on the DPS (Figure 38A). In the second (protocol B), the cRE was placed on the end part of the microfluidic paper-based platform that did not contain any KCl, thus eliminating any paper-based reference electrode element from the potentiometric cell (Figure 38B), similarly to what has been done previously [9,10,89,144]. For K^+ -ISEs, the slopes in protocol A (Figure 38A) and B (Figure 38B) were 60.0 ± 2.0 and 57.2 ± 2.9 mV dec⁻¹, respectively. In both protocols, the LDLs of K^+ -ISEs were similar ($10^{-4.1}$ M) and comparable with those obtained when the paper substrate was combined with nRE (Figure 37A). For Na^+ -ISEs, the slopes in protocol A and B were 62.2 ± 1.0 and 58.4 ± 0.8 mV dec⁻¹, respectively. In protocol A, the LDL was approx. $10^{-3.2}$ M, and was similar to the one obtained when the paper substrate was combined with nRE (Figure 37B). However, the LDL at approx. $10^{-4.1}$ M 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 LDL is driven by the paper-ISE interactions rather than the application of the nRE or reference electrode arrangement onto the paper platform.

Figure 38. Potentiometric response of both potassium ($\circ$) and sodium ($\blacksquare$) ISEs in $10^{-7.0}$ - $10^{-1.1}$ M corresponding standard solutions utilising protocol A (A) and protocol B (B).

8.5. Real samples ion determination.

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 pre-treatment was done to the samples, except that the samples were allowed to warm up to room temperature before the actual ion analysis. 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. For potentiometric determinations, the calibrations of K⁺-, Na⁺- and Cl⁻-ISEs were performed in the potentiometric paper cells in $10^{-4.0}$ - $10^{-1.0}$ M KCl, NaCl and KCl ranges, respectively. Then samples were dropped onto the sample introduction zone of the paper (Figure 36). 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.

Table 20 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

The ion concentrations determined in all analysed samples are listed in Table 20. 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 the analysis done with these methods, potentiometric measurements were performed without any pre-treatment, using unfiltered sludge samples. Thus the solids in sludge (containing organic and inorganic residues of bio digestion) may have actively participated in the equilibration of ions in the solution phase [179,186,187]. The differences in the measured ion concentrations might result from the complexity of the samples, in terms of possible ligands availability in the solution [188–190]. Finally, solvent evaporation from the paper substrate could cause pre-concentration of analytes in liquid samples[145]. Taken the complexity of both samples, the paper substrate coupled with a new reference electrode seems to be a promising tool for analysis of environmental and clinical samples.

8.6. Conclusion.

In this chapter, 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.

CHAPTER 9 CONCLUSIONS AND FUTURE PROSPECTS

9.1. Conclusions.

- Explored inorganic salts/acid pre-treatment onto paper substrates for microfluidic paper-based potentiometric heavy metal ion sensing
- Applied PU sponge substrate for microfluidic sponge-based potentiometric heavy metal ion sensing
- Investigated Au modification on paper substrates to reduce biofouling on microfluidic paper-based potentiometric ion sensing in clinical samples
- Explored a novel concept of a paper-based solid reference electrode

Paper-based potentiometry brings many benefits such as portability, low-cost, ability to analyse small sample volumes, simplicity of operation. Through this study, the crystalline membrane solid-state Cd^{2+} - and solid-contact Pb^{2+} -ISEs were used to investigate the possibility to facilitate the use of paper-based microfluidic sampling coupled with potentiometric sensors for the determination of heavy metal ion concentration. Solid-contact type of ISEs was used here for the very first time and their use was found to be compatible with paper-based sampling. To reduce the hindering effect of the paper substrate, paper pre-treatment was applied to reduce the metal ions interaction between the standards/the sample solutions and the paper substrates, thus eliminating unfavourable super-Nernstian response of the ISEs. It was found that a relatively high concentration of inorganic salt (10^{-3} M) solution was necessary to modify the paper substrates prior to their use for paper-based sampling coupled with ISEs. Further on, when the paper substrates were pre-treated with interfering ions, only a low concentration of 10^{-6} M Pb^{2+} solution was applicable when coupled with Cd^{2+} -ISEs. Then, acidification onto the paper substrates was investigated in order to find a simpler paper modification protocol to eliminate the super-Nernstian response of ISEs. The pH 2 acidification of paper substrates successfully eliminated the super-Nernstian response. Moreover, by ICP-MS analysis, it was found that paper substrates are very good sorbents of metal ions, with a stronger binding force given to Pb^{2+} over Cd^{2+} . The

acidification of paper substrates reduced the heavy metal adsorption onto the paper substrates. Lastly, the new area of material used for microfluidic solution sampling was investigated, e.g. application of polyurethane sponge substrates to use as prepared solution sampling substrates in heavy metals detection. The microfluidic sampling allowed measurements of ions in samples with low volume of liquids and high content of solids. Thus, the sponge substrate not only allowed the transport of ions from the sample to the electrode surface for ion analysis but also protected the electrode from mechanical damages. It was found that sponge-based microfluidic solution sampling integrated with ISEs was found compatible with ion-selective electrodes sensitive to Cd^{2+} and Pb^{2+} . Furthermore, owing to low heavy metal sorption capacity of polyurethane sponge, out of three investigated sponge types, only polyurethane-based sponge was found suitable to be used as the substrate for solution sampling. Contrary to previous findings on paper-based microfluidic solution sampling, the application of sponge-based microfluidic sampling allowed measurements of heavy metals without prior modification of the sampling substrate.

Then, the modification of paper by AuNPs was compared with unmodified paper as sampling matrix for the potentiometric determination of Na^+ , K^+ and Cl^- in clinical samples, namely sweat, saliva, artificial tears and artificial serum. The unmodified paper showed better precision (lower standard deviation between measurements) but lower accuracy, while the gold-modified paper substrates showed better accuracy (values closer to those of ICP-OES and IC) but lower precision of the analytical results. The improved analytical accuracy obtained with gold-modified paper can be related to lower biofouling as a result of the adsorption of proteins onto AuNPs.

Finally, 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 stabilised the potential of the Ag/AgCl reference element. It was found that the

reference electrodes equipped with substrates offering higher KCl levels were characterised 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, e.g. 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 utilising the nRE would have to be subjected together with the indicator electrode to calibration before actual use. Nonetheless, after further optimisation, the novel approach of the reference electrode may open new possibilities for conducting the 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.

9.2. Future prospects.

In the future, a better understanding of the interactions between different samples and functional groups on the paper substrate would aid to implement proper paper modification protocols to suit any sort of ion sensing application with improved LDLs [144]. Although the determination of the clinically relevant ions using paper-based analytics is somewhat in an advanced stage, the estimation of environmentally relevant ions, e.g. heavy metals, is still a subject of potentiometric cell optimisation. The lack of research progress is especially apparent for microfluidic paper-based sampling coupled with ISEs, where sensors exhibit significantly higher LDL as compared to a sensor response in conventional potentiometric beaker-based measurements. By now, the lowering of the LDL for ISEs coupled with microfluidic paper-based sampling has not yet been fully understood. Among possible reasons embedding improvement of LDL in

the paper-based potentiometric analysis are [9,89,165]: (i) anionic nature of the paper that may affect ion equilibration at a sensor | paper interface, e.g. through engaging in ion-exchange or complexation reactions with the analyte and/or (ii) possibility for the ion physical and chemical adsorption onto the paper substrate during the measurement. In this regard, it is vital to study the factors affecting LDL for ISEs coupled with microfluidic paper-based sampling, especially for determining heavy metals at low analyte concentrations. It is important to mention that the physio-chemical nature of paper can be highly influenced by its papermaking process. For example, depending on a specific preparation approach, paper differently influences the measurements of various samples, especially if the samples are weakly buffered, e.g. environmental samples. So far, the papermaking process has not been tuned for the designing and manufacturing of paper with physio-chemical properties adequate for analytical purposes. Thus, it is clear that in order to unveil the possibility of using microfluidic paper-based sampling with ISEs for detection of heavy metals at low analyte concentration and pH, the design and development of the paper and papermaking process have to be addressed. On the other hand, efforts have been made previously like (i) investigations of conducting polymer doped with primary ion-complexing agents to reduce the primary ions leaching and contamination at membrane | sample interface[68], (ii) use of hydrophobic materials to avoid water layer formation [191], and (iii) application of a galvanostatic current to establish a primary ion transport gradient from the sensing membrane into the conducting polymer, thereby, reducing primary ion transport into the sample [192]. Furthermore, novel membranes[193] and conditioning methods were also proposed [194].The ISEs with improved LDLs from all these methods can be investigated for paper-based sampling using modified/acidified paper. Also, other strategies such as doping chemical agents into the ISM to reduce paper effects on paper | ISM interface and optimization of the potential reading time can be explored.

Also, fabrication of a user-friendly platform is needed. A calibration-free and smart automatic calculation system should be applied for users without any related knowledge.

The current paper-based sampling coupled with potentiometric cells used for ion sensing is supposed to do the calibration and calculate the slope, then the measurements is carried out and the concentration can be assessed using calibration curve. To simplify the measurement calibration-limited or calibration-free systems would be required. For that reliable potentiometric sensor are needed. This is currently explored in standard potentiometry. Furthermore, for the current paper-based sampling coupled with potentiometric cells setup, the electrodes are manually pressed onto the paper substrates. A novel design to integrate the electrode onto paper should be applied for easier maintenance of the setup.

Additionally, the signal transmission, such as radio frequency identification (RFID) or interfacial potential detector (IPD), and data processing to facilitate the presentation of the results e.g. concentration to a smartphone, has to be studied more extensively in paper-based potentiometric sensors for ion determination. By now, RFID system has been successfully demonstrated in conventional baker-based potentiometric setup [195], while Bluetooth Low Energy (BLE) transmitter has very recently exploited in a paper-based potentiometric sensor [196,197]. Finally, standardised validation protocols must be implemented to evaluate sensor reliability in real-life decision-making applications considering that different applications may have different requirements, e.g. clinical analysis is more restricted than physiological activity monitoring.

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