Ion-selective electrodes and potentiometric sensing schemes for protein assays

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To those all

who helped me along the way
Preface

The research work presented in this thesis was mainly carried out at the MTA-BME Research Group for Technical Analytical Chemistry at Budapest University of Technology and Economics. Fundings from the Hungarian Scientific Fund (OTKA NF 69262), the New Hungarian Development Plan (TÁMOP-4.2.1/B-09/1/KMR-2010-0002), the Lendület program of the Hungarian Academy of Sciences (LP2013-63/2013), the Swiss National Foundation, the Johan Gadolin Scholarship of Åbo Akademi University, and the Academy of Finland are gratefully acknowledged.

First and foremost, I am deeply thankful to my supervisors Prof. Róbert E. Gyurcsányi and late Prof. hab. Klára Tóth for giving me the opportunity to work at the laboratory and for their support throughout the years. I thank them for guiding me into the world of research and helping me to develop my research abilities. I would like to thank Prof. Gyurcsányi for all the challenges he faced me during the years, which helped me in every aspect to become the person who I am today, that truly taught me a lot.

I would also like to thank Prof. Ernő Pretch from ETH Zürich for welcoming me in his group and by that giving me the possibility to redirect my PhD work towards potentiometry, which finally ended up to be the main topic of this thesis. Furthermore, I also thank him for the chance to spend a wonderful half a year in Switzerland.

I give my deepest thanks to Prof. Tom Lindfors, for giving me the opportunity to work at the Laboratory of Analytical Chemistry at Åbo Akademi University, where I have become a chronic visitor. I’m very thankful to him for his scientific and practical guidance, for the always supportive atmosphere, and to everybody in the lab for ever making me feel very welcome. It was always very inspiring to work in Turku, and a great pleasure to return. Meanwhile I’ve fallen in love with the relaxed environment, and not only.

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Dia, Szilvi, Zoli, thank you for keeping me going.

These long years of PhD have been significant in my life also outside science. I am very lucky to have such wonderful people, friends around me. Thank you for being there and listening to me in good times and in bad times, to keep company, to share
memorable moments, to cheer up life. Thank you for your invaluable support and motivation. Thank you for believing in this day to come even when I did not.

I would also like to express my deepest appreciation to my family. To my mother and father for their never-ending unconditional love, for their priceless encouragement and support in every way possible. All I have achieved I owe to you! Finally, my warmest thanks goes to my husband, Bright, for sharing life, the most exciting experiment possible with me.

There is so much to be thankful for and so many people who have helped me in so many ways. I’m truly thankful for you all who helped me onward the long way, I dedicate this work to you.

Thank you for your support!

Nyúl, August, 2015
Abstract

Nowadays, there is a growing demand for low-cost, easy-to-use analytical devices, especially in the field of diagnostic assays. As such point-of-care diagnostic devices and *in vitro* diagnostic platforms undergone a vigorous development and expansion. The cost-effective fabrication of these devices, the simplification of the analytical methodologies and the use of robust reagents are essential for their wide spread application in the everyday life. Accordingly, the focus of my doctoral research was to explore various new sensing methodologies, chemical reagents and materials to enable such cost-effective assays for protein targets of diagnostic relevance.

We explored the opportunities to use potentiometry for readout which instrumental-wise necessitates only a high impedance voltmeter (pH meter) ubiquitous in any analytical laboratory. Potentiometry offers several essential advantages, such as cost-effectiveness, simple and fast readout, it is compatible with modern miniaturization/microfabrication technologies; and it is suitable for measurements in minute volumes. The challenge was to adapt this technique, by developing novel ion-selective electrodes (ISEs) and general sensing schemes, to the detection of protein targets. Although the gold standard of protein detection, enzyme-linked immunosorbent assay (ELISA), is conventionally coupled with optical detection, potentiometry is more suited for meeting the portability requirements of point-of-care testing or field detection of biomarkers.

Two different potentiometric measuring schemes were developed for immunoassay detection.

In the first approach a potentiometric ELISA assay was worked out to detect human prostate specific antigen (PSA) in real serum samples in microtiter plate wells. The sandwich assay involved the potentiometric detection of an anion, 6,8-difluoro-4-methylumbelliferone (DIFMU), product of the hydrolysis reaction catalysed by the galactosidase (GAL) enzyme label of the tracer antibody. A simple and cost-effective anion-exchanger based minielecetrode could be used for this purpose.

The second approach involved an affinity assay for human IgE using gold nanoparticle as label of the tracer aptamer. The analyte concentration dependent signal was obtained ultimately by the potentiometric measurement of silver ions released by oxidative dissolution from a silver layer autocatalytically deposited on gold nanoparticle-conjugated bioreagents. The silver enhancement and the potentiometric detection of silver were integrated in a microfluidic paper-based platform. Our study represents most likely the first approach to adapt potentiometric detection to the fashionable paper-based assays.
At the fundament of potentiometric bioassays we explored various possibilities to fabricate solid-contact silver ion-selective electrodes (AgSCISEs) with low detection limits, good long-term potential stability and reproducible electrode-to-electrode $E^0$ standard potentials. All these parameters are important premises for a successful use in diagnostic platforms. To achieve ultratrace detection limit and exceptional selectivities solid-contact silver-selective electrodes were fabricated using silicone rubber-based ion-selective membrane (ISM). To accomplish good device-to-device reproducibility and long-term stability three-dimensionally ordered (3D) PEDOT(PSS) conducting polymer, loaded with the lipophilic redox mediator 1,1’-dimethylferrocene (DMFe), as large surface area solid-contact in silver ion-selective electrodes was found to be beneficial.

Taking advantage of nanosphere lithography, also used in the latter work, nanostructured surface-imprinted conducting polymer nanostructures were created as well.

Therefore, the doctoral research summarized in this thesis aimed to:

1. explore the feasibility of potentiometric detection in bioaffinity assays;
2. improve the analytical performance of solid-contact ion-selective electrodes used for the potentiometric detections; and
3. fabricate and utilize novel 3D nanostructured conducting polymer layers both as ion-to-electron transducer and as biorecognition element.

The results demonstrated the applicability of potentiometric detection both for the classic microtiter plate format and for the paper-based protein assays. They proved to be a viable alternative to the conventionally used optical detection having comparable or better analytical performance parameters.
Összefoglalás

Napjainkban egyre növekvő igény van olcsó és egyszerűen használható bioanalitikai eszközökre, különösen a diagnosztikai vizsgálatok területén. Ennek megfelelően a különféle gyorstesztetek, betegágy melleti és in vitro diagnosztikai készülékek hatalmas fejlődésen mentek át az utóbbi időben és gyorsan terjednek. Ezen eszközök olcsó előállítása, az alkalmazott analitikai módszerek egyszerűsítése és robusztus reagensek használata elengedhetetlen a mindennapi életben történő széles körű alkalmazhatóságukhoz. Éppen ezért doktori munkám fókuszában az ilyen, diagnosztikai fontossággal bíró fehérjék olcsó meghatározását lehetővé tevő, új érzékelési elvek, valamint kémiai reagensek és anyagok vizsgálata állt.

Munkám során a potenciometriás méréstechnika alkalmazhatóságát vizsgáltam erre a célra, melyhez egy minden analitikai laboratóriumban megtalálható, nagy bemeneti ellenállású voltméterre van szükség. A potenciometriás méréstechnika számos további alapvető előnytel rendelkezik: egyszerű, olcsó és gyors mérési módszer, amely kompatibilis a modern miniaturizálási ill. mikrofabrikációs eljárásokkal, valamint alkalmas kis térfogatban történő mérésekre. Ennek megfelelően célul tűzem ki, hogy a potenciometriás detektálást, megfelelő ion-szelektív elektródk (ISE), valamint általános érzékelési sémák fejlesztésével alkalmassá tegyem fehérjék kimutatására. Habár a fehérjék meghatározására ma legszelesebb körben használt ELISA módszer (enzimhez kapcsolt immunoszorbens vizsgálat) hagyományosan optikai érzékelési eljárásokon alapszik, a potenciometriás méréstechnika sokkal jobban megfelel a diagnosztikai eszközök által támasztott követelményeknek, például a hordozhatóság tekintetében.

Munkám során kétféle potenciometriás detektálási sémát fejlesztettem ki fehérjék meghatározására.

Az elsőben humán prosztata specifikus antigén (PSA) valós szérum mintákból, mikrotitter tálcában történő kimutatására potenciometriás ELISA assayt dolgoztam ki. A szendvics assay a jelölő molekulaként használt galaktozidáz enzim (GAL) anionos hidrolízis termékének (DIFMU, 6,8-difluoro-4-methylumbelliferone) mérésén alapul. Erre a célra egyszerű és olcsó, anioncserehő-alapú minielektródok használhatóak. A második egy affinitás assay human IgE meghatározására, arany nanorészecskével jelölt aptamer, mint felismerő ágens segítségével. Az analát koncentrációjával arányos jelet a jelölésre használt arany nanorészecskék felületére szelektíven leválasztott fém ezüst réteg oxidatív visszaoldásával generált ezüst-ionok potenciometriás mérése biztosította. Az ezüst alapú jelerősítés és az ezüst potenciometriás detektálása papíralapú mikrofluidikai assay-be integrálva történt. Tudomásunk szerint ez az első kísérlet a divatos papíralapú assay-k potenciometriás detektálására.
A potenciometriás bioassay mérések alapján különféle szilárd belső-elvezetésű ezüst ion-szelektív elektródokat (AgSCISE) is vizsgáltam, a kis kimutatási határ, hosszú távú potenciál stabilitás és elektródok között reprodukálható \( E^0 \) standard potenciál elérése érdekében. Ezek a paraméterek az ion-szelektív elektródok diagnosztikai készülékekből történő használatának fontos követelményei. Kis kimutatási határ és rendkívüli szelektivitás értékek elérésére szilárd belső-elvezetésű ezüst-szelektív elektródok esetében szilikont matrixt alapú membránt (ISM) alkalmaztam. Az elektródok közötti jó reprodukálhatóság és hosszú távú potenciál stabilitás megvalósításához pedig a nagy felületű szilárd belső-elvezetésként használt és redox mediátorral módosított három dimenziósnál rendezett PEDOT(PSS) vezető polímer használata bizonyult előnyösnek.

Az utóbbi vizsgálatnál alkalmazott nanogömb litográfiai eljárást fehérjék felismerésére használható felületi lenyomatú vezető polímer nanostruktúrák kialakításánál is alkalmaztam.

Mindezeknek megfelelően a disszertációban bemutatott munka az alábbi 3 fő cél köré csoportosítható:

1. bioaffinitás vizsgálatok potenciometriás detektálásának lehetőségei;
2. a potenciometriás mérésekhez használt szilárd elvezetésű ion-szelektív elektródok analitikai tulajdonságainak javítása; valamint
3. olyan nanostrukturált vezető polímer rétegek kialakítása, melyek szilárd belső-elvezetésként és fehérjék felismerésére egyaránt alkalmasak lehetnek.

Az eredmények bebizonyították, hogy a potenciometriás detektálás mind a klasszikus, mikrotiter tálca alapú, mind pedig a papír-alapú fehérje kimutatási módszer esetében alkalmazható. A potenciometria a hagyományos optikai érzékelési módszerek vetélytársának bizonyult, azókéval összehasonlítható, vagy azt még túl is szárnyaló analitikai teljesítményjellemzőkkel.
List of publications

This thesis is based primarily on the following publications, which are referred to in the text by their Roman numerals. The original publications are appended.


Contribution of the Author:

Paper III. The author did the fabrication and characterization of silver ion-selective electrodes.

Paper V. The author did the optimization of the polymer layer thickness by using atomic force microscopy measurements.
List of other publications related to the topic†

Oral presentations:


†Note: The presenter of the publication is underlined, while the Author is marked bold.
Poster presentations:


Proceedings:

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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning (usual units)</th>
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<tr>
<td>µPADs</td>
<td>microfluidic paper-based analytical devices</td>
</tr>
<tr>
<td>3DOM</td>
<td>three-dimensionally ordered macroporous carbon</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APP4</td>
<td>aminophenyl phosphate</td>
</tr>
<tr>
<td>Apt‒AuNP</td>
<td>aptamer–gold nanoparticle conjugate</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflexion</td>
</tr>
<tr>
<td>AuNP</td>
<td>gold nanoparticles</td>
</tr>
<tr>
<td>CIM</td>
<td>colloid-imprinted mesoporous carbon</td>
</tr>
<tr>
<td>CP</td>
<td>conducting polymer</td>
</tr>
<tr>
<td>CWE</td>
<td>coated-wire electrode</td>
</tr>
<tr>
<td>DIFMU</td>
<td>6,8-difluoro-4-methylumbelliferone</td>
</tr>
<tr>
<td>DIFMUG</td>
<td>6,8-Difluoro-4-methylumbelliferyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>DL</td>
<td>detection limit</td>
</tr>
<tr>
<td>DMA</td>
<td>decyl methacrylate</td>
</tr>
<tr>
<td>DMFe</td>
<td>1,1’-dimethylferrocene</td>
</tr>
<tr>
<td>DOS</td>
<td>bis(2-ethylhexyl) sebacate, commonly referred also as dioctyl sebacate</td>
</tr>
<tr>
<td>EDOT</td>
<td>3,4-ethylenedioxythiophene</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>EIS</td>
<td>electrochemical impedance spectroscopy</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMF</td>
<td>electromotive force (mV)</td>
</tr>
<tr>
<td>ETH500</td>
<td>tetradecylammonium tetrakis(4-chlorophenyl)borate</td>
</tr>
<tr>
<td>FIM</td>
<td>fixed interference method</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>GAL</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>GC</td>
<td>glassy carbon</td>
</tr>
<tr>
<td>HOMO</td>
<td>highest occupied molecular orbital</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HS-TEG</td>
<td>(1-mercaptoundec-11-yl)tetra(ethylene glycol)</td>
</tr>
<tr>
<td>IDA</td>
<td>interdigitated microelectrode array</td>
</tr>
<tr>
<td>IDA</td>
<td>isodecyl acrylate</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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xlv
1. Introduction

Nowadays, there is a growing demand for low-cost, easy-to-use bioanalytical devices especially in the field of clinical diagnostics. Point-of-care diagnostic devices, in vitro diagnostic platforms undergo a vigorous development and expansion. As research moves into this direction, scientists are faced with the challenge of developing effective methods for detecting proteins. Methods that enable sensitive, selective and rapid detection of proteins, even in ultra-low levels.

Proteins are generally detected via binding to an affinity ligand. Conventionally antibody-antigen interactions are preferred due to their exquisite selectivity. Owing to their higher sensitivity and exceptional selectivity caused by their double recognition process sandwich-type assay formats are the most popular choice. The primary (capture) antibody is mostly immobilized to a solid surface; in case of immunoassays most often to the wells of a microtiter plate whereas in case of immunosensors to the surface of the transducer. The label on the second (tracer) antibody ensures quantitative analysis and via the possible signal amplification high sensitivity as well.

Enzyme-linked immunosorbent assay (ELISA) remains up to now the gold standard in protein detection, with detection limits in the picomolar range. Conventionally it is coupled with optical detection methods which enable high sample throughput via the greatly parallel measurements in the microtiter wells.

Although the optical detection is wide-spread in immunoassays the utilization of electrochemical methods has a number of advantages. Electrochemistry enables fast, simple and economical measurements, and it has minimal power requirements. It is compatible with modern miniaturization/microfabrication technologies, applicable for measurements in small volumes, and furthermore ideally suited for meeting the portability requirements of point-of-care testing or field detection of bioreagents.

The electrochemical sensors with the longest history and probably with the largest number of applications are the potentiometric ion sensors, or as better known ion-selective electrodes (ISEs).

Since the first pioneering work of Cramer in 1906 on the ion-selective glass membranes much has been done on the field of ISEs. From the use of liquid ion-exchanger membranes the technique evolved via the introduction of poly(vinyl chloride) (PVC) as membrane matrix, until finally the plasticized PVC based ion-selective membrane (ISM) was inaugurated and modern ion-selective electrodes were created as we know it nowadays.

At the end of the 1990s the ISE field experienced a new boost inspired by the significant improvements of the lower detection limit (LDL) leading to better
understanding of the sensing mechanism, initiation of new membrane matrices, and introduction of solid-contact ion-selective electrodes (SCISEs).
Thanks to the latter remarkable improvements, potentiometry became rediscovered as a viable alternative for bioanalytical measurements as well. The use of potentiometric detection in protein assays can lead to the development of cost-effective, miniaturized bioanalytical systems based on ISEs.

The aim of the work summarized in this thesis was threefold:

1. Utilizing the feasibility of potentiometric detection to replace the conventional optical detection methods in bioaffinity assays;
2. Improving the analytical performance of solid-contact ion-selective electrodes used for the potentiometric measurements;
3. Fabricating and utilizing novel 3D nanostructured conducting polymer layers both as ion-to-electron transducer and as biorecognition element.
2. Ion-selective electrodes

2.1. Introduction

Ion-selective electrodes are electrochemical sensors that allow the potentiometric determination of the activity of certain ions in aqueous solutions in the presence of other ions.\(^1\)

![Figure 1: Schematic representation of a potentiometric measuring cell consisting of an ion-selective electrode (ISE) and reference electrode (RE). Electrochemical interfaces are represented as | and the liquid junction as II. A high input impedance voltmeter is used in the measuring circuit.](image)

The setup for a potentiometric measurement is shown in Figure 1, including an indicator electrode (the ISE), a reference electrode and a millivolt potentiometer for measuring the potential difference, or electromotive force (EMF) between them. Theoretically the measurement is conducted under zero-current conditions, practically a high input impedance voltmeter (\(10^{13} \Omega\) or higher) is used in the measuring circuit to keep the current flowing in the picoampere range. This system can be considered as a galvanic cell. The ISE is a galvanic half-cell consisting of an ion-selective membrane that is in electrical contact with an internal reference electrode and the sample. The electrical contact can be accomplished through an inner filling solution (liquid-contact electrodes) or by a direct solid contact (solid-contact electrodes). In this work, both electrode types were used, but solid-contact electrodes will be discussed more into detail. The indicator electrode should respond quickly and selectively to changes in the free ion activity of the analyte in the sample. The other half-cell is the external reference electrode, which has a constant potential under zero-current conditions. Ideally, the reference electrode should provide a constant and stable potential irrespective of the composition of the sample solution, so that changes in the EMF can
attributed to the changes in the activity of the target ion, i.e., potential change of the ion-selective electrode[2] [3].

2.2. Response mechanism: The phase boundary potential

The response mechanism of the ISEs is described by the so-called phase boundary potential, which was introduced in 1929 and 1930 by Guggenheim and further developed by Teorell, Meyer and Sievers.

The measured potential difference, the electromotive force (EMF) is the sum of several local potential differences arising at every electrochemical interface. Since for a given electrode assembly and temperature, only the membrane potential ($E_M$) and the liquid junction potential of the reference electrode ($E_J$) are sample-dependent, all other sample-independent terms can be combined in one constant potential ($E_{const}$), and the EMF can be simplified to:

$$EMF = E_M + E_J + E_{const}$$  \hspace{1cm} (1)

The membrane potential may be divided into three separate contributions: the two phase boundary potentials at the membrane/sample solution interface ($E_{PB}$) and the membrane/inner solution interface ($E_{PB*}$), as well as the transmembrane diffusion potential ($E_D$). In practically relevant cases the last term is negligibly small and $E_{PB*}$ is considered to be sample-independent at steady state, so that $E_M$ can be described as:

$$E_M = E_{PB} + E_{const}$$  \hspace{1cm} (2)

The main contribution to the overall cell potential comes from the phase boundary potential of the membrane/sample interface and the liquid junction potential so Eq. (1) can be written as[2] [4]:

$$EMF = E_{PB} + E_J + E_{const}$$  \hspace{1cm} (3)

The liquid junction potential (or diffusion potential in conventional reference electrodes) arises due to the different mobilities of the ionic species in the sample and the bridge electrolyte of the reference electrode. It can be kept close to constant by using a relatively concentrated bridge electrolyte solution of ions with similar mobilities (e.g. 1 M KCl, NH$_4$NO$_3$ or LiOAc). However, variations in $E_J$ at the sample interface should always be considered and the approximate values can be obtained from the Henderson equation, by assuming that the ion activities in the junction are equal to the concentrations and that the concentration profiles are linear throughout the junction[3] [5] [6];
\[ E_j = \frac{\sum_i |z_i| \frac{u_i}{u_{i,a}} (C_{i,\beta} - C_{i,\alpha})}{\sum_i |z_i| u_i (C_{i,\beta} - C_{i,\alpha})} \frac{RT}{F} \ln \frac{\sum_i |z_i| u_i C_{i,\alpha}}{\sum_i |z_i| u_i C_{i,\beta}} \]  

where \( z_i \) is the charge number; \( u_i \) is the mobility and \( C_i \) is the molar concentration of species \( i \); \( \alpha \) and \( \beta \) are the two electrolyte phase; \( R \) is the universal gas constant; \( T \) is the absolute temperature; and \( F \) is the Faraday constant.

The phase boundary potential arises from the unequal distribution of ionic species at the phase boundary of two phases. The phase-boundary model readily states that \( E_{PB} \) is the main factor determining the potentiometric response of the ISE, which can be derived from basic thermodynamic considerations, with the help of chemical and electrochemical potentials, \( \mu_i \) and \( \mu_i^\alpha \), respectively. Consequently the electrochemical potential of species \( i \) in phase \( \alpha \), \( \mu_i^\alpha \), is given by:\[ \mu_i^\alpha = \mu_i^\alpha + z_i F \varphi^\alpha = \mu_i^0 + R T \ln(a_i^\alpha) + z_i F \varphi^\alpha \]  

where \( \mu_i^\alpha \) is the chemical potential, \( z_i \) is the charge number, \( \mu_i^0 \) is the standard chemical potential, and \( a_i^\alpha \) is the activity of species \( i \); \( \varphi^\alpha \) is the electrical potential of phase \( \alpha \).

From Eq. (5) one can formulate the expression for \( \mu_i^\alpha \) in the aqueous phase (sample solution, S) and in the organic phase (membrane phase, M). Since the electrochemical equilibrium should prevail at the aqueous/organic interface, i.e. sample/ISM interface, the electrochemical potential must be equal in both phases, i.e. \( \mu_i^S = \mu_i^M \), and the phase boundary potential can be expressed as follows:\[ E_{PB} = \Delta \varphi = \varphi^M - \varphi^S = \frac{\mu_i^0S - \mu_i^0M}{z_i F} + \frac{RT}{z_i F} \ln \frac{a_i^S}{a_i^M} \]  

where \( \varphi^S \) and \( \varphi^M \) are the electrical potentials in the sample and membrane, respectively; \( \mu_i^0S \) and \( \mu_i^0M \) are the standard chemical potentials and \( a_i^S \) and \( a_i^M \) are the activities of species \( i \) in the respective phases; \( z_i \) is the charge number of species \( i \).

If interferences from other ions are not considered, and assuming that \( a_i^M \) is constant and sample-independent so that it can be included into the constant \( E_i^0 \), Eq. (6) simplifies to the classical Nernst equation:\[ E_{PB} = E_i^0 + \frac{RT}{z_i F} \ln a_i^S \]  

where \( E_i^0 \) includes all the constant potential contributions to \( E_{PB} \) for species \( i \), and is constant for a given ion, but varies from ion to ion\[ z_i \) is the charge number of species \( i \); \( a_i^S \) is the activity of species \( i \) in the sample.
Hence, according to the phase boundary model, and changing from the natural logarithm to the 10-base logarithm, at 25 °C Eq (3) can be written as:

\[
EMF = E_i^0 + 2.303 \frac{RT}{z_iF} \log a_i^S + E_j + E_{const} = \\
= E_i^0 + \frac{0.05916}{z_i} \log a_i^S + E_j = E_i^0 + s_i \log a_i^S
\]  

where \(E_i^0\) is the standard potential for species \(i\), i.e. the sum of all the species-dependent and sample-independent potential contributions to the EMF (except \(E_j\)), i.e. \(E_i^0 = E_i^0 + E_{const}\). \(E_i^0\) corresponds to the intercept of the linear (vs log \(a\)) response function and is unique for an ISE in a given measurement setup. \(s_i = 2.303RT/(z_iF)\) is the Nernstian slope of the ISE response function, which is 59.16/z\(_i\) mV at 25°C. This shows that the EMF of a potentiometric cell is related to the activity of the ion in the solution and that in the linear response range, at 25°C, a 10-fold change in the activity of a monovalent ion should result in a 59.16 mV change in the EMF. When the calibration plot of EMS vs. log \(a_i\) shows a slope of 59.16 mV/z\(_i\) the ISE is said to exhibit a so-called Nernstian behaviour\[3\].

2.3. Selectivity

So far the discussion has focused on the situation when the potentiometric response of the ISE is exclusively connected with the analyte of interest, i.e. the primary ion \(i\). However, in practice this is seldom the case. Unfortunately the ISM never responds ideally for the primary ion alone, it can only be designed to prefer it in some extent to other, interfering ions, i.e. to be selective for \(i\). The selectivity of an ISE membrane is its capability to differentiate between various ions\[1\]. It is influenced by the membrane material, as well as by the lipophilicity of the ions involved, however, the factor that has the greatest influence on the selectivity of ISMs is the ionophore\[2\].

As in the above derivation of the Nernst equation for ion \(i\) in Eq. (7), it can also be written for an interfering ion \(j\), with \(E_j^0\) as the intercept of the linear response function. Traditionally the response to both the primary and interfering ion (of the same charge) has been described by an extended Nernst equation, the semiempirical Nikolskii-Eisenman equation:

\[
EMF = E_i^{0'} + \frac{RT}{z_iF} \ln \left( a_i + \sum_{i\neq j} K_{i,j}^{pot} a_j^{z_i/z_j} \right) \]  

(9)

where \(E_i^{0'}\) is the standard potential and includes all the constant potential contributions (including \(E_j\)); \(z_i\) and \(z_j\) are the charge numbers, and \(a_i\) and \(a_j\) are the activities of the primary and the interfering ions (of the same charge), respectively, in the same solution.
The *potentiometric selectivity coefficient*, \( K_{i,j}^{pot} \), defines the ability of the ISE to discriminate against interfering ions, the response function of an ISE can be predicted with the help of it. It is a constant characteristic of a given ion-selective electrode. The selectivity coefficient, is obtained from:\(^8\)\(^9\):

\[
\log K_{i,j}^{pot} = \frac{z_i F}{2.303 RT} \left( E_i^{0'} - E_j^{0'} \right) = \frac{z_i F}{2.303 RT} \left( E_j^{EMF} - E_i^{EMF} \right) \log \frac{a_i}{a_j} \tag{10}
\]

\[
\log K_{i,j}^{pot} = \frac{E_i^{0'} - E_j^{0'}}{s_i} = \frac{E_j^{EMF} - E_i^{EMF}}{s_i} + \log a_i - \frac{z_i}{z_j} \log a_j \tag{11}
\]

where \( E_i^{0'} \) and \( E_j^{0'} \) are the standard potentials of species \( i \) and \( j \), respectively, and include all the constant potential contributions (including \( E_j \); \( E_i^{EMF} \) and \( E_j^{EMF} \) are the measured EMFs in solutions, according to Eq. (8), containing only the ion activities either \( a_i \) or \( a_j \) alone, with the charge numbers \( z_i \) and \( z_j \) respectively; \( s_i \) is the Nernstian slope of the ISE response function of the primary ion.

In order for Eq. (10) and (11) to be valid, the ISE must show a Nernstian response for both the primary and the interfering ions\(^8\)\(^9\). According to Eq. (11) the potentiometric selectivity coefficient is actually the difference between the EMF responses, extrapolated to unity activity, of a solution containing either the primary or the interfering ion.

In general, the classical Nikolskii-Eisenman equation, Eq. (9), is only valid for mixed ion responses for the case when the primary and interfering ion has identical charge numbers, i.e. \( z_i = z_j \), due to the power term \( a_j^{z_i/z_j} \)\(^10\). However, to describe the response of ionophore based polymeric membrane ISEs, based on the phase-boundary model, a new equation has been proposed that is valid for any number of mono-, di-, and trivalent ions\(^11\):

\[
EMF = E_i^{0'} + \frac{RT}{F} \ln \left[ \frac{1}{2} \sum_{j_1} K_{i,j_1}^{pot 1/z_i} a_{j_1} \right]^\frac{z_i}{2} + \left( \frac{1}{2} \sum_{j_1} K_{i,j_1}^{pot 1/z_i} a_{j_1} \right)^\frac{z_i}{2} + \sum_{j_2} K_{i,j_2}^{pot 2/z_i} a_{j_2} \right] \tag{12}
\]

where \( I \) is the analyte with charge number \( z_i \), which does not have to be identical to the primary ion \( i \); and \( j_1 \) and \( j_2 \) indicate monovalent and divalent ions, respectively,
including \( I \) for which \( K_{i,1}^{pot} = 1 \). This equation will simplify to the Nikolskii-Eisenman formulism when all the involved ions are of the same charge.

\[
\log K_{i,j}^{pot} / s_i = \frac{Z_i}{Z_j} \log a_i / a_j
\]

**Figure 2** Determination of potentiometric selectivity coefficients according to the separate solution method (SSM). The \( \log K_{i,j}^{pot} \) corresponds to the potential difference between the separately measured response functions for the two ions in their pure salt solution extrapolated to \( \log a = 0 \) \( (E_J^{0r} - E_I^{0r}) \) and divided by the slope of the response function of the primary ion \( (s_i) \).

There are different methods to determine \( K_{i,j}^{pot} \), of which the separate solution method (SSM) is the most often used. According to the SSM, the unbiased values of the selectivity coefficient is obtained by measuring the unbiased response function of the ISE, first in the solution of the discriminated (interfering) ion, \( j \), and then in the solution of the primary ion, \( i \). To avoid sub-Nernstian electrode response caused by the primary ion or the more preferred interfering ion leaching out from the membrane, a certain protocol\(^{[12]}\) has to be followed. An ISM that has never been in contact with the primary ion is used, and the measurement sequence goes from the most to the least discriminated ions, and at the end the response to the primary ion is recorded. This, of course, implies that some prior knowledge of the selectivity. The measurement should be done at two different ion activities for each ion at high enough concentration \( (>10^{-4} \text{ M}) \), but not too high to avoid coextraction of counterions. In Figure 2, the determination of selectivity coefficients is graphically represented.

There are also other methods, besides the SSM, for determining the selectivity of ISEs. The fixed interference method (FIM) is based on measuring a calibration curve for the primary ion with a constant background of the interfering ion, \( a_i \). The selectivity is determined by calculating \( a_i \) from the intersection of the extrapolated Nernstian part of the calibration curve with \( a_j \), and using the equation\(^{[8]}\): 

\[
\log K_{i,j}^{pot} = \log a_i / a_j^{z_i/z_j}
\]

Another, more empirical method is the matched potential method (MPM). With this
method a certain activity of primary ion, $\Delta a_i$, is added to a starting solution and the resulting potential change is recorded. After this interfering ions, $\Delta a_j$, are added to an identical starting solution until a corresponding potential change is observed. The selectivity factor is simply $\Delta a_i / \Delta a_j$, which is highly dependent on experimental conditions and do not have any predictive ability, only practical significance$^8$.

2.4. Detection limit

Another important parameter of ISEs is the detection limit (DL). The specific response of the sensor to the analyte is limited by a lower (LDL) and an upper detection limit (UDL)$^{13}$. For most applications, i.e. for bioassays, the LDL is of more interest. Generally in analytical chemistry, the lower detection limit is defined as the concentration for which the same analytical signal is measured as in a blank solution plus three times the standard deviation of the background noise. In potentiometry, however, the detection limit is generally defined differently. According to the IUPAC recommendations$^{13}$, the detection limits are defined by the cross sections of the two extrapolated segments of the linear parts of the calibration curve (Nernstian and non-Nernstian), as shown schematically in Figure 3.

![Figure 3](image_url)  

Figure 3 Calibration curve of an ISE and the definition of the lower and upper detection limits (LDL and UDL) according to the IUPAC definition as the cross section of two extrapolated linear parts of the calibration curve.

From new findings on lowering the detection limit for polymeric membrane electrodes another alternative for determining the DL has also been proposed$^{14}$. It defines the DL as the $a_i$ where the measured potential response starts to deviate from the Nernstian part of the calibration curve by $(RT/z_iF)ln2$ in either directions. This approach was proposed mostly for electrodes with a super-Nernstian response, since the IUPAC definition has no applicability in that case, and it gives approximately an identical DL to the IUPAC definition for Nernstian ISEs. The upper detection limit is governed by the coextraction of primary ions together with counterions from the sample solution.
into the membrane as the concentration increases\cite{15}. Thereby the membrane loses its permselectivity and the concentration of counterions in the membrane phase increases, this is the so-called Donnan failure. Since the concentration of analyte ions in the membrane phase ($a_i^M$) rises with increasing activity in the sample ($a_i^S$), the electrode gives a less than Nernstian response (Equation (6)). The UDL decreases with increasing lipophilicity of the counterions in the sample and with increasing stability of primary ion–ionophore complexes in the membrane, since stronger complexes will facilitate coextraction. The degree of coextraction, and hence the upper detection limit, naturally depends on the amount of ionic sites, i.e. lipophilic salt, in the membrane. The lipophilic salt, on the other hand, will have a deteriorating effect on the selectivity and LDL, due to increased ion fluxes, if added in too large quantities to the ISM\cite{2} \cite{15}. The so-called static lower detection limit ($LDL_{i}^{static}$) is caused by interfering ions in the sample solution (insufficient selectivity to the primary ion), it is achieved when the interfering ions give the same potential response as the primary ion. $LDL_{i}^{static}$ can be expressed with the help of the selectivity coefficient ($K_{i,j}^{pot}$) and the activity of the interfering ion ($a_j$)\cite{2}:

$$LDL_{i}^{static} = K_{i,j}^{pot} \frac{a_i^{z_i}}{a_j^{z_j}}$$

(13)

where the $z_i$ and $z_j$ are the charge numbers of the primary and interfering ions, respectively. As can be seen from equation (13), the better the selectivity the lower the detection limit. However, not even for highly selective ISEs can the lower detection limit predicted by Eq. (13) be reached in practice. In practice, the lower DL will be dictated by the primary ion due to the zero-current ion fluxes in the membrane. The outward (from the membrane to the sample) flux of primary ions causes a biased LDL that can be significantly higher than the unbiased (static) one\cite{16} (see Figure 4).

**Figure 4** Representation of the lower detection limit caused by the presence of a certain activity of an interfering ion ($a_j$) as background (static LDL) and by leaching of the primary ion (biased LDL).
2.4.1. Lowering of the detection limit

Usually the lower detection limit of most ISEs is approximately $10^{-5} – 10^{-7}$ M, modifications to the electrode design and adjustment of the measurement procedures and techniques has to be done to lower it. If the LDL is determined by zero-current ion fluxes, than the outward directed flux of primary ions needs to be avoided or controlled, to obtain an LDL better by several orders of magnitude. Various strategies have been employed in order to reduce these unwanted fluxes. In general there are two basic approaches: either decreasing the ion fluxes in the membrane or increasing them in the sample solution.

The radical improvement of the DL started\cite{17} with adjustment of the inner solution to match the sample solution more closely. By equalizing the concentration of the primary ion on both sides of the membrane, the driving force of the leaching is decreased\cite{14} \cite{16} \cite{18} \cite{19}. This can be achieved by partly exchanging the primary ions in the inner surface layer of the membrane by interfering ones. For this purpose an appropriate inner solution is used, which contains both primary and interfering ions. This technique, however, is quite inconvenient since ideally it should be adjusted to match each sample, otherwise a too strong inward flux can cause a super-Nernstian response, whereas an outward flux will lead to a non-ideal DL\cite{20}. The other option to reduce the bias is decreasing the concentration gradient in the membrane. By reducing the total amount of ions in the membrane, i.e. lowering the concentration of ionic sites\cite{20} or adding lipophilic particles to the membrane\cite{21}, this difference will be smaller too, hence leaching of primary ions from the membrane decreases. Unfortunately this affects also the UDL and the selectivity. External current control has also been used to counterbalance the ion fluxes in the membrane\cite{22} \cite{23}, which seems to be more convenient than adjusting the inner solution, however, ideally the current should be adjusted for each sample and it is unlikely to be applied for practical measurements\cite{24}.

Another method to reduce the bias is by decreasing the diffusion coefficients in the membrane or the thickness of the diffusion layer. On one hand, the ionic diffusion coefficients in the membrane can be reduced by changing the membrane composition: using higher PVC content\cite{16} \cite{25} \cite{26}, which, however, will also increase the membrane resistance and conditioning times; using other more viscous polymers instead of the generally used PVC (see below); or by covalently binding the ionophore\cite{27} \cite{28}. On the other hand, the thickness of the diffusion layer can be decreased: by decreasing that of the aqueous Nernst layer in order to increase the transport of the primary ion from the membrane surface, by strong stirring\cite{16}, using rotating electrode\cite{20} \cite{30}, wall jet\cite{22} \cite{31}, or flow-through\cite{16} systems.

Solid-contact ISEs are promising in lowering the detection limit. With SCISEs, any leaching of primary ions due to coextraction is eliminated because there is no inner solution from which ions could be extracted into the membrane. This design may
significantly help in lowering transmembrane ion fluxes as it replaces the inner reservoir of highly concentrated solution with a solid-contact conducting polymer layer\textsuperscript{[32]}\textsuperscript{[33]}. Additionally, the conducting polymer film may be doped with a compound that complexes the primary ion, thus promoting a supplementary driving force for it to enter the membrane\textsuperscript{[34]}\textsuperscript{[35]}. However, the drifts of the SCISEs due to possible formation of water layers in the structure\textsuperscript{[33]} and/or spontaneous redox reactions of the polymer\textsuperscript{[36]} still needs to be solved.

Even though most of the mentioned methods suffer from some drawbacks, they have successfully been applied for constructing ISEs with LDL in the order of $10^{-8} - 10^{-12}$ M\textsuperscript{[11]}\textsuperscript{[37]}, the most impressive ones in picomolar range, for Ag\textsuperscript{+}\textsuperscript{[19]}\textsuperscript{[38]} and Pb\textsuperscript{2+}\textsuperscript{[18]}\textsuperscript{[21]}\textsuperscript{[33]}.

2.5. The components of the ion-selective membrane

Originally ionophore-based membranes constituted liquid solutions of ionophores or lipophilic ion-exchanger salts in organic solvents immiscible with water and mechanically supported with a thin porous film, e.g. filter paper or sintered glass\textsuperscript{[39]}. These electrodes were rather inconvenient to use and sluggish, so they were replaced by solvent polymeric liquid membrane electrodes, based typically on highly plasticized PVC\textsuperscript{[40]}\textsuperscript{[41]}. For a long time the most widely used polymeric liquid membrane consisted of about 66 wt % plasticizer, 33 wt % high-molecular weight PVC, 1 wt % ionophore and a small amount of some lipophilic additive\textsuperscript{[2]}. Commonly polymeric liquid membranes are prepared by casting from a solution, or so-called membrane cocktail, containing all the membrane components dissolved in an organic solvent, such as tetrahydrofuran (THF). For the preparation of solid-contact ion-selective electrodes the membrane cocktail is normally drop-cast directly on top of the solid-contact.

For an adequate performance of the ISE the following membrane components are usually used:

2.5.1. Polymer matrix

The polymer matrix provides the necessary physical properties of the membrane, such as the mechanical stability, elasticity and most importantly the immiscible phase from the sample solution. The polymer matrix also has a slight influence on the membrane properties, such as the polarity or the adhesion on the electrode surface in case of a solid contact.

*Plasticized poly(vinyl chloride)*

The most common polymer matrix for potentiometric ion-selective electrodes is poly(vinyl chloride) which is used with an adequate membrane solvent generally in a 2:1 wt % ratio. It gained popularity due to its good compatibility with ionophores, easy
handling and chemical inertness\textsuperscript{[21]}. The ion mobility in PVC membranes containing two thirds of plasticizer is about 1000 times lower than in water\textsuperscript{[42]}. Although in most of the cases it is assumed to be inert, minor amounts of its ionic impurities can act as ionic sites and affect the performance of the ISEs\textsuperscript{[43]} \textsuperscript{[44]}.

Despite its popularity, the use of plasticized PVC membranes have several downsides: impairment of the sensor response due to the slow leaching of the ionophore\textsuperscript{[45]}, biocompatibility issues due to leaching of the plasticiser \textsuperscript{[46]}, extraction of selectivity-altering lipophilic components into the organic membrane phase\textsuperscript{[47]}, high water uptake\textsuperscript{[48]}, insufficient detection limits\textsuperscript{[25]} due to the high ion mobility in PVC\textsuperscript{[49]} \textsuperscript{[50]} \textsuperscript{[51]}, and poor adhesion to a number of substrates\textsuperscript{[46]}.

\textit{Poly(methacrylates) and poly(acrylates)}

To overcome the above mentioned shortcomings several polymers consisting of different methacrylate and acrylate monomers have been explored\textsuperscript{[52]} as alternative materials for ISMs. While a homopolymer of methyl methacrylate (MMA) needs to be mixed with a plasticizer\textsuperscript{[53]}, a copolymer of MMA with some other monomer, such as decyl methacrylate (DMA)\textsuperscript{[33]} \textsuperscript{[54]} \textsuperscript{[55]} \textsuperscript{[56]}, n-butyl acrylate (NBA)\textsuperscript{[53]} \textsuperscript{[57]} or isodecyl acrylate (IDA)\textsuperscript{[58]} \textsuperscript{[59]} are so-called self-plasticized materials. These plasticizer-free PA based membranes are more biocompatible. The apparent ion mobility in these materials is about 3 orders of magnitude lower (~10\textsuperscript{-11} cm\textsuperscript{2}/s)\textsuperscript{[60]} than in plasticized PVC, these reduced ion fluxes make PA materials probably the best candidates for preparing low detection limit ISEs. However, the methacrylic-acrylic polymer based ISMs have their drawbacks too. It has been shown with FTIR-ATR measurements that the equilibrium water uptake of different PA membranes is much higher than for plasticized PVC\textsuperscript{[61]}. They are generally polymerized by the user which means a great variety of materials with different properties\textsuperscript{[55]}, and they have high resistance due to their low ion mobility\textsuperscript{[57]} \textsuperscript{[59]} \textsuperscript{[60]} \textsuperscript{[62]} which requires long conditioning times\textsuperscript{[55]} \textsuperscript{[62]}.

\textit{Silicon rubber}

Silicon rubber (SR) emerges as a good candidate to replace PVC as well, especially in solid-contact electrodes. It has superior adhesion\textsuperscript{[63]} to different electrode substrates, excellent mechanical characteristics, better biocompatibility than PVC membranes\textsuperscript{[64]} \textsuperscript{[65]} \textsuperscript{[66]} \textsuperscript{[67]}, lower nonspecific adhesion of proteins from biological samples. Its water repellent properties ensure a much lower water uptake of the silicone rubber based ISMs than that of PVC or PA membranes\textsuperscript{[61]}. Although the use of silicon rubber as membrane matrix was reported already in 1973\textsuperscript{[68]} \textsuperscript{[69]}, it has been studied and used only in a limited range of ISEs, which is mainly explained by some of its drawbacks, such as the poor solubility of the membrane constituents in most of the SRs\textsuperscript{[70]} \textsuperscript{[71]} and the high electrical resistance (bulk impedance) of the SR-based ISEs. Although the pure SRs themselves possess a low glass transition temperature\textsuperscript{[71]}, by the addition of
plasticizer the solubility of the membrane components can be increased\(^\text{[70]}\)\(^\text{[71]}\) to overcome the above mentioned problem. Lately the one-component, room temperature vulcanizing (RTV) silicon rubbers, commercialized as an insulating coating in the electrical industry, emerged as a viable alternative. Especially Dow Corning RTV 3140, which is a silanol-terminated poly(siloxane), consisting of >60 wt % dimethyl siloxane, 10-30 wt % trimethylated silica and 5-10 wt % methyltrimethoxysilane. It can be dissolved in THF and cures at room temperature through a methanol-evolving moisture activated condensation process when the silicone prepolymers form a clear and flexible high molecular weight rubber. RTV 3140 has been successfully used for ISEs, both with and without plasticizer, for ions such as Na\(^+\)\(^\text{[70]}\)\(^\text{[72]}\)\(^\text{[73]}\)\(^\text{[74]}\)\(^\text{[75]}\)\(^\text{[76]}\), K\(^+\)\(^\text{[72]}\)\(^\text{[73]}\)\(^\text{[74]}\)\(^\text{[77]}\), Ca\(^{2+}\)\(^\text{[67]}\)\(^\text{[71]}\)\(^\text{[72]}\)\(^\text{[73]}\)\(^\text{[74]}\)\(^\text{[77]}\), H\(_3\)O\(^+\)\(^\text{[72]}\)\(^\text{[74]}\)\(^\text{[77]}\), NH\(_4^+\)\(^\text{[66]}\)\(^\text{[77]}\) and CO\(_3^{2-}\)\(^\text{[74]}\). These sensors commonly showed an enhanced lifetime and sensor-to-sensor reproducibility\(^\text{[70]}\)\(^\text{[71]}\)\(^\text{[74]}\). Due to its low water uptake, SR could be a promising material for the fabrication of SCISEs.

2.5.2. Membrane solvent or plasticizer

The plasticizer is the actual solvent in which the membrane constituents are dissolved, and as such it must be compatible with all membrane components, otherwise it will exude and the membrane composition will become unstable\(^\text{[2]}\)\(^\text{[42]}\). It is a water-immiscible organic solvent which is required to achieve optimal physical properties, i.e. to lower the glass transition temperatures below room temperature to obtain better elasticity of the membrane, and to ensure relatively high mobilities of the membrane components\(^\text{[78]}\). In a classic plasticized PVC membrane plasticizer makes up two thirds of the membrane (2:1 w/w ratio, 60-66%) so it has a strong effect on the ISM properties\(^\text{[42]}\)\(^\text{[78]}\). The membrane solvent should be highly lipophilic and provide an optimal supporting matrix for the selectivity behaviour of the ionophore as it can modify the membrane selectivity according to its polar and dielectric constant\(^\text{[2]}\). Generally it can be stated that less polar plasticizers prefer monovalent ions over monovalent ions of the same radius\(^\text{[78]}\). From the two most widely used plasticizers, o-nitrophenyl octyl ether (o-NPOE) and bis(2-ethylhexyl) sebacate (DOS) the former is used in ISMs when divalent- and the latter when monovalent ions are to be measured\(^\text{[2]}\).

2.5.3. Lipophilic ion-exchangers

To achieve a theoretical Nernstian electrode response function with an ISE, the so-called permselectivity of the membrane must be ensured, the ISM must have ion-exchanging properties. In case of neutral ionophore based ISMs this is realized by incorporation of highly lipophilic ion-exchangers, so-called ionic sites, of opposite charge to the primary ion into the membrane. These charged ionic sites will prevent...
coextraction of counterions from the solution and provide for a fairly constant activity of the primary ion in the membrane\cite{12} \cite{15}. It means that no significant amount of the counterions from the sample enter the membrane phase. This is the so-called Donnan exclusion.

ISE membranes without additional lipophilic ion-exchangers may also give a Nernstian response because of a certain amount of charged carriers\cite{79} and ionic impurities in the polymer matrix\cite{44} \cite{43} \cite{80} or other membrane constituents\cite{81}. Typically the impurities concentration in PVC membranes is about 0.01-0.05 mM\cite{82}, therefore to secure the required electrode response, the concentration of intentionally added sites has to be 1 mM or higher\cite{41}. But as the lipophilic ion-exchanger can influence the selectivity of the ISM, normally the concentration should not exceed that of the ionophore\cite{42}.

![Figure 5](https://example.com/f5.png) Structural formulas of two lipophilic ion-exchangers: (A) NaTFPB and (B) TDMA-NO₃, as well as of the lipophilic additive (C) ETH500.

The charged ionic sites not only prevent coextraction and provide for a fairly constant activity of the primary ion in the membrane\cite{2} \cite{81} \cite{15} \cite{83}, but they also lower the electrical resistance of the membrane, can have a slight influence on the selectivity\cite{84} and lower detection limit\cite{20}, or can reduce the response time and the activation barrier for the cation-exchange reaction\cite{85}.

In the membrane the lipophilic ion-exchanger dissociates into one lipophilic ion and one hydrophilic ion, with the hydrophilic ion being exchanged to the primary ion during the conditioning step\cite{2}. In cation-selective membranes, tetraphenylborate derivatives, such as sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB), are used as ionic sites, while the anionic permeselectivity is ensured with lipophilic tetraalkylammonium salts, such as tridodecylmethylammonium nitrate (TDMA-NO₃) \cite{2} \cite{15}, see Figure 5A and B. Furthermore, an inert lipophilic additive without ion-
exchanger properties can also be added to the ISM in order to decrease the resistance of the membrane\textsuperscript{[86]} \textsuperscript{[87]}. Tetradecylammonium tetrakis(4-chlorophenyl)borate, better known as ETH500 (Figure 5C), will dissociate into two lipophilic ions, thus increasing the amount of cationic and anionic sites equally.

2.5.4. Ionophore

The most important constituent responsible for the selective and reversible binding to the target ion is the lipophilic complexing agent, the so-called ionophore or ion carrier. It is this selective complexation process, leading to a charge separation when ions are moved between the aqueous and organic phase, that gives rise to the measurable change in the phase-boundary potential at the ISM/sample interface.

Membranes containing only a lipophilic ion exchanger, no ionophore, will follow a selectivity order depending on the partitioning of the ions between the aqueous and organic phase, according to their chemical potentials. This selectivity sequence is in agreement with the so-called Hofmeister series\textsuperscript{[88]} that has traditionally been associated with the lipophilicity of ions\textsuperscript{[89]}. Ion-exchangers have mainly been utilized for the construction of anion-selective electrodes, due to the lack of adequate ionophores for anions, and typically lipophilic quaternary ammonium, phosphonium or borate salts have been used\textsuperscript{[2]}.

There is a vast number of ionophores available for more than 60 analytes, with several eligible ionophores for each analyte depending on the measuring conditions\textsuperscript{[90]}. The uncomplexed form of the ionophores can either be electrically neutral or charged, yet the most commonly used ionophores for cations are neutral carriers. Historically many neutral ionophores were based on natural macrocyclic molecules like valinomycin (K\textsuperscript{+}), nonactin (NH\textsubscript{4}\textsuperscript{+}) or monensin (Na\textsuperscript{+}), or synthetic crown compounds such as crown ethers (alkali and alkaline earth metal ions)\textsuperscript{[90]}. However, nowadays many other cyclic and noncyclic ionophores are available. The structural formula of a few ionophores used in this thesis can be seen on Figure 6.

Although the choice of plasticizer or polymer matrix can affect the preference of the different ions for the organic phase, in comparison with other membrane constituents, the ionophore has the greatest impact on the membrane selectivity. It is responsible for the selective and reversible extraction of the target analyte into the membrane. It should bind the analyte strongly but reversibly and all the interfering ions weakly. In an ideal case, the complex formation constant \( \log \beta \), for the ion-ionophore complexes is higher by several orders of magnitude for the primary ion than for interfering ones. Too strong complexation would lead to irreversible binding and coextraction (Donnan failure). Ultimately it is the complex formation ability of the ionophore with the different ions in a sample matrix that will ensure a selective response for the analyte and determine the selectivity sequence of the ISE\textsuperscript{[2]}. 
Besides the complexing center, the ionophore molecule contains several apolar groups, such as alkyl chains to ensure sufficient lipophilicity in order to prevent the leaching of the ionophore into the aqueous phase.

Figure 6 The structural formula of three of the ionophores used in this thesis: (A) copper (II) ionophore, (B) CSV321 ionophore, both used as silver ionophores and (C) calcium ionophore IV
3. Solid-contact ion-selective electrodes

3.1. Introduction

In conventional ISEs (*liquid contact electrodes*, see Figure 7A) the electrode has a symmetrical design with the sensing membrane being intermediated between the sample solution and the internal reference half-cell, i.e. the internal electrolyte solution. Owing this well-defined pathway for the ion-to-electron transduction with stable and reversible phase-boundary potentials throughout the whole system, liquid contact electrodes have potential stabilities hard to surpass in many routine applications. On the other hand, though, this design proved to be limiting in a number of situations. These electrodes only work in positions which ensure a direct contact between the sample and the internal electrolyte, i.e. not in upside-down orientation; they have poor tolerance for high-pressure environments, such as deep-water measurements or high-pressure sterilization; they require periodical service, i.e. refilling of the internal electrolyte, making autonomous remote monitoring more difficult to achieve. As already discussed before, the inner filling solution can act as a reservoir for the primary ions[^31], and the leaching of the primary ions can have a detrimental effect on the lower detection limit. Furthermore, very importantly, it is difficult to miniaturize electrodes of the classical design[^91], strongly restricting their use for studying small samples, which is especially substantial for the analysis of biological samples.

![Figure 7 The different designs of ion-selective electrodes: (A) liquid contact ISE; (B) coated-wire electrode (CWE); and (C) solid-contact ISE (SCISE)](image)

**Polymeric liquid membrane based solid-state electrodes** (Figure 7B and C) offer solution for these shortcomings; they are easy to miniaturize[^92][^93] and compatible with microfabrication technologies[^94]; their greatest potential lies in the facile construction of robust, inexpensive, maintenance-free, or single-use miniaturized sensors, e.g. in the field of homecare or bedside diagnostics, environmental analysis, and quality control checking. These electrodes are asymmetric sensors, where the internal reference electrode and the inner filling solution is replaced by a solid material which should be able to provide the same function. While in the ion-selective membrane there
is an ionic conductivity, the solid substrate electrode (reference element) is electron-conducting.

The first important step towards modern solid-state electrodes was made by Cattrall and Freiser\cite{95}, when they constructed the coated-wire electrode (CWE, Figure 7B), an extremely simple approach, a PVC sensing membrane directly coated onto a metal (Pt) conductor\cite{96}. As the membrane/metal interface, which should provide ion-to-electron transfer between the polymeric membrane and the underlying metal layer, is “blocked”, these electrodes are characterized by ill-defined phase boundary potentials, and sensitivity to the measuring conditions\cite{97}. Subsequently CWEs exhibit limited long-term stability\cite{98} with potential drifts of up to a few hundred µV/h range.

Many efforts have been made to solve this problem, various intermediate layers (solid contacts, Figure 7C) that permit charge transfer between the ion-selective membrane and the solid electron-conducting substrate have been investigated. There are three main requirements on a solid contact to exhibit a stable inner phase boundary potential and therefore a stable and reproducible potential response\cite{99}:

1. reversible and stable transition from ionic conduction in the membrane to electronic conduction in the metal substrate, i.e. ion-to-electron transduction at the interface
2. the potential of the electrochemical reaction ensuring this transfer has to be constant, stable, and must not depend on the sample composition, i.e. ideally nonpolarizable interface with high exchange current density which is not influenced by the input current of the amplifier
3. the SC must not influence the membranes analytical performance, no side reactions should occur besides the main electrode reaction, i.e. SC materials with stable chemical composition.

As a thin aqueous layer beneath the membrane\cite{100} can lead to a drifting electrode potential\cite{101}, the above mentioned three criteria can be complemented with the requirement of

+1) hindering the formation of an aqueous layer by using hydrophobic solid-contact materials and/or membrane matrices\cite{48} [61] [102] [103] [104] [105].

Conducting polymers are good candidates to fulfil these requirements, as they possess both electronic and ionic conductivity due to their ion transport-coupled redox (doping) reactions\cite{106} \cite{107}. Furthermore, they show sufficiently high redox capacitance\cite{108} and absence of side reactions\cite{109}. However, depending on the polymer and the measuring conditions, they can suffer from light sensitivity, and other undesirable redox processes, caused by oxygen, CO₂ or pH change.

Lately the field of SCISEs became dominated by conducting polymers as solid contact materials, this thesis also focuses on this topic, although alternative SC materials will be also briefly discussed.
3.2. Conducting polymers

Traditionally polymers are known to have good insulating properties, for instance they are used to coat metal wires as electric insulators. This view was changed drastically in 1976 by Heeger, MacDiarmid, and Shirakawa when they discovered that a polymer, polyacetylene (PA) can actually be made conductive almost like a metal[^110], and that upon doping polymers can have the ability to conduct electricity[^111]. They were awarded with the Nobel Prize in Chemistry in 2000 “for the discovery and development of electrically conductive polymers”. Although many of the conducting polymers (CP) were actually known already before (e.g. polyaniline and polypyrrole), their unique properties were not realized.

Electrically conducting polymers or, more precisely, intrinsically conducting polymers are organic polymers that, via their mobile \( \pi \)-electrons, conduct electricity due to the conjugated structure of the polymer. The key feature for their conductivity is therefore the regular structure of alternating single- and double-bonded \( sp^2 \)-hybridized atoms, where the unpaired \( \pi \)-electrons are delocalized over several carbon atoms along the backbone due to the partially overlapping \( p_z \)-orbitals. Since this conjugated system consist of regularly alternating shorter and longer bonds, \( \pi \) and \( \sigma \)-bonds respectively, CPs have a fully occupied \( \pi \)-band (or valence band) and a totally empty \( \pi^* \)-band (or conduction band) separated by an energy gap (band gap, \( E_g \approx 0.8 - 4 \, eV \))[^112]. The band gap is the energy difference between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). This band gap is one of the main factors responsible for the electrical and optical properties of conducting polymers. Without any further modification conducting polymers behave as insulators or semiconductors (\( 10^{-10} - 10^{-5} \) S/cm conductivity[^113]), as their energy bands are either fully occupied or totally empty and their band gap is too high for thermally activated conduction[^112]. However, the band structure of CPs can be modified, the conjugated backbone can be “doped” by partial oxidation (\( p \)-doping) or partial reduction (\( n \)-doping). This removes electrons from the full energy band or adds them to the empty one, both resulting in partially filled energy bands, highly mobile electrons in the delocalized orbitals, and consequently an increased conductivity (\( 0.1 - 10^5 \) S/cm for different polymers[^114]). During the doping process local charge carriers are formed, cations for \( p \)-doping, and anions for \( n \)-doping, often referred to as polarons and bipolarons, and the movement of these charged defects along the backbone endows the conductivity[^112] [^115] [^116].

By controlling the doping level of the polymer, anywhere between non-doped to fully doped state, its conductivity can be reversibly be adjusted within its whole conductivity span, ranging over several orders of magnitude[^117]. Most CPs require \( p \)-doping, however several can undergo \( n \)-doping as well, e.g. PEDOT[^118]. The doping is done either chemically or electrochemically. During electrochemical doping, the doping level can be accurately defined in a reproducible and reversible manner. Oxidation of
a conducting polymer is accompanied by simultaneous incorporation of counteranions form the electrolyte solution to compensate the charge, whereas counter-cations are incorporated during reduction, these counterions are known as dopants[114].

CPs can be synthesized either chemically or electrochemically, with each having advantages and disadvantages. In the industrial community chemical polymerization is normally preferred as this technique fits better for large scale production and post-processing methods. On the contrary, the scientific community favours electrochemical polymerization for its simplicity and reproducibility. The straightforward electrochemical polymerization offers the possibility for producing well controllable, thin films directly on the electrode surface; the ease of synthesis with the polymerization and doping happening simultaneously; and the possibility of entrapping different molecules in the polymer[114]. The polymerization is usually performed in a conventional three-electrode electrochemical cell, containing the monomer and the electrolyte salt (dopant). A number of important variables has to be considered, including deposition time and temperature, solvent system, electrolyte and deposition charge. Each of these parameters has an effect on film morphology, mechanics and conductivity[114].

Although conducting polymers are first of all known for their electrical conductivity, spanning over more than fifteen orders of magnitude[114], their structure is also responsible for exceptional modifiable optical and chemical properties widely used in electrochromic devices (e.g. OLEDs, displays, smart windows), in solar cells and sensors. Additionally they are light, mechanically flexible, and one of their main advantage is their processability, mainly by dispersion. With the availability of stable and reproducible dispersions, PEDOT and PANI have gained some large scale applications (see below). PEDOT is mainly used in the form of PEDOT(PSS) dispersions.

Owing their dual electronic and ionic conductivity, CP materials are ideal ion-to electron transducers, e.g. SC materials in SCISEs. Furthermore, CPs are organic in nature, making them more likely to be biocompatible[114]. In this thesis a PANI dispersion was used for the preparation of low detection limit SCISEs (Paper III); whereas PEDOT(PSS) was used for the construction of a 3D nanostructured conducting polymer solid contact (Paper IV), and for the manufacturing of surface-imprinted polymer film for selective protein recognition (Paper V).

3.2.1. PEDOT(PSS)

PEDOT, poly(3,4-ethylenedioxythiophene), shown on Figure 8A, is so far one of the most stable conducting polymer, attracting interest both in academia and industry. It is based on 3,4-ethylenedioxythiophene (EDOT) monomers, and was developed in the late 1980s[119], by attaching an ethylenedioxy substituent to the 3- and 4-positions of a thiophene ring. Due to its heterobicyclic nature it possesses many excellent properties
in its oxidized (conductive) state: good optical transparency\textsuperscript{120,121}, good chemical and thermal stability\textsuperscript{122}, high conductivity (maximum is around 600 S/cm\textsuperscript{120,121}), a moderate band gap (E\textsubscript{g} \approx 1.6 eV\textsuperscript{123}) and low oxidation potential (with the half-wave potential E\textsubscript{1/2} \approx 0.0 V vs. Ag/Ag\textsuperscript{+} \textsuperscript{120}). Because of its low oxidation potential PEDOT is readily oxidized in air and have a transparent light blue colour. PEDOT belongs to the group of conducting polymers that are both p- and n-dopable, however, its conductivity is orders of magnitude lower in the less stable n-doped state\textsuperscript{118}. Unlike many other CPs it is widely used in the industry, due to its environmental stability and transparency, for example; as an antistatic coating on photographic films or on packaging materials; as a transparent electrode in displays, touchscreens, OLEDs and electronic papers; as organic electrochemical transistor; or as conductive coating. It can even be used to manufacture windows or mirrors that can become opaque or reflective upon the application of potential\textsuperscript{124}.

![Figure 8](image)

**Figure 8** The structural formulas of (A) poly(3,4-ethylenedioxythiophene) (PEDOT) and (B) poly(styrenesulfonate) (PSS)

Perhaps the most common use of PEDOT up to date, though, is in a mix with sodium poly(styrenesulfonate) (NaPSS) known as PEDOT(PSS). PSS, shown in Figure 8B, is a sulfonated polystyrene, with part of the sulfonyl groups deprotonated and carrying negative charge. This polyanion offers high conductivity and good water solubility. PEDOT on the other hand carries positive charge, together these charged macromolecules form a polymer salt (ionomer complex) that is a flexible conductive material retaining the excellent properties of PEDOT\textsuperscript{125,126}. A major disadvantage of PEDOT on its own is its poor solubility, which can be overcome with the use of PEDOT(PSS), that is commercially available as aqueous solution dispersion or as ready-to-use formulation mixed with solvents. If treated with water or with a simple solvent, the conductivity of PEDOT(PSS) is not much changed. The prefabricated PEDOT(PSS) polymer has good film forming properties, high conductivity (10-1000 S/cm), good thermal and light stability, and after drying becomes insoluble in most common solvents\textsuperscript{120}. Furthermore, PEDOT(PSS) has an inherently high biocompatibility\textsuperscript{127} due to structural similarity with natural compounds such as
melanin\textsuperscript{128}. PEDOT(PSS) can also be used as an ink via variants on low cost inkjet printers and in screen printing technology.

PEDOT can be both chemically and electrochemically synthesized. Electrochemically it can be synthesized from EDOT monomer by using standard polymerization methods in organic, aqueous and even in aqueous micellar electrolytes\textsuperscript{120} \textsuperscript{121}. The rapid and efficient polymerization produces well adhering light blue conducting polymer films. PEDOT(PSS) is prepared the same way by template-guided synthesis of PEDOT with the water soluble PSS as counterion\textsuperscript{126}. PEDOT(PSS) seems to emerge as a promising SC material, due to its water layer hindering ability\textsuperscript{105}, very good electrochemical stability\textsuperscript{129}, and insensitivity for room light\textsuperscript{129}.

3.2.2. PANI

Although polyaniline itself was discovered more than 150 years ago (1862), only since the early 1980s captured intense attention. Because of its rich chemistry, PANI is one of the most investigated conducting polymers\textsuperscript{130}. It is mostly synthesized by oxidative chemical or electrochemical polymerization of aniline\textsuperscript{130}, which is especially attractive because the aniline monomer is inexpensive, polymerizes easily and with a high yield, with the resulting polymer showing an excellent environmental stability\textsuperscript{115} \textsuperscript{130}. Furthermore, PANI was the first conducting polymer commercially available in large scale in conducting, stable, and solution processable form\textsuperscript{115}, nowadays several water and organic dispersion products can be found on the market. PANI has three distinct oxidation states with different colours (see Figure 9) and has an acid/base doping response. This latter property makes PANI attractive for acid/base chemical vapour sensors. PANI is utilized for actuators, supercapacitors and electrochromics, widely used for printed circuit board manufacturing, but it can be found in electrically conducting yarns or antistatic coatings as well.

![Figure 9](image)

**Figure 9** The general structural formula of polyaniline (PANI), representing its three different oxidation states; the fully reduced form or leucoemeraldine (y=1), white/clear; the fully oxidized form or (per)nigraniline (y=0), blue/violet; and the half-oxidized form, the emeraldine (y=0.5), green/blue\textsuperscript{113}

In this thesis an organic PANI dispersion, Ormecon D1003 (Ormecon GmbH, Ammersbek, Germany), was used as SC material. It consists of 9.7 wt % PANI particles with an average size of 8 nm dispersed in xylene, with a conductivity of 1.8
× 10⁻³ S/cm measured in vacuum on a spin-coated film. It has good film forming and mechanical properties, and excellent pH stability even up to pH 12. This is quite exceptional, since generally the conducting emeraldine salt form is stable at pH ≤ 4 and totally converted into the insulating emeraldine base form at pH ≥ 7-8, however, the pH dependency of PANI is strongly affected by the synthesis conditions. This PANI dispersion allows the easy preparation of SCISEs exhibiting reproducible standard potentials, good potential stability, and no-light-sensitivity.

3.3. Conducting polymers as solid contact materials

After several important early approaches to find sufficient solid contact materials for solid-state electrodes, e.g. silver-epoxy solid contacts or incorporation of silver-ligand complexes in the membrane, conducting polymers became the most established solid-contact materials. Although conducting polymers were already introduced in 1987 and 1992 as ion-to-electron transducer materials, the concept became widely accepted within the ISE community only after it has been shown in 2004 that the lower detection limit of such electrodes could be comparable with those of corresponding optimized liquid-contact electrodes. This opened up the research for SCISEs with LDL in the subnanomolar range.

As discussed before, the elimination of the inner filling solution can avoid leaching of the primary ion and hence, via lowering the transmembrane ion fluxes, lower the detection limit. Possible leakage, however, from the ISM itself still remains. In this respect, it is especially important to use membranes characterized by low ion diffusion rates, such as PA materials or silicone rubber. Low ion diffusion rates, furthermore, also provide stable potentials at low primary ion concentrations.

Besides the rate of ion transport, the water uptake of the membrane is also important for the attainable LDL and potential stability. It was shown that a higher water uptake of the ISM result in a higher accumulation of the primary ion in the membrane phase prohibiting measurements at low sample concentrations. The water layer or scattered islands of water forming at the substrate/SC and/or at the SC/ISM interface has a tremendous effect not only on the detection limit and potential stability but on the good mechanical stability (adhesion) between the interfaces as well. The sensor will actually behave as conventional ISEs with an extremely small inner electrolyte. When such an SCISE, conditioned with the primary ion, is put into a solution with an interfering ion a quite fast potential increase will be observed, after the initial potential drop. Changing back to the primary ion will be followed by a slow potential decrease, after the immediate potential increase. This is the so-called potentiometric aqueous layer test. Water layer formation could be the reason for the commonly observed drifting potential response of CWEs. There are two main ways, to avoid the formation of a water layer, either by using hydrophobic solid-
contact materials and/or by minimizing the water uptake of the ISM, since the aqueous layer formation mainly originates from water penetrating the membrane.

A large number of CPs were tested as solid contact materials, of which the most commonly used are derivatives of poly(pyrrole) (PPy) \(^{[138]}\), poly(3-octylthiophene) (POT) \(^{[142]}\) \(^{[143]}\), PANI \(^{[144]}\), and PEDOT(PSS) \(^{[142]}\).

Conducting polymers, as intermediate layers, were particularly useful to stabilize the interfacial potential and to offer a very straightforward and controllable fabrication methodology based on localized electrochemical deposition of the respective polymeric films.

### 3.4. Other solid contact materials

A different method for solid-contact design is to use carbon materials with high surface area, intrinsic hydrophobicity and electric conductivity as solid contact interlayers: single \(^{[145]}\) \(^{[146]}\) - and multi-walled \(^{[147]}\) \(^{[148]}\) carbon nanotubes, fullerene \(^{[149]}\), reduced graphene oxide \(^{[150]}\), three-dimensionally ordered macroporous carbon (3DOM) \(^{[151]}\) \(^{[152]}\) \(^{[153]}\) and colloid-imprinted mesoporous carbon (CIM) \(^{[154]}\). The ion-to-electron transduction mechanism in these materials is capacitive in nature and hence differs from the redox reaction responsible for the transduction mechanism in the electroactive conducting polymers \(^{[7]}\).

Due to their large interfacial contact areas and subsequently high capacitances 3DOM and CIM carbon-based SCISEs have shown remarkable long-term potential stabilities with the lowest potential drifts reported so far, 11.7 µV/h \(^{[151]}\) and 1.3 µV/h \(^{[154]}\), respectively. These materials consist of a glassy carbon skeleton with interconnected macro- (420 nm) and mesopores (24 nm) that can be infiltrated with the ISM to form a bicontinuous structure, in which electrons are conducted through the carbon framework while ions move through the infused ISM. However, in spite of these appealing characteristics, 3DOM carbon contains significant amounts of redox-active surface functional groups \(^{[153]}\) that may lead to large changes in the \(E^0\) potential, while the cleanliness of the carbon material is very critical to eliminate aqueous layer formation, and the monolithic nature of the material is problematic in view of mass production as well. The use of CIM carbon has overcome these problems but the fabrication and handling of the material is not as simple as drop casting or localized electropolymerization of CPs. The synthesis of CIM is rather time consuming, about 5 days in total, and complicated with steps among others like carbonization at 900 °C in a \(N_2\) atmosphere, soaking in 6 M KOH for days at 180 °C.

### 3.5. Current directions of SCISE research

Much work has been invested lately to overcome the shortcomings of CPs as solid contacts. The interference from a number of ambient factors such as light intensity
changes\textsuperscript{[129]}, oxygen or carbon dioxide partial pressure changes, or pH sensitivity were effectively solved. While PANI, PPy, and PEDOT have been extensively studied as solid contacts in ISEs, the possibility of improving SCISE performance by using CP nanostructuring is largely unexplored\textsuperscript{[155]}. In a very preliminary study polypyrrole microcapsules\textsuperscript{[156]} have been employed as solid contacts in ISEs to control the electrolyte composition of the microcapsules, thus providing a means to fine-tune the LOD. Several new conducting polymers\textsuperscript{[157]} \textsuperscript{[158]} were also tested as solid contact material besides the above mentioned ones. Mainly because of the water-layer problem, the highly hydrophobic POT was rediscovered. The electropolymerized POT was shown\textsuperscript{[143]} to exist in a mixed surface/bulk oxidation state in the intermediate layer ensuring the unusually stable potential readings for this type of electrodes.

Of particular interest is the lowering of the detection limit down to the nanomolar level to have SCISEs for trace level analysis.

With a few exceptions the electrode-to-electrode reproducibility of the EFM response of solid-contact ISEs is not particularly good. Recently it was shown\textsuperscript{[159]} \textsuperscript{[160]} that the reproducibility of $E^0$ values can be significantly enhanced by incorporating a hydrophobic redox couple, with adjusted ratio of the oxidized and reduced forms, in the membrane to adjust the inner phase boundary potential. Another study suggested\textsuperscript{[161]} that the standard potential of SCISEs can be shifted by applying a potential that deviates from the open-circuit potential of the electrode in the chosen electrolyte solution or by applying current pulses in the nA range.

However, in spite of extensive research in this area, obtaining solid-contact ISEs with reproducible device-to-device $E^0$ standard potentials and obtaining long-term potential stabilities is still a great challenge, in these terms SCISEs can still hardly surpass their conventional liquid contact based counterparts.
4. Potentiometric immunoassays

4.1. Introduction

Immunoassays that rely on the remarkably specific antibody-antigen interactions provide a promising means of analysis for the growing demand of fast and simple measurement of clinical, biomedical or environmental analytes. The introduction of enzyme immunoassays in the early 1970s\textsuperscript{162} \textsuperscript{163} made it possible to move immunodiagnostics from specialized radioisotope laboratories to general chemistry laboratories. The popularity of immunoassays started to grow tremendously after, owing the low detection limits and high selectivities for analysing complex samples that could be achieved with relatively simple procedures and instrumentation. The availability of selective antibodies for an increasingly wide variety of important analytes was also an important factor in this success\textsuperscript{164}. Lately though, significant efforts have been made to replace both the delicate biological-origin recognition and labelling molecules, i.e. antibodies and enzymes, respectively, with synthetic analogues providing similar or even enhanced properties but increased robustness and decreased assay cost.

Although various assay formats and methods of detection, each with its advantages and disadvantages, have been used to match the requirements of sensitivity, selectivity, and massively parallel determinations, optical detection methods are clearly dominating the filed. Another promising method to fulfil the above mentioned criteria is electrochemical detection possessing advantages especially in terms of increased sensitivity\textsuperscript{165} \textsuperscript{166} \textsuperscript{167}. Furthermore, electrochemical detection enables fast, simple, and economical measurements; it has minimal power requirements; and it is compatible with modern miniaturization/microfabrication technologies ensuring cost-effective mass production of the electrodes. Electrochemistry is an interfacial process in which the relevant reactions take place at the electrode/solution interface, rather than in bulk solution. Therefore, electrochemical immunoassays\textsuperscript{168} offer an added bonus of detecting analytes in very small volumes\textsuperscript{169}.

Voltammetry, amperometry, and more recently electrochemical impedance spectroscopic measurements are among the electrochemical detection techniques often used in conjunction with immunoassay systems, leading to their respective categories according to the type of signal measured. Although these techniques will be also briefly discussed, this thesis focuses on novel potentiometric immunoassay detection methods.
4.2. Immunoassays

4.2.1. Antibody-antigen interaction

Immunoassays rely on the inherent ability of an antibody (Ab) to recognize and bind to a specific structure of an antigen (Ag). Antibodies are a family of glycoproteins known as immunoglobulins (Ig) having five distinct classes (IgA, IgG, IgM, IgD and IgE) with IgG being the most abundant and most widely used in immunoassays. The “Y”-shaped IgG is approximately 150kDa, containing of two larger weight i.e. heavy chains, and two smaller i.e. light chains held together by disulphide linkages. As shown in Figure 10 both heavy and light chains are divided into constant (C) and variable (V) domains based on their amino acid sequence variation, with V_L and C_L domains for the light, and V_H, C_H1, C_H2 and C_H3 domains for the heavy chain, respectively. In general, the antibody is divided into two main fragments: the variable domains, V_H and V_L form the antigen-binding fragment (F(ab’)₂) which is responsible for the antibody-antigen binding; while the constant domains construct the non-antigen binding fragment (Fc). There are three distinct subregions of high sequence variability, known as hypervariable regions, on both V_H and V_L, forming the six hypervariable loops, known as complementarity determining regions, which constitute the antigen binding site. The diversity in this region is estimated to form about $10^8$ different antibody specificities, which allows the production of a high affinity antibody against a very wide range of antigens\[169\].

![Figure 10](image)

Figure 10 A schematic illustration of the structure of an antibody

The binding affinity for the antibody – antigen interaction ($Ab + Ag \rightarrow AbAg$) can be described by the equilibrium constant, $K = \frac{[AbAg]}{[Ab][Ag]}$, where $K$ is the affinity constant for the interaction and $[AbAg]$ is the immunocomplex. Typical values of $K$ range from
10^6 - 10^{12} \text{ M}^{-1}$, whereas only antibodies yielding a high $K$ value ($\geq 10^8 \text{ M}^{-1}$) will exhibit low cross-reactivity.

Antibodies are thus ideal biological recognition components in immunoassays, in addition, the use of monoclonal antibodies are particularly suitable. Monoclonal antibodies are antibodies produced by a cloned cell line and thus have the same epitope specificity and affinity. This homogenous population can be made in large numbers. Therefore, they offer superior specificity and homogeneity compared to polyclonal antibodies, reducing the need for laborious purification [169].

4.2.2. Sandwich immunoassay and ELISA

Immunoassays are the quantitative methods of analysis where antibodies are the primary binding agents for the analyte of interest, i.e. the antigen. To achieve quantitative analysis a label is used on one of the interacting species, i.e., on the antigen or on the tracer antibody, thus the measured signal caused by the label can quantitatively be related to the amount of analyte present in the sample solution. The result of an immunoassay is principally the investigation of the binding between the antibody and the antigen, determined by the $K$ value, in other words, immunoassays depend on measuring the fraction occupancy of the recognition sites [170]. This leads either to assessment of occupied sites (non-competitive immunoassay format), or indirectly, to measuring the unoccupied sites (competitive assay format).

In a competitive immunoassay the sample analyte is mixed with labelled analyte, which both compete for a limited number of antibody-binding sites, causing an inversely proportional thus smaller signal than in the non-competitive format.

![Diagram](image)

**Figure 11** General scheme for a conventional sandwich-type enzyme-linked immunosorbent assay

In the non-competitive or, most often, sandwich-immunoassay (for an assay scheme see Figure 11) the sample analyte is captured by an excess of a so-called capture antibody, separating it from the bulk solution. The captured analyte is than exposed to an excess of second, so-called tracer antibody, which is labelled, and which will only bind to the already existing capture antibody-analyte complex. As a result the antigen is sandwiched between two antibodies hence the name sandwich immunoassay. It is a heterogeneous assay format that uses solid phase immobilized capture antibody and
washing steps to remove non-bound material. For immunoassays the capture antibody is immobilized on a solid supporting material, such as to the well of a microtiter plate, magnetic bead or polystyrene bead, while for an immunosensor it is immobilized on the surface of an electrochemical transducer. In an ideal sandwich assay no signal would be produced in the absence of the analyte as there are no appropriate sites for the tracer antibody binding. However, in practice, this is not the case because of the non-specific adsorption, and thus the undesirable interactions should be reduced by the use of a blocking reagent. The main advantage of this assay format is its superior specificity owing to the double recognition step. On the other hand, however, it can only be used for analytes with two antibody binding sites (epitopes) that can be simultaneously recognized, which in practice means mostly large antigens are suited for sandwich assays. This assay format is also relatively labour intensive and time consuming because of the multiple washing and incubation steps.

Enzyme-linked immunosorbent assay (ELISA) uses an enzyme as reporter label. The most commonly used enzymes are alkaline phosphatase (ALP), horseradish peroxidase (HRP), and β-galactosidase (GAL). The enzyme label provides great signal amplification as each enzyme molecule can generate 10-1000 product molecules/s (1.8×10^4–1.8×10^6 molecules in 30 min which is the most common incubation time) leading to an extremely low detection limit.

4.2.3. Replacing biological assay components with synthetic analogues

In conventional sandwich ELISA immunoassays the biological recognition of the analyte is based on antibodies, while the signal transduction and amplification of the antigen-antibody recognition events is provided by the enzyme label. However, for actual technological applications, the use of proteins (antibodies and enzymes), which are delicate, biological-origin molecules with narrow pH and temperature optimum, high chemical sensitivity and subsequently short shelf-life, not to mention the relatively high preparation cost, and restricted amount that can be generated is often limiting. Therefore, in the past decades increasing efforts were done to replace antibodies and/or enzyme labels with in vitro generated, synthetic analogues in immunoassays. These analogues provide the same basic functions as their biological counterparts, but they are more robust and cost-effective. They provide easily adjustable properties, often expanded with novel characteristics.

4.2.3.1 Synthetic receptors

Although antibodies offer a wide range of applications for almost half a century and thus have become indispensable in most diagnostic tests, they are often limited in terms of ruggedness and cost-effectiveness. General understanding of the molecular recognition processes in the past decades permitted to imitate biorecognition, resulting
in affinity reagents including RNA and DNA aptamers, peptides, and a variety of protein scaffolds. Unlike antibodies, they are synthesized via an *in vitro* process, hence their properties can easily be changed on demand, they can be modified chemically, and their accurate and reproducible synthesis ensures little or no batch-to-batch variation. As no cells are involved in this process, toxins or molecules that do not elicit a good immune response can be target molecules as well. These artificial recognition sites may be substituted for antibodies in bioaffinity assays.

In this thesis two synthetic receptor families were applied, aptamers and molecularly imprinted polymers

**Aptamers**

Aptamers are *in vitro* generated, short, single stranded oligonucleotides (both DNA and RNA) that selectively bind to target compounds ranging from small molecules to macromolecules. Their name is derived from the Latin word ‘aptus’ meaning ‘to fit’.

The identification method of oligonucleotide sequences with unique binding properties to target molecules, a technique called SELEX, was described in 1990. SELEX, systematic evolution of ligands by exponential enrichment, is a technique for screening very large, $10^{12} - 10^{14}$ random sequence, combinatorial libraries of oligonucleotides by an iterative process of *in vitro* selection and amplification. This technique made the isolation of aptamers with the capacity to recognize virtually any class of target molecules with high affinity and specificity possible.

The dissociation constant of aptamer-ligand complexes is similar to that of antibody-antigen interactions, however, aptamers are superior to antibodies in many aspects. Besides the obvious advantages of the *in vitro* selection, aptamers, owing to their nucleic acid composition, are amenable to well controlled chemical modifications, have good chemical stability and are easy to handle. Their main advantage over monoclonal antibodies is their resistance to thermal denaturation. They are robust and easy to synthesize. Aptamers have great potential in bioanalysis, most of the routine immunanalytical methodologies can be easily adapted to detect aptamer-ligand interactions and thus aptamers can potentially fulfill molecular recognition needs in immunoassays.

A possible limitation of their use as diagnostic receptors can be, however, that oligonucleotides are susceptible for the ubiquitously present nuclease enzymes. Therefore, lately various attempts were done to generate aptamers built from modified nucleotides with enhanced nuclease resistance. Spiegelmers, based on the enantiomers of natural RNA and DNA molecules, i.e. L-ribose or L-2'-deoxyribose units, are an aptamer variety showing complete resistance to enzymatic degradation.
Molecularly imprinted polymers

A promising technique to generate fully synthetic recognition sites is molecularly imprinted polymers (MIPs). Molecular imprinting is a method to generate materials with “molecular memory” by performing a polymerization of suitable functional monomers in the presence of the target molecule, acting as a template. The concept was established in 1972 by Wulff and Sarhan when introducing template molecules during polymerization what they then called “host-guest polymerization”[179]. The preassembly of the functional monomers around the template is conserved by the polymerization, hence, after the template is removed, recognition sites complementary with shapes, sizes and orientation to the template remain within the polymer matrix. This straightforward strategy enables chemists to generate synthetic polymers, molecularly imprinted polymers that are selective towards a specified template via complementary non-covalent binding sites, i.e. ionic, hydrophobic or hydrogen bond interactions[180].

Molecularly imprinted polymers could easily compete with antibodies in terms of cost and complexity of synthesis, stability and mechanical properties as well as the range of target molecules. However, molecular imprinting still faces challenges when imprinting large, delicate biomacromolecules, such as proteins[181], and often fails to work as synthetic antibody[182]. So far the affinity and catalytic activity of MIPs have in general been lower than those of their biological counterparts[183].

Although the bulk synthesis method produces excellent results in generating MIPs for recognition of low molecular weight compounds[184] [185] [186] [187], the macromolecules become entrapped in the polymer material, which hinders both removal and rebinding of the target. Thus, an essential prerequisite of protein imprinting is to create binding sites accessible for the target from the solution phase, i.e. to have binding sites confined to the surface of the MIPs. To realize this requirement “surface imprinting” techniques have been emerged lately, based on polymeric micro- and nanostructures. A few examples include protein surface imprinting of nanometer thin films[188] [189], microrods[190] [191] or microbands[192]. Surface imprinting is inherently advantageous to minimize non-specific interactions as well, because it reduces the contact area between macromolecular targets and bulk polymeric material.

4.2.3.2. Synthetic labels

Similarly to antibodies, the enzyme label can also be replaced by synthetic materials. The attractive amplification and multiplexation properties of metal and semiconductor nanoparticles make them ideal labels for bioaffinity assays. The unique and attractive properties of nanomaterials have paved the way for the development of highly sensitive electrochemical diagnostic devices.
Nanoparticle-labelled affinity reagents

Nanoparticle labels provide excitingly new possibilities for advanced development of new analytical tools and instrumentation, especially on the field of electrochemical immunoassays. Antibodies or other affinity reagents labelled with nanoparticles (antibody – nanoparticle or affinity reagent – nanoparticle conjugates, respectively) can retain their bioactivity due to the similar dimensions of nanoparticles and protein and aptamer molecules and interact with their counterparts. Based on the detection of these nanoparticles, the amount of the analyte can be determined\[193]\.

Various nanomaterial labels are used in the sandwich-type immunoassays, including noble metal nanoparticles, semiconductor (quantum dot, QD) nanoparticles, metal oxide nanostructures, carbon nanomaterials, hybrid nanostructures and marker loaded nanocarriers (e.g. silica nanoparticles, apoferritin, and liposome beads)\[194]\). Metal, and/or semiconductor nanostructures, i.e. gold and silver nanoparticles as well as quantum dots have been directly used as electroactive labels to amplify the signal in the electrochemical detection of proteins, so this part of the thesis will focus on these labels.

One important goal of using nanoparticle labels in immunoassays is to achieve signal amplification complying with or surpassing that of the enzyme labels (see Table 1), while utilizing more robust and chemically stable molecules enabling new and advanced functions, e.g. the detection of multiple targets. One major advantage lies in the possibility to control and tailor the properties of nanoparticles to meet the needs of specific applications, for example, to provide unique chemical and physical properties (electrical, electrochemical, optical and magnetic)\[193] [195]. In particular, nanomaterial labels are showing the greatest promise for developing ultrasensitive electrochemical immunoassays\[196]\.

**Table 1** The number of generated ions and the corresponding amplification effect of gold and silver nanoparticles of various sizes

<table>
<thead>
<tr>
<th>np diameter</th>
<th>Au atoms/np</th>
<th>Ag atoms/np</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 nm</td>
<td>3862</td>
<td>3836</td>
<td>$\times 10^4$</td>
</tr>
<tr>
<td>10 nm</td>
<td>30896</td>
<td>30689</td>
<td></td>
</tr>
<tr>
<td>15 nm</td>
<td>104273</td>
<td>103575</td>
<td>$\times 10^5$</td>
</tr>
<tr>
<td>20 nm</td>
<td>247118</td>
<td>245510</td>
<td></td>
</tr>
<tr>
<td>25 nm</td>
<td>482750</td>
<td>479512</td>
<td></td>
</tr>
<tr>
<td>30 nm</td>
<td>834183</td>
<td>828596</td>
<td>$\times 10^6$</td>
</tr>
</tbody>
</table>

Quantum dots were initially used as fluorescent biological labels in 1998 (CdSe core with ZnS shell)\[197]\, and the first use of QD labels for electrochemical monitoring of DNA hybridization was reported in 2002 (CdS)\[198]\. The combination of the intrinsic redox properties of QDs with the sensitive electrochemical stripping analysis of the metal components of semiconductor nanoparticles led to very sensitive detection when
using quantum dot labels in the electrochemical immunoassays, and enabled the simultaneous multiplexed measurement of protein targets utilizing different semiconductor nanoparticle label traces (ZnS, CdS, PbS, CuS)\textsuperscript{199}. Each biorecognition label yielded to a distinct voltammetric peak whose peak potential and current reflected the identity and concentration, respectively, of the corresponding analyte.

The application of colloidal gold/silver as amplification in immunology dates back to the early 1970s when the development of silver-enhanced methods allowed gold labels to provide high-resolution, specific and sensitive immunocytochemistry\textsuperscript{194}. The use of colloidal gold as electrochemical immunoassay label, however, for voltammetric monitoring of protein interactions was pioneered only in 2000\textsuperscript{200} \textsuperscript{201}. To further enhance the sensitivity of gold nanoparticle label-based immunoassays various techniques have been developed. For example, the nanoparticle-promoted precipitation of silver on gold nanoparticle labels\textsuperscript{202} \textsuperscript{203} \textsuperscript{204}, where the silver ions were detected by voltammetric\textsuperscript{202} \textsuperscript{204} or potentiometric\textsuperscript{203} method after dissolution of the metallic silver. A common problem in Ag enhancement can be the high background signal as a result of non-specific precipitation of silver onto the substrate. Various techniques have been developed to reduce this non-specific deposition to further increase the sensitivity of this method.

4.3. Electrochemical immunoassays

The combination of the high selectivity of molecular recognition with the low detection limit of modern electrochemical techniques could result in a powerful analytical tool, however electrochemical immunoassays did not gain as much attention, especially in the early times, as the optical detection techniques. Although immunoassays emerged decades ago there are still vigorous research efforts and tremendous progress in the development of electrochemical immunoassays\textsuperscript{170} \textsuperscript{205}. As most antibodies and antigens are intrinsically unable to act as redox partners, an appropriate label, e.g. an enzyme or nanoparticle, is usually conjugated to a particular component of the immunocomplex to promote an electrochemical reaction. The electrochemical signal produced is then used to relate quantitatively to the amount of analyte present in the sample solution. These methods are free from problems like sample turbidity, quenching, or interferences from the many absorbing and fluorescing compounds in typical biological samples that plague spectroscopic techniques\textsuperscript{206}. The relatively simple instrumentation requirements are really advantageous as well, just like their compatibility with modern miniaturization and microfabrication techniques\textsuperscript{91}.

Although one of the first immunoassays using a label was electrochemically based\textsuperscript{207}, the state of the art of electrochemistry back in 1951 could not meet the analytical demands of such an assay yet. A few years later, Yallow and Berson developed
immunoassays based on the radioisotopic label\textsuperscript{[208]} (radioimmunoassay, RIA) gaining very low detection limits, and thus resulting in an enormous growth rate in the use of RIA in both the clinical and research laboratories. RIA, however, has the disadvantages that accompany radioisotope handling, together with an inability to distinguish label which is bound from that which is not. There has therefore been an enormous effort to find suitable replacements for the radiolabel. The most successful of these have been enzyme linked immunosorbent assay (ELISA)\textsuperscript{[162]} [163]. The popularity of the method started to grow tremendously after, allowing the application of such assays also by unspecialized users and in field applications.

Electrochemically-based immunoassays have also mostly used ELISA and thus enzyme amplification. Enzyme labels are easy to connect with electrochemical detection because of their ability to yield an electroactive product following the catalytic conversation of a non-electroactive substrate. The most common substrate – enzyme combinations utilized were NAD/NADH and glucose-6-phosphate dehydrogenase\textsuperscript{[209]}; phenyl phosphate/phenol or later aminophenyl phosphate (APP)/aminophenol and alkaline phosphatase\textsuperscript{[210]} [211], respectively\textsuperscript{[165]}. These early assays used mostly \textit{amperometric techniques} for detection, quantitatively relating the magnitude of current, arising under controlled potential conditions, from the redox reaction of the product to the amount of analyte present. Later, several other suitable alternative substrates were also investigated to replace the common APP, such as 1-naphthyl phosphate\textsuperscript{[212]} or ascorbic acid 2-phosphate\textsuperscript{[213]}. These substrates increased the efficiency of the readout and minimized non-specific interferences by possessing lower detection potentials; hence improved the sensitivity; ensured lower-assay cost; and enabled faster enzymatic reaction. Amperometric detection methods remained up to now the most commonly used electrochemical transduction techniques, due to their fast detection, broad linear range, and low detection limit\textsuperscript{[205]}. They also have the advantage that unspecific binding of molecules other than the labelled immunoreactant will not contribute to the signal. The main drawback is, however, when using any of the above mentioned substrates that they can cause passivation of the surface of the sensing electrode, leading to small amperometric responses and poor reproducibility.

\textit{Voltammetric techniques}, which are extremely sensitive, were also shown to be the electrochemical methods of choice in enzyme immunoassays very early on. In these methods the potential is varied while measuring the current change related to the analyte. An interesting example is the use of interdigitated microelectrode arrays (IDAs), consisting of pairs of microelectrode fingers that are held at different potentials to achieve redox “cycling” of the electroactive species to be detected\textsuperscript{[214]}. Voltammetric sensing, however, possesses several drawbacks as well, unless ultramicroelectrodes are used, the current signal is strongly dependent on mass transport conditions.
Upon the specific molecular recognition of the antigen by the immobilized antibody, there are changes in the interfacial charge, capacitance, resistance, mass, and thickness at the sensor surface. Thus there is lately an emerging interest in exploring electrochemical techniques that follow these interfacial changes enabling direct, label-free, and sensitive real-time monitoring of the antibody-antigen interactions\[^{170}\]. Electrochemical impedance spectroscopy (EIS) can be an effective method for probing the features of an electrode surface modified by immunocomplexes\[^{215}\]. These *impedimetric methods* relay on measuring the change in impedance as a result of the antibody-antigen interaction\[^{170}\]. Another technique, based on similar principles, is *conductimetric immuno sensing*, where the change in conductance is measured upon the interaction\[^{216}\]. *Capacitance detection* of immunoassays exploits the change in dielectric properties or thickness of dielectric layer, i.e. the inorganic insulator and the immobilized molecules, at the electrolyte-dielectric interface due to the antibody-antigen interaction. During the interaction the thickness of the immobilized bioactive layer changes, causing an alteration in the capacitance\[^{217}\].

4.3.1. Potentiometric immunoassays

Potentiometry has the simplest measurement technique and instrumentation from the electrochemical detection approaches, and furthermore it is free of the problems mentioned before concerning other electrochemical techniques. In potentiometric detection principle, the change in the membrane potential of an ion-selective electrode, occurring after the specific antibody-antigen binding, is measured. A logarithmic relationship between the electrode potential and the concentration of the detected species exists, as given by the Nernst equation. The research in potentiometric immunoassays started already in the 1970s, with pioneering contribution by the group of Rechnitz\[^{218}\] [219] [220] and Janata\[^{221}\] [222].

*Direct, label-free potentiometric detection* of antibody-antigen interactions is also possible with this method, by immobilizing one of the participants of the immunoreaction on the indicator electrode. In a so-called ionophore modulation immunoassay\[^{223}\] a K\(^+\)-selective ionophore was covalently linked to the target antigen, and the presence of the antibodies in the sample was found to alter the EMF of the electrode. Another alternative is the use of polycation-selective electrodes for competitive homogenous assays\[^{224}\], where the potentiometric response of a synthetic polycation-analyte substrate is suppressed when binding to the antibody occurs. A third direct potentiometric method\[^{225}\], not using ion-selective electrodes though, is based on the change of the charge when the binding of an antigen at the surface of an Ag-electrode with immobilized antibody occurs. Using the potentiometric transduction capabilities of single-walled carbon nanotubes in combination with the recognition capabilities of protein specific RNA aptamers disease-related proteins were as well measured with direct potentiometry from blood\[^{226}\]. Label-free
potentiometric detection is rapid and simple, with no separation steps required. A main disadvantage is, however, that the change in potential due to the antibody–antigen interaction is relatively small, and interferences from the sample matrix may prevent this small signal from being successfully detected, therefore the reliability and sensitivity of these assays have been limited[217].

Early efforts about the potentiometric detection of labelled immunoreagents mainly focused on using potentiometric gas sensors, such as ammonia or CO₂ electrodes, in conjunction with urease[227][228][229]–, asparaginase[228]–, adenosine deaminase[228][230]–, and chloroperoxidase[231]–label. There were also a few attempts to measure the pH change caused by the urease enzyme label[232][233]. Another research direction aimed at generating I⁻ and F⁻ ions, by enzymatic reaction of the commercially more available horseradish peroxidase[234][235] and alkaline phosphatase[236], to detect them with all-solid-state iodide[234]– and fluoride[235][236]–selective electrodes. Although these detection methods seemed promising, the limited availability of commercial reagents needed for the unconventional ELISA systems, as well as the poor detection limits and dynamic range of the early potentiometric ion or gas sensors clearly limited their practical utilization.

Recent improvements on the field of solid-contact electrodes and the improvements in the lower detection limit of the ion-selective electrodes motivated researchers to revisit potentiometry as a detection method in immunoassays. The few interesting approaches published in the past decade used nanoparticle-label. In the first one a miniaturized Ag⁺-selective electrode was successfully used to detect silver ions, released by the oxidative dissolution of silver enhancement of gold nanoparticle labels of a sandwich immunoassay[203]. Following the same principle, potentiometric immunoassays were built on the detection of Cd²⁺ released from CdSe quantum-dot-labelled tracer antibodies[237] or aptamers[238]. The most recent works in the field reported lately, i.e. after the research described in this thesis was published, will be briefly summarized in the respective outlook part.

Although ion-selective potentiometry is not affected by mass transport limitations, passivation of the electrode surface or limited selectivity that concerns other electrochemical detection techniques, it seems that little follow-up work was done on most of the early approaches. Potentiometric immunoassay detection provides a simple measurement method with widely available instrumentation, and establishes miniaturization and low-cost microfabrication of the electrodes. Since ion-selective electrodes are already extensively used in commercial point-of-care sensors and automatic blood-gas analysers the limited number of potentiometric immunoassays reported up to now is rather surprising.
5. Materials and methods

5.1. Chemicals and reagents

5.1.1. Components for ion-selective electrodes

The components for ion selective membrane preparation were all of Selectophore® grade obtained from Fluka (Buchs, Switzerland), unless otherwise mentioned.

(i) **Ionophores**: potassium ionophore I (valinomycin), potassium ionophore III (2-Dodecyl-2-methyl-1,3-propanediyl bis[N-[5’-nitro(benzo-15-crown-5)-4’-yl]carbamate], BME 44), sodium ionophore X (4-tert-butylcalix[4]arene-tetraacetic acid tetraethyl ester), calcium ionophore IV, silver ionophore CsV321 (synthesized by the Department of Organic Chemistry and Technology at Budapest University of Technology and Economics), copper(II) ionophore I used as silver ionophore (o-xylene-bis-(N,N-diisobutylthiocarbamate))

(ii) **Lipophilic additives**: tridodecylmethylammonium nitrate (TDMA-NO₃), sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB), tetradodecylammonium tetrakis(4-chlorophenyl)borate (ETH500)

(iii) **Plasticizers**: 2-nitrophenyl octyl ether (o-NPOE), bis(2-ethylhexyl) sebacate (DOS)

(iv) **Membrane matrices and solvents**: high molecular weight poly(vinyl chloride) (PVC), room temperature vulcanizing silicone rubber (RTV 3140) (obtained from Dow Corning), tetrahydrofuran (THF)

(v) **Solid contact materials**: The polyaniline dispersion (PANI (D1003)) was obtained from Ormecon GmbH (Cookson Electronics) and had a mean particle size of 8 nm. The size of 90% of the particles was <14 nm and the conductivity in vacuum was given as 1.8×10⁻³ S/cm. The dispersion had a solid content of 9.7 wt% and was stored in a closed glass bottle. The average molecular weight of the PANI dispersion was unfortunately not provided by the manufacturer and cannot be reported. Poly(sodium 4-styrenesulfonate) (NaPSS, MW ~70 kDa), 3,4-ethylenedioxythiophene monomer (EDOT, >97 %), 1,1’-dimethylferrocene (DMFe) were purchased from Sigma-Aldrich (St. Louis, MO, USA).
5.1.2 Proteins

The capture anti-human prostate specific antigen (PSA, SPRN-1) and tracer anti-human PSA (SPRN-5) antibodies were purchased from Medix Biochemica (Kauniainen, Finland). The latter was biotinylated by the Institute of Isotopes Co. Ltd. (Budapest, Hungary), which also provided the prostate specific antigen standards, controls, serum samples, and washing solutions. Galactosidase conjugates, β-galactosidase from Escherichia coli biotin labeled (GAL-biot) and streptavidin-galactosidase (Str-GAL) were from Sigma and Thermo Fisher Scientific Inc. (Rockford, IL, USA), respectively.

Human immunoglobulin E (IgE) was purchased from Abcam (Cambridge, UK) and mouse IgG from Sigma-Aldrich.

Avidin, NeutrAvidin® were obtained from Thermo Fisher Scientific. Avidin-FITC, Streptavidin, ExtrAvidin®, lysozyme (Lys) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich.

5.1.3. Nanoparticle preparation and conjugation

Chemicals for the preparation and modification of gold nanoparticles, hydrogen tetrachloroaurate(III) (HAuCl₄), trisodium citrate, 1-mercaptopoundec-11-yl)tetra(ethylene glycol (HS-TEG) were purchased from Sigma-Aldrich. Oligonucleotides with terminal thiol functional groups and spacer of 4 thymine (tttt) were custom synthesized at 200 nmol scale and HPLC purified. The following sequences were used: thiol labeled IgE aptamer[^239] (Apt: 5’- gggg cacg tta tccg tccc tct cttg gctg gcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
ROMIL (Cambridge, UK). The monomer 3,4-ethylenedioxythiophene (EDOT) and poly(sodium 4-styrenesulfonate) (NaPSS, MW~70 000) were from Sigma Aldrich.

5.1.5. Other reagents used in protein assays

SuperBlock (TBS) Blocking Buffer Dry Blend, Protein-Free (TBS) Blocking Buffer were obtained from Thermo Fisher Scientific Inc. 6,8-Difluoro-4-methylumbelliferyl-β-D-galactopyranoside (DiFMUG), 6,8-difluoro-4-methylumbelliferone (DiFMU) from Invitrogen (Carlsbad, CA, USA), 2- and 4-nitrophenyl-β-D-galactopyranoside (pNPG), the reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and (1-mercaptoundec-11-yl)tetra(ethylene glycol) (HS-TEG) were purchased from Sigma-Aldrich. The instant skimmed milk powder used as blocking agent was from Bedeco (Zirc, Hungary). The silver enhancement solution LI Silver was obtained from Nanoprobes (Yaphank NY, USA).

All other reagents and chemicals such as inorganic compounds, buffers and buffer components were of the highest analytical grade from Sigma-Aldrich. Solutions were prepared with 18.2 MΩ×cm specific resistance ultrapure deionized water (Millipore, Bedford, MA, USA).

5.2. Preparation of the ion-selective electrodes

5.2.1. Anion exchanger-based minielectrodes for potentiometric enzyme immunoassay

The typical composition of the ion-selective membranes was PVC (40 wt%), plasticizer (60 wt%), and TDMA-NO₃ (10 mmol/kg). After dissolving all components in THF, master membranes were cast in glass rings (Ø 30 mm). Miniaturized electrodes were fabricated using 10 ml pipette tips as shown in Figure 12. These electrodes were designed for measurements in 150 µl sample volumes accommodated in the wells of a microtiter plate. To improve the adhesion of the ion-selective membrane to the inner pipette wall, the pipette tips were first cleaned by repeatedly dipping them into THF and draining out the solvent. Membrane segments were cut out from the master membrane and dissolved in THF (dry weight 7.7 wt%, 1:13 w/w). The pipette tips were dipped into this membrane solution until the column of the liquid reached 4–5 mm. Then, the pipettes were placed in a holder and the THF was left to evaporate.
overnight yielding solvent polymeric membranes of ca. 300–500 mm thickness situated at the very end of the pipette tip. The pipette was filled with a solution containing 1 mM phosphate buffer (PBS), 1 mM MgSO₄, 10⁻² M KCl, and 10⁻⁵ M DiFMU. An inner reference electrode was also prepared from a 10 ml pipette tip whose narrower opening was obstructed by a porous plug made from a hydrophobic polypropylene membrane (Celgard). After filling it with 10 mM KCl and inserting an Ag/AgCl wire, the other opening of the pipette was sealed with Parafilm. This pipette tip was then placed into the pipette tip containing the sensing membrane and secured with Parafilm® (Figure 12Aa). A small orifice was made in the side of the latter tip to avoid overpressure during the assembly, which could damage the ion-selective membrane. A miniature reference electrode was fabricated exactly as described above for the inner reference electrode (Figure 12Ab).

To measure in 150 µl sample volume in the wells of a microtiter plate, a special electrode holder was prepared to accommodate the reference electrode and concentrically up to five ion sensors, which could be used simultaneously in a single well (Figure 12B).

![Figure 12](image.png)

**Figure 12** (A) Scheme of the (a) working and (b) reference minielectrodes and (B) the electrode arrangement that allows simultaneous multi-electrode recordings in one microtiter well, i.e. in 150 µl sample volume

### 5.2.2. Solid-state minielectrodes for paper-based potentiometric bioassay

In the respective study silver ions generated by oxidative dissolution in the nitrocellulose membrane were detected with a silver selective electrode against
a pseudo-reference calcium selective electrode. Both ISEs were coated wire electrodes prepared by drop casting the respective membrane cocktails onto glassy carbon substrates (CHI104, CH Instruments, Inc., Austin, TX, USA). The Ag$^+$-selective membrane comprised: 2.0 wt % silver ionophore (CsV321), 1.0 wt % NaTFPB, 29.3 wt % PVC and 67.7 wt % oNPOE while the Ca$^{2+}$ selective membrane: 1.0 wt % calcium ionophore IV, 0.4 wt % NaTFPB, 43.0 wt % PVC and 55.6 wt % oNPOE. After dissolving all components in THF (dry weight 10 wt%) 30 µl membrane cocktail was applied onto the electrodes. After drying, the electrodes were conditioned overnight in a stirred solution of 10$^{-5}$ M AgNO$_3$ and 10$^{-5}$ M Ca(NO$_3$)$_2$. Prior to the measurements they were placed into a stirred solution containing 10$^{-9}$ M AgNO$_3$ and 10$^{-5}$ M Ca(NO$_3$)$_2$ for 10 min.

For potentiometric readout paper strips were sandwiched between two silicone sheets and the two electrodes, as depicted in Figure 13.

![Figure 13](image-url)  
*Figure 13* Potentiometric setup for the detection of silver ions generated in paper. The paper strip is sandwiched between two silicone rubber sheets with the dot positioned in the midst of the silver-selective electrode and the pseudo-reference calcium-selective electrode.

5.2.3. Silicon rubber-based solid-contact silver-selective electrodes

Silicon rubber-based solid-contact silver-selective electrodes (AgSCISEs), and silver-selective coated-wire electrodes (AgCWEs) were prepared by drop casting.

The SC layer was prepared by applying 3 µl of PANI on GC electrodes (polyether ether ketone (PEEK) body). The SC layer was allowed to dry
overnight before 25 µl of the outer SR based Ag⁺-selective membrane solution (dry weight: 31 %) was applied on the top of it. The SR membrane solution was prepared with a rather high viscosity to avoid the presence of excess THF that could dissolve the PANI solid-contact, which is soluble in THF. The SR based ISM was composed of 88.55 wt% SR, 0.8 wt% (17.1 mmol/kg) ionophore (o-xylene-bis-(N,N-diisobutylthiocarbamate)), 0.65 wt% (6.8 mmol/kg) NaTFPB and 10 wt% DOS as plasticizer to facilitate the solubilisation of the silver ionophore. The AgCWEs were prepared in the same way but without the SC intermediate layer. The SCISEs and the CWEs were then allowed to dry overnight. Note, after weighing in the SR, it was dissolved immediately in THF in order to avoid the premature start of the curing as it comes in contact with atmospheric humidity.

The AgISEs were conditioned in stirred 1 mM AgNO₃ solution for 2 days and then in 10⁻⁹ M AgNO₃ for 1 day.

For the PVC based AgSCISEs and AgCWEs, the membrane cocktails (dry weight: 31 %) consisting of 0.87 wt% (17.1 mmol/kg) ionophore, 0.63 wt% (6.9 mmol/kg) NaTFPB, 55.45 wt% DOS and 43.05% PVC were deposited similarly as described for the SR membranes and allowed to dry for 2-3 hours.

5.2.4. Electrodes with 3D nanostructured conducting polymer solid contact

In the respective study ordered conducting polymer nanostructures were fabricated by nanosphere lithography as high surface area solid-contact layer. PEDOT(PSS) films with 746 nm interconnected pores were electrosynthesized as the intermediate layer between a glassy carbon electrode and a silver-selective membrane.

*Preparation of the 3D ordered solid contact by nanosphere lithography*

*Figure 14* Scheme of the (A) 3D ordered and (B) compact PEDOT(PSS) film synthesis
Glassy carbon disk working electrodes (0.07 cm$^2$) with Teflon body were used as substrate for template unmodified polystyrene bead (ø 746 nm) deposition (Figure 14A). The substrate was wet polished with alumina suspension of 1 µm and 0.05 µm particle size followed by rinsing with water and ethanol. The glassy carbon surface was then delimited with a 470 µm thick silicone ring of 3 mm inner diameter in which 6 µl of the aqueous dispersion of polystyrene nanoparticles (2.6 wt %) was drop cast and left to dry slowly under a controlled relative humidity of 75%.

The potentiostatic deposition of PEDOT(PSS) within the voids of the particle array was done at 0.86 V (reference electrode: Ag/AgCl/3M KCl/1M KCl, counter electrode: GC) in an aqueous solution of 10 mM EDOT and 25 mM NaPSS as the supporting electrolyte. Prior to polymerization, the monomer solution was allowed to infiltrate the interparticle voids for 20 min to avoid mass transport limitation during the electropolymerization process. During this time the monomer solution was kept under nitrogen to protect it from oxygen. The amount of deposited polymer (thickness of the film, see also Table 6 on page 78) was controlled based on the electrical charge passed during the electropolymerization.

![Figure 15](image.png)

*Figure 15* Image of the electrodes with (A) 3D ordered and (B) compact PEDOT(PSS) film synthesized with different polymerization charges on their surface

After the electropolymerization, the PS template was dissolved away through several washing cycles in toluene and subsequently in ethanol. While for the thinnest film already 1 washing cycle was sufficient (ca. 6 min) the thickest films required up to 5 cycles (60 min). The electrodes were finally rinsed with ethanol and DI water, and dried. The ordered nanostructure of the polymer visualized in a holographic shiny surface (A).
Compact PEDOT(PSS) films were prepared the same way (same polymerization charge) but in absence of the PS nanosphere template (Figure 14B and B).

In case of solid contacts with incorporated redox couple for $E^0$ adjustment, the conducting polymer films were infiltrated with an equimolar ratio of the oxidized and reduced form of 1,1'-dimethylferrocene. The ratio of the two forms was used adjusted by coulometry, i.e. the reduced form was oxidized by using the theoretical amount of charge. This procedure led to a practical ratio of the two forms of 1.1 as determined by redox potential measurements. The infiltration was made by drop casting, i.e., applying 3 times 10 µl 0.1 mM DMFe in acetonitrile onto the SC (1 mmol/kg relative to the ISM applied in the next step). The solutions were allowed to dry overnight after the last drop before 10 µl of the Ag$^+$-selective membrane cocktail was drop cast.

Silver-selective electrode fabrication

The PVC-based membrane cocktail (dry weight 33 wt%) consisted of 0.8 wt % (15.4 mmol/kg) ionophore (o-xlylyenebis(N,N-diisobutyl dithiocarbamate)), 0.6 wt % (7.1 mmol/kg) NaTFPB, 55.4 wt % DOS, and 43.1 wt % PVC. The silicone rubber-based ISM (dry weight 33 wt%) contained 88.4 wt % RTV 3140, 0.8 wt % (15.6 mmol/kg) ionophore (o-xlylyenebis(N,N-diisobutyl dithiocarbamate)), 0.6 wt% (6.9 mmol/kg) NaTFPB, and 10.2 wt % DOS as plasticizer to facilitate the solubilization of the silver ionophore. After weighing in, the silicone rubber was dissolved immediately in THF in order to avoid the premature start of the curing process of the SR exposed to atmospheric humidity. The membrane solutions were prepared with a rather high viscosity to minimize the redissolution of the redox couple in the underlying SC layer. The PVC-based ISMs were allowed to dry overnight and the SR-based ISMs for 24 h. The electrodes were conditioned in 1 mM AgNO$_3$ solution.

Ag$^+$-selective solid-contact ISEs (AgSCISEs) and, for comparison, coated-wire electrodes (AgCWEs) were also prepared. As shown in Figure 16 the following 6 types of electrodes were prepared:

(i) without redox couple GC/3D PEDOT(PSS)/ISM, GC/compact PEDOT(PSS)/ISM, GC/ISM, and

(ii) with redox couple GC/3D PEDOT(PSS)/redox couple/ISM, GC/compact PEDOT(PSS)/redox couple/ISM, GC/redox couple/ISM.

Each different electrode type was prepared in triplicate.
Figure 16 Scheme of the different electrode types prepared: AgSCISEs with (A) 3D ordered and (B) compact SC layer, and (C) AgCWEs; (left) without and (right) with redox couple.

5.3. Potentiometric measurements

In all potentiometric measurements the potential response curves were recorded with a 16-channel high-input impedance ($10^{15} \Omega$) voltmeter (Lawson Labs Inc., Malvern, PA) at room temperature, mostly in stirred solutions (except for the paper-based immunoassay). Since electrolyte concentrations never exceeded 1 mM, the ionic strength of the solutions being <10 mM, all calculations were done using concentrations instead of activities.

The external reference electrode for the anion detection study was the custom made miniaturized reference electrode already discussed before with the inner filling solution of $10^{-2}$ M KCl (see Figure 12Ab on page 42). A special electrode holder was designed to accommodate the reference electrode and concentrically up to five working electrodes for simultaneous measurements in microtiter plate wells (see Figure 12B on page 41). In the paper-based immunoassay study a pseudo-reference electrode, i.e. a calcium selective CWE was used against the silver-selective working electrode. To provide constant potential a constant $10^{-5}$ M Ca(NO$_3$)$_2$ background was established in all solutions during the EMF measurements. The working and pseudo-reference CWEs were placed on the two opposite surfaces of the wet paper strip as shown in Figure 13 (page 42). For the two solid-contact silver-selective electrode studies the external reference electrode consisted of a double-junction Ag/AgCl electrode with 3 M KCl inner electrolyte and 1 M KNO$_3$ bridge electrolyte.
Unbiased selectivity coefficients were determined with ISMs, which had not previously been in contact with their primary ions. The selectivity coefficients were calculated by the separate solution method (SSM) using nitrate salt solution of the primary ion (i=Ag) and interfering cations (j).

The solutions below $10^{-5}$ M were always prepared freshly for the calibration measurements. In case of the potentiometric immunoassays the calibrations were carried out in the respective volume, i.e. in 150 µl in the wells of the microtiter plate and in the paper wetted by 30 µl solution, respectively. For the silicon rubber-based AgSCISE study hydrophobic polypropylene beakers of 500 ml (or 100 ml Teflon beakers) were used that had been fully equilibrated with the respective ion. One beaker was dedicated for one specific concentration and the solution was remade in the same beaker by weighing before each measurement. In this way the use of volumetric flasks, transfer pipettes and any volumetric glassware that have been shown to adsorb or release ions was avoided.

5.4. Preparation of the surface-imprinted polymer

In the respective study selective recognition sites for avidin binding on the surface of PEDOT(PSS) film were created by nanosphere lithography. A layer of avidin-conjugated nanoparticles was imprinted in the conducting polymer film with thicknesses on the order of the bead radius.

![Figure 17](image1.png)

_Figure 17_ Electrosynthesis of surface-imprinted polymer films by nanosphere lithography for selective recognition of avidin. The avidin-modified beads are deposited on the surface of the QCM crystal followed by the electropolymerization of PEDOT(PSS). The removal of the beads in two successive steps, i.e., removal of avidin by cleaving the disulphide bond of the crosslinker and dissolution of the polymeric bead, leaves behind cavities bearing the imprint of avidin molecules.

5.4.1. Synthesis of the avidin-nanoparticle conjugates

The Polybead® Amino stock solution was diluted 10 times with PBS followed by the addition of 100 µl heterobifunctional crosslinker NHS-SS-biotin
(10 mM) in DMSO. The suspension was incubated under continuous shaking for 60 min and then centrifuged at 16660 × g for 10 min. The supernatant was discarded, and the particles were resuspended in 900 μl PBS. This procedure was repeated five times to separate the biotinylated nanoparticle from the free reagent. Further modification with avidin was made by mixing 100 μl of 10 mg/ml avidin in PBS with 900 μl biotinylated bead suspension for 60 min. The beads were collected by centrifugation (at 16660 × g for 10 min) and washed five times with water, followed by resuspension in 1000 μl water to give a final concentration of ca. 10¹⁰ particles/ml.

5.4.2. Preparation of the avidin-imprinted polymer film

The surface of the gold electrode (0.205 cm²) on 10 MHz AT-cut gold-coated quartz crystal resonator (Gamry Instruments, Warminster, PA, USA) was pretreated in a UV ozone cleaner (Novascan PSD Pro UV Ozone System) for 15 minutes. An aqueous suspension of non-modified or avidin-modified beads (0.13 w/v%) was drop cast onto the surface to provide a calculated surface coverage of 8.98×10⁸ beads/cm² and slowly dried at a controlled relative humidity of 75 % (t=23ºC). The potentiostatic deposition of PEDOT(PSS) within the voids of the particle array was done at 0.9 V (reference electrode: Ag/AgCl/3M NaCl, counter electrode: Pt) in an aqueous solution of 10 mM EDOT and 25 mM NaPSS. The amount of film deposited (thickness of the layer) was controlled based on the electrical charge passed during the electropolymerization. The polystyrene beads were dissolved away in toluene. The quartz crystals were then rinsed with ethanol and DI water, and dried. The bare gold surfaces exposed upon removal of the microspheres were blocked with HS-TEG (1 mM in PBS for 30 min) in order to reduce non-specific adsorption. Non-imprinted polymers (NIPs) were prepared in the same way, except that unmodified beads were used.

5.5. Protein assays

5.5.1. Potentiometric immunoassays

5.5.1.1. Potentiometric enzyme immunoassay for the determination of prostate specific antigen in serum

Sandwich immunoassays were performed on commercial microtiter plates (Immuno Module F8 Maxisorp Loose, No. 469949, Nunc, Roskilde, Denmark)
at room temperature. All solutions were prepared with the working buffer, i.e. 1 mM PBS containing 1 mM MgSO₄ at pH 7.7.

First, the microtiter plates were modified with the capture antibody: 100 µl of capture anti-human PSA antibody (5 µg/ml) in the working buffer was placed in the polystyrene microwells, which were then sealed and incubated at 4 °C overnight. The wells were rinsed with 400 µl aliquots of washing buffer three times, whereupon the surface was blocked using 400 µl of blocking buffer (i.e. either SuperBlock, Protein-Free, or BSA (20 µg/ml) solution). After drying, the modified plates were stored at 4 °C if not used immediately.

Figure 18 Schematics of the sandwich PSA assay

The assay started by adding 100 µl of different concentrations of human PSA (hPSA) serum solutions into the wells and incubating for 1 h. Following this step, a solution (100 µl) of biotin-conjugated tracer Ab (5 µg/ml) in the working buffer was injected and incubated for 1 h. After the formation of the sandwich immunocomplex was completed, a solution (100 µl) of streptavidin conjugated β-galactosidase (5 µg/ml) in the working buffer also containing Protein-Free blocking buffer (1 %, v/v) was applied for 30 min. After removing this solution and rinsing the wells, freshly prepared 0.5 mM DiFMUG substrate in the working buffer (150 µl) was added and incubated at room temperature for another 30 min. Finally, the enzymatic reaction was stopped with 2 mM of CuSO₄ (15 µl) and the released DiFMU was detected potentiometrically.
5.5.1.2. Paper-based potentiometric bioassay

For the quantitative determination of human IgE a paper-based assay format, i.e. a dot-blot assay was used. IgE aptamer-gold nanoparticle conjugates were used to trace the target protein. These nanoparticle conjugates than further amplified the signal by catalysing the deposition of silver nanoparticles. The resulting brown to black dots of metallic silver were detected optically, or by oxidative dissolution silver ions were generated for potentiometric readout.

Preparation of aptamer-modified gold nanoparticles

In the first step the aptamer-gold nanoparticle conjugates were synthesized. Gold nanoparticles (AuNPs) were prepared based on a protocol from Liu and Lu[240] reported to result in 13 nm diameter nanoparticles. Briefly, 10 ml 38.8 mM sodium citrate was added to 100 ml of 1 mM boiling HAuCl₄ solution under stirring. The mixture was allowed to reflux for another 20 min and then, apart from the original protocol, after cooling and before further modification the gold nanoparticle solution was centrifuged at 3000 rcf (relative centrifugal force) for 5 min. The nanoparticle-oligonucleotide conjugates were synthesized by injecting 8 µl of 100 µM thiol labeled IgE aptamer to 4 ml solution of the gold nanoparticle solution. After stirring at room temperature for 20 h, 200 µl of 0.5 mM HS-TEG solution was added and the reagent mixture was stirred for another 2 h. The nanoparticles were separated by centrifugation at 12000 rcf for 20 min and the clear supernatant was removed. The resulting DNA nanoparticle conjugate was resuspended in PBS-Mg buffer (PBS (pH 7.4, 10 mM phosphate buffer containing 2.7 mM KCl and 137 mM NaCl) with 1 mM MgCl₂). The aptamer-gold nanoparticle (Apt-AuNP) conjugate was stored at 4 °C.

The same recipe was used for the synthesis of AuNP modified with HS-TEG (TEG-AuNP) and T44 (T44-AuNP).

Dot-blot assay

Dot blot assays were performed at room temperature on nitrocellulose paper strips having 0.2 µm pore size (Bio-Rad, Hertfordshire, UK). 10 µl of IgE containing sample or control solution was spotted with a micropipette onto the surface of the nitrocellulose paper. For calibration purposes IgE in PBS-Mg buffer was applied at different concentrations (0-250 µg/ml). Care was taken to minimize the wetted area, i.e., to better localize the spot by applying the
samples slowly, without pressure, letting the solution to move laterally by capillary action. After the strip dried the surface was blocked for 10 min by immersing it into 2.5 ml 5 wt.% freshly prepared instant skimmed milk powder solution in PBS buffer. Like all incubation steps, the blocking was carried out in a glass beaker of ca. 5 ml volume placed on an orbital stirring platform operated at a constant 400 rpm. The excess of blocking agent was rinsed with PBS-Mg buffer for 2 min. The freshly prepared AuNP conjugate (1 ml) was added and allowed to incubate for 30 min. The strip was then thoroughly rinsed with deionized water and dried. The light pink to reddish blots were then scanned by using a commercial flatbed scanner at 1200 dpi resolution (HP ScanJet 3800).

![Figure 19 Schematics of the dot-blot assay](image)

To amplify the signal a two component silver enhancement solution was freshly prepared by mixing equal volumes of the initiator and enhancer and applied onto the nitrocellulose paper. The silver enhancement solution is nucleated by the gold nanoparticles in the paper, resulting in the precipitation of metallic silver and formation of a dark brown to black signal within 15 min. Finally the strip was rinsed with deionized water and the image of the dried strip was acquired again for optical evaluation.

**Potentiometric dot-blot assay**

The potentiometric dot-blot assays were performed in the exact same conditions as described for optical detection; however, the deposited metallic silver was
dissolved with H₂O₂ to generate silver ions, as shown in . The silver ions generated by oxidative dissolution in the nitrocellulose membrane were detected with a silver selective electrode against a pseudo-reference calcium selective electrode.

For potentiometric readout the membrane was cut into 0.5 × 2.0 cm strips containing one dot each. The dried strips were then sandwiched between two silicone sheets and the two electrodes, as already depicted in Figure 13 (page 42), with the blot facing the AgISE. For the dissolution of the silver deposit within the spot a freshly prepared 30 µl 1% H₂O₂ solution with 10⁻⁵ M Ca(NO₃)₂ background was applied through a hole in the upper silicon rubber sheet. The solution was taken by capillary action to the blot while the silver ion activity was monitored continuously. The maximum EMF change of the potential transients was used for quantitative determinations.

5.5.2. Protein detection with surface-imprinted polymer

Avidin binding to the synthetic avidin receptors imprinted in the surface of the PEDOT(PSS) film was measured with quartz crystal microbalance (QCM) by Júlia Erdössy (nee Bognár).

The MIP- and NIP-coated quartz crystals were mounted into a flow cell of 120 µl (Als Co. Ltd, Tokyo, Japan) and connected to Gamry eQCM 10M electrochemical quartz crystal microbalance. After stabilization of the frequency, increasing concentrations of avidin were injected in the carrier buffer and the frequency change was monitored in real-time. The protein solutions (250 µl) were injected into the carrier solution flown at a rate of 60 µl/min. The avidin binding of NIPs was recorded in similar conditions but in a separate experiment.

5.6. Characterization techniques

5.6.1. Electrochemical techniques

**Impedance measurements**

Electrochemical impedance spectroscopy (EIS) is an excellent tool for characterizing the electrical properties of materials and interfaces. The minimal perturbation of the system, normally ≤ 10 mV, makes it an excellent technique for the study of conducting polymers, whose electrochemical properties are very sensitive to changes in potential.
Commonly a single-frequency ac voltage, i.e. voltage phasor, is applied to the system and the phase shift and amplitude of the resulting alternating current, i.e. current phasor is measured. The impedance (Z) is the ratio between the voltage phasor and the current phasor, and hence a complex number. Z is given by:

\[ Z = Z' - jZ'' = |Z|e^{j \phi} \]  

(14)

where \( Z' \) and \( Z'' \) are the real and imaginary parts of \( Z \), respectively, \( j = \sqrt{-1} \), \( |Z| = (Z'^2 + Z''^2)^{1/2} \) and \( \phi \) is the phase shift, or phase angle between the voltage and current phasor. The measurement is commonly repeated over a wide frequency range spanning several orders of magnitude, typically 1 mHz – 1 MHz. In accordance with Eq. (14) the obtained impedance data is mostly displayed as \(-Z''\) vs. \( Z'\) i.e. Nyquist plots, and as \( \log|Z| \) and \( \phi \) vs. the log of the frequency, i.e. Bode plots. The mathematical treatment of the impedance data can be very demanding, hence it is common practice to model the impedance response of the electrochemical system by an equivalent circuit.

All electrochemical impedance spectra in this work were measured with an Autolab PGSTAT 12 potentiostat/galvanostat equipped with FRA2 impedance module (Metrohm Autolab B.V., Utrecht, The Netherlands). Nova software was used to plot and evaluate the data.

For the silicon rubber-based solid-contact electrode characterization impedance spectra were measured within the frequency range of 100 kHz to 10 mHz in 1 mM AgNO\(_3\) solutions. The excitation potential (\( \Delta E_{ac} \)) was 100 mV.

For the 3D ordered solid contact characterization the typical frequency range was 100 kHz - 30 mHz. The excitation potential (\( \Delta E_{ac} \)) was 5 mV. For aqueous systems a 1mM AgNO\(_3\) solution was used as the electrolyte with a double junction Ag/AgCl reference electrode with 3 M KCl and 1 M KNO\(_3\) in the inner and outer compartments, respectively. For non-aqueous systems a 1 mM solution of ETH500 in acetonitrile was used as electrolyte, and Ag/AgCl wire was used as reference electrode. In both cases, a glassy carbon rod served as the counter electrode.

**Chronopotentiometry measurements**

The potential stability of the electrodes with 3D ordered solid-contact were evaluated by chronopotentiometry. A constant current of +1 nA was applied
on the working electrode for 60 s followed by a current of -1 nA for another 60 s, under simultaneous recording of the electrode potential. The slope of the E-t curves at longer times gives a direct measure of the potential stability of the electrodes and is related to the low-frequency capacitance of the solid contact ($\Delta E/\Delta t = i/c$). The potential jump in the chronopotentiometric E-t curves can be used to estimate the total bulk resistance ($R$) of the membrane ($R = E/i$). The electrodes were conditioned in stirred 1 mM AgNO$_3$ solution overnight before this measurement. For constant-current chronopotentiometry experiments the same Autolab instrumentation and electrodes were used as described above for aqueous EIS measurements.

5.6.2. Other characterization methods used

The possible post-diffusion of PANI into the SR membrane was studied with spectroelectrochemical microscopy (SpECM). A blank SR membrane (without PANI) was contacted with a SR membrane containing 2 wt% PANI, and the UV-vis spectrum over a cross section of the interface between the two membranes was measured for 24 h. The membranes had cured for 24 h before the measurements.

The morphology of the 3D ordered PEDOT(PSS) solid-contact polymer films were investigated with scanning electron microscopy (SEM) (LEO, Carl Zeiss, Oberkochen, Germany).

The modification of PS beads with avidin and their removal from the PEDOT(PSS) surface was confirmed by using fluorescent labelled avidin (Av-FITC) and a hyperspectral optical imaging system consisting of an Olympus IX71 inverted epifluorescence microscope and a Paris$^\text{®}$ hyperspectral imaging system (LightForm Inc., Asheville, NC, USA). The fluorescence intensities were recorded in at least 240 different spots on the surface using an LCPLFL20XPH/0.40 long working distance objective and a U-MNB2 fluorescence filter cube (narrow band excitation (470-490 nm), 500 nm cut-off dichroic mirror and a long pass 520 nm emission filter).

The morphology of the imprinted PEDOT(PSS) surfaces was investigated with an easyScan2 atomic force microscope (Nanosurf AG, Liestal, CH) in dynamic force mode.
6. Results and discussion

6.1. Potentiometric immunoassays

6.1.1. Potentiometric enzyme immunoassay

The aim of this work was to explore the feasibility of designing enzymatic schemes, possibly based on the most widely used enzyme labels of ELISA, to generate organic anions which can be detected potentiometrically. The measuring scheme was based on the idea that if sufficiently lipophilic anions are generated by an enzymatic reaction, a simple anion-exchanger electrode could be used for their detection (see chapter 2.5.4.). The choice of measuring anions was determined by the fact that cations in bioassays often get adsorbed and/or complexed by biomolecules\(^{[203]}\) \(^{[237]}\), leading to decreased ion activities and thus to insufficient detection limits.

The proof of concept for the feasibility of this potentiometric detection method was demonstrated by a sandwich immunoassay for the determination of prostate specific antigen (PSA) from serum samples. PSA is a 33 kDa single-chain glycoprotein that is produced by cells of the prostate gland and released at very low concentrations in the blood of healthy males. PSA tests are widely used for prostate cancer screening and post cancer treatment monitoring. Although the PSA level can be influenced by many factors, the clinically relevant elevated PSA levels are >4 ng/ml for middle aged men and >2.5 ng/ml for young males.

To create a widely utilizable detection method, while designing the measuring scheme we focused only on the three most commonly used enzyme labels, i.e. alkaline phosphatase, horseradish peroxidase and galactosidase, and their commercially available substrates. As mentioned before, one of the main hindering of the early potentiometric immunoassays was the limited availability of commercial reagents for the unconventional assay systems/reagents used. For optimal potentiometric detectability the following criteria should be complied:

(1) The enzyme reaction has to result in an organic anion which is lipophilic enough for the determination by an anion-exchanger based electrode, while its solubility in aqueous solutions is still sufficient.

(2) It’s also very important, that the product is in anionic form around the pH optimum of the enzyme, which for most of the organic anions practically
means having a $pK_a$ smaller by at least 2 logarithmic units than the working pH. Another alternative to keep the product in anionic form, is to stop the enzymatic reaction by concentrated NaOH, however this works only for endpoint-type detection.

(3) The commercial availability,
(4) low toxicity, and
(5) chemical stability of both the enzyme substrate and the product anion is essential as well.
(6) Preferably the organic anion should form with high turnover rate
(7) in a one-step enzymatic reaction.

While HRP provides limited flexibility in terms of anion-generating substrates, there is an excess of commercially available substrates for ALP and GAL enzymes. Remarkably, the anionic products generated by these two hydrolysing enzymes are practically the same since the artificial substrates differ only in having either an inorganic phosphate group or a $\beta$-D-galactopyranoside to be cleaved. The use of ALP, however, was immediately ruled out since the substrate also is an anion and there is little difference in the lipophilicities of the substrate and the product.

One substrate of galactosidase, i.e. 6,8-difluoro-4-methylumbelliferyl-$\beta$-D-galactopiranoside (DIFMUG) was found to fulfil all the above mentioned criteria. Although GAL is the least popular of the three most common enzyme labels in ELISA owing to its higher molecular weight (464 kDa), it is widely utilized, has a high turnover number, good compatibility with an extremely large range of buffers, and unlike HRP and ALP it is not present in the mammalian tissue, which helps to lower the chance of non-specific signals.
GAL has its pH optimum between 7 and 8, needs 1 mM Mg$^{2+}$ for its activity as a cofactor, and can be inhibited by several metal ions, e.g. by Cu$^{2+}$. The enzymatic reaction of DIFMUG, as shown in Figure 20, yields in the ionisable hydrolysis product 6,8-difluoro-4-methylumbelliferone (DIFMU), which has a p$K_a$ of 4.9 and a calculated log $P$ of 1.83.

Table 2 Potentiometric selectivity coefficients ($log K_{DIFMU^-j}^{pot}$) of various membranes, measured by the separate solution method at 1 mM level, for detecting DIFMU. In the case of 4-methylumbelliferone anion (MU$^-$) and p-nitrophenolate (PNP$^-$) the pH was adjusted with concentrated NaOH, to *pH 9.0 and **pH 10.0, respectively, to ensure the dominance of the anionic form.

<table>
<thead>
<tr>
<th>Plasticizer</th>
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<td>10</td>
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<td>PNP$^-$**</td>
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</tbody>
</table>

When using an anion-exchanger-based membrane for potentiometric detection in a heterogeneous ELISA immunoassay, the main interference to be expected arise from the inorganic anions of the working buffer solution. This buffer should ensure the optimal pH and ionic conditions for the galactosidase enzyme reaction. Potentiometric selectivity coefficients for the commonly encountered inorganic anions were measured by the separate solution method at 1 mM level, $log K_{i,j}^{pot}$ ($i =$ DIFMU$^-$) values for the different membrane constitutions tested are summarized in Table 2.

A compromise between the selectivity and the suitability of the buffer solution in terms of optimal enzyme function resulted in the use of 1 mM phosphate buffer containing 1 mM MgSO$_4$ at pH 7.7 as working buffer, and the use of an ISE membrane composition of 40 wt% PVC, 60 wt% o-NPOE as plasticizer, and 10 mmol/kg TDMA-NO$_3$ as anion-exchanger.

To obtain the potentiometric detection of PSA immunoassay in microtiter plates miniaturized liquid junction electrodes were prepared. The conventional, but miniaturized reference electrode was surrounded by 5 anion-exchanger-based working electrodes, as shown in Figure 12 (page 41), allowing the performance comparison of 5 electrodes simultaneously. The electrodes were first tested in 5 ml working buffer, than the DIFMU$^-$ calibration curves were
recorded in 150 µl volume in the microtiter plate wells. The electrodes gave Nernstian response with a detection limit of $8 \times 10^{-5}$ M. This LDL was in good accordance with the theoretical detection limit determined by the selectivity coefficients (Figure 21 black). This indicates that no contamination or adsorption occurs during the measurement. Since a classic sandwich ELISA is an endpoint-type assay, i.e. the DIFMU$^-$ detection is performed after the enzymatic reaction is terminated with a stop reagent, calibrations were done in the presence of both 1 mM NaOH (Figure 21 blue) and 2 mM CuSO$_4$ (Figure 21 red). (According to our preliminary measurement, when incubating GAL with 2 mM CuSO$_4$ the enzyme activity drops to only 0.9 % of the initial value.) Although the use of NaOH seems to be advantageous in terms of keeping the product in anionic form via the resulting pH, OH$^-$ is less discriminated than SO$_4^{2-}$, and it led to sub-Nernstian response slopes (possibly due to anion interference) and much higher LDL. In contrast, the addition of CuSO$_4$ resulted in a linear super-Nernstian response of DIFMU$^-$ ($\sim105$ mV/decade), which has proved to be reproducible, and which considerably increased the sensitivity of the determination.

![Figure 21](image)

**Figure 21** Calibration curves for DIFMU$^-$ in microtiter plates, i.e. in 150 µl sample volume: (black) in the working buffer (1 mM phosphate buffer with 1 mM MgSO$_4$ at pH7.7), (blue) in the same working buffer but with 1 mM NaOH and (red) in the same working buffer but with 2 mM CuSO$_4$.

The determination of human PSA was performed according to the scheme shown in Figure 18 (page 49). The microtiter plates were modified with capture antibody having an affinity constant of $1.0 \times 10^{10}$ M$^{-1}$ [243], to which the PSA bound from human serum samples. After successive incubation in biotinylated tracer antibody, streptavidin-GAL conjugate and 0.5 mM DIFMUG, the
enzymatic reaction was stopped by 2 mM CuSO₄, and the captured PSA was detected potentiometrically by measuring the generated DIFMU⁻ anion in the solution. Each concentration was measured in 3 replicate wells, with 3-5 working electrodes.

All the assay steps were optimized in order to use the lowest concentration of the bioreagents providing maximal or close to maximal response, and thereby to lower the non-specific adsorption. The lowest concentration needed of the capture antibody, the tracer antibody and the streptavidin-GAL conjugate, was determined one-by-one by potentiometric DIFMU⁻ detection, using 6-7 different concentration of the respective reagent, three parallel from each, and keeping the rest at the maximum (e.g. 50 ng/ml PSA, 20 µg/ml enzyme conjugate) or at the already optimized level. The ideal concentration was found to be 5 µg/ml for all the three components (for diagrams see Paper I).

The assay was also optimized in terms of reducing non-specific adsorption with a number of blocking agents (Protein-Free and SuperBlock blocking buffers, and 20 µg/ml BSA in the working buffer) and two different washing methods (distilled water and washing buffer). The goal was to minimize both the potential response of the blank sample, i.e. the potential difference between the 0.5 mM DIFMUG solution without enzymatic treatment and the 0 ng/ml PSA well (Figure 22A), and to maximize the potential range of the assay, i.e. the potential difference between the 0 ng/ml and 50 ng/ml PSA samples (Figure 22B). While the use of washing buffer minimalizes the extent of non-specific adsorption for PSA detection in potentiometric ELISA, in terms of (A) minimizing the potential response for the blank sample and (B) maximizing the potential range of the assay. Different blocking methods: 20 µg/ml BSA in the working buffer, Protein-Free blocking buffer (PF) and SuperBlock blocking buffer (SB) for 1 h and for 10 min were tested; and distilled water (H₂O) and washing buffer (WB) was used in the washing steps.

![Figure 22](image-url)
adsorption, the potential range increases spectacularly when blocking with 20 µg/ml BSA solution.

Figure 23 Calibration curves for PSA in human serum background using ELISA assays with (A) optical absorbance and (B) potentiometric detection.

The potentiometric ELISA detection of PSA from human serum gave linear semilog calibration in the range of 0.1–50 ng/ml, as shown in Figure 23B. It provides a sufficiently low detection limit (≤0.1 ng/ml), which complies with the requirements of in vitro diagnostic PSA assays. The detection limit of the assay is determined by the selectivity of the electrodes, so further enhancements in this matter could improve the performance of the anion-exchanger based potentiometric immunoassays. When comparing these results with the conventional optical detection of the same assay using HRP labelling (Figure 23A), there was no significant difference at the 95 % confidence level between the two methods. Error propagation calculations, after fitting the experimental data with dose-response function, show that the uncertainty of the optical measurement in terms of concentration in the middle of the measuring range is 28.1 %, while the potentiometric assay has a relative error of 8.9 %. Although the procedure cannot compete in terms of analysis time with the highly parallel microtiter plate readers, it is by far superior to the previously published nanoparticle-based potentiometric immunoassays\(^ {203} \)\(^ {237} \). The results confirm the applicability of anion-exchanger based potentiometric detection in diagnostic PSA assays.
Outlook

Following this work, recently, several publications by the group of Wei Qin utilized the same concept, the potentiometric detection of enzymatically generated lipophilic molecules with a simple ion-exchanger-based polymeric membrane. First it was shown[244], that reactive cationic intermediates rather than stable reactants or products can also induce large potential responses on appropriately formulated membranes with a cation exchanger. In many cases both substrates and products of enzymatic reactions are non-ionic, so these reactions are not considered in designing potentiometric biosensing schemes. However, reaction intermediates having different pK_a values from substrates or products can be transferred into the membrane from the aqueous phase with the right ion-exchanger. This method was used for monitoring enzymatic reactions of HRP, and the peroxidase mimetic G-quadruplex DNAzymes[245] [246] [247]. This way horseradish peroxidase has been detected with a detection limit at least two orders of magnitude lower than those obtained by spectrophotometric techniques. Different HRP substrates were utilized for the detection, such as phenol[245], o-phenylenediamine[246] and even the widely used TMB (3,3’,5,5’-tetramethylbenzidine)[247].
6.1.2. Paper-based potentiometric bioassay

Microfluidic paper-based assays\textsuperscript{[248]} represent a relatively new approach to address the need for simple and cost-effective medical diagnostics. They use paper as a cheap and conveniently disposable platform and integrate the already existing paper-based bioanalytical methods with cheap paper microfluidics. To preserve the advantages of the platform the detection technique should match in terms of easy methodology and low cost. Although a simple visual inspection often provides a yes-or-no type response, for a quantitative bioassay conventionally reflectance-based methods are employed, using a digital camera or a flatbed scanner combined with image analysis. The aim of this work is to explore the feasibility of using potentiometric detection instead, by measuring generated ions directly in the paper. It would still preserve the cost-effectiveness of the methodology, but provide advantages in terms of quantitative determination and sensitivity. Detecting ions in paper potentiometrically makes the methodology very much different from the previous bioassay investigation performed in the wells of a microtiter plate. The goal is to show the proof of concept of potentiometric transduction for quantitative paper-based bioassays by placing the ISEs directly on the surface of the wet paper (see Figure 13 on page 42) and measuring ions in the solution phase entrapped within the paper.

As proof of principle a dot-blot model assay based on using IgE aptamer – gold nanoparticle conjugates was developed for the quantitative determination of human IgE. The gold nanoparticle conjugates further catalysed the deposition of silver nanoparticles that by oxidative dissolution generated silver ions.

To generate ions whose concentration is proportional with the amount of target analyte the label of the tracer reagent is used. Whereas in the previous study enzyme labels were utilized, in this case metallic nanoparticles seem to offer a better alternative. Silver nanoparticles with the diameter of 25-30 nm are easy to prepare\textsuperscript{[249]} and if adequately modified very stable as well. Their natural bright yellow colour can be optically detected. Although with oxidative dissolution theoretically 0.5–0.8×10^6 silver ions can be generated from each nanoparticle, which compares to the amplification effect of the enzymes (see chapter 4.2.2.), under the experimental conditions it is often not sufficient due to the contingent adsorption and/or complexation of cations in bioassays. As the preparation of larger silver nanoparticles is problematic, to increase the number of generated silver ions the oxidative dissolution of silver enhancement
of gold nanoparticle labels\textsuperscript{[203]} \textsuperscript{[204]} \textsuperscript{[250]} offers a viable alternative ($2\text{AgNP} + \text{H}_2\text{O}_2 + 2\text{H}^+ = 2\text{Ag}^+ + 2\text{H}_2\text{O}$). Gold nanoparticles act as catalysts to reduce silver ions to metallic silver. From a silver enhancement solution AuNPs nucleate the deposition of dense silver particles on their own surface and grow rapidly to 100 nm in diameter. Silver enhancement is one of the most sensitive immunodetection systems. It is time dependent (see also Figure 28 on page 67) and for the first time period the reaction is highly specific for gold nanoparticles. This way after the silver dissolution practically one order of magnitude more silver ions are available for the potentiometric detection.

![Figure 24](image)

Figure 24 Images of the dots spotted with different amounts of IgE after applying (A) the IgE aptamer – AuNP conjugates and (B) further amplification of the signal by using silver enhancement reagent for 15 min.

For the IgE detection a dot-blot type assay was used (Figure 19, page 51). Dot-blot assay is a simple and fast paper-based assay format for detecting, identifying and analysing proteins, without separating them electrophoretically but spotting them directly onto a paper substrate with high protein binding capacity. (E.g. the particular nitrocellulose membrane used in this study has a protein binding capacity of 800-100 µg/cm$^2$.) As no capture antibody is utilized in this assay format, and the first binding step is non-specific and therefore competitive, dot-blot assays are generally less sensitive than sandwich ELISAs. This is challenging in terms of the selectivity of these assay as well. The target IgE protein is detected in a subsequent step by the labelled, highly specific IgE aptamer – gold nanoparticle conjugate. As gold nanoparticles are used to label the tracer reagent, light pink to reddish dots are developed in proportion to the protein concentration, perceptible even by the naked eye (see Figure 24A). Conventionally these dots can be detected by a reflectance-based technique. When further applying the silver enhancement solution on the paper, it is nucleated by the gold nanoparticles on the surface, resulting in the precipitation of metallic silver and consequently the formation of dark brown to black dots (Figure 24B). On one side this amplifies the optical signal, increases the sensitivity and considerably lowers the detection limit (see also Figure 26). On
the other hand by oxidative dissolution silver ions can be generated from the metallic silver enabling the potentiometric detection. This assay format is very simple, and it does not only offer significant savings in time but also the possibility to compare and evaluate the conventionally used optical detection side-by-side with the potentiometric detection. During the two different detection methods the exact same dots are measured consecutively, and thus the differences in analytical performance reflects solely the performance differences of the respective techniques.

Figure 25 Specific and non-specific aptamer-modified gold nanoparticle binding on the IgE containing dots and on the substrate, after using different blocking agents: (A) 1 mg/ml BSA in PBS buffer, (B) protein free blocking buffer, (C) 5 mg/ml casein in PBS and (D) 5% skim milk solution in PBS.

To reduce the non-specific adsorption of the Apt–AuNP conjugate, and thereby the non-specific silver deposition on the background, after applying the IgE on it the surface has to be blocked. Different blocking agents were tested, i.e. 1 mg/ml BSA in phosphate buffer, protein free blocking buffer, 5 mg/ml casein in PBS and 5% skim milk solution in PBS (Figure 25). The best specific vs. non-specific signal was measured when utilizing 5% skim milk solution.

Figure 26 Eight bit greyscale values versus the IgE amount in the calibration spots recorded with a flatbed scanner after application of the IgE aptamer linked AuNP and further amplification with the silver enhancement reagent. The data were fitted with a 4-parameter logistic curve.
During the silver enhancement process the silver staining effect decline as the surface area of the nanoparticles increases, until finally self-nucleation occurs and silver precipitates spontaneously on the background. At 24 °C the enhancement solution should be stable for at least 35 minutes, however at the experimental conditions a uniform background staining was observed after 20 min, and deposition in random spots after 17 min. To obtain high contrast and very low background signal the silver enhancement was performed for 15 minutes in all assays.

Figure 26 shows the reflectance-based evaluation results of the IgE dot-blots. This detection technique is quantitative for the spotted protein amounts, with the smallest detectable IgE amount being 50 fmol.

For the potentiometric detection solid-contact minielectrodes were prepared with a diameter of 3 mm. To measure the generated silver ions directly in the wet paper matrix a setup was designed in which the paper is sandwiched between two silicone rubber sheets and the working and reference electrode as shown on Figure 13 (page 42). As silver ions are measured, conventional Ag/AgCl reference electrodes cannot be used to avoid contingent Cl⁻ contamination, and furthermore the use of a solid-contact reference would prevent diffusion potential related uncertainties as well. To find the best pseudo-reference electrode candidate, silver selectivity of different ionophores (i.e. Na X, valinomycin, BME-44, and Ca IV ionophores) was studied. From the results shown in Table 3 the calcium selective electrode seemed to provide the best alternative by having the highest silver selectivity of \( \log K_{Ca,Ag}^{pot} = -4.3 \). As the silver ionophore (CsV321) had a sufficiently high calcium selectivity as well, \( \log K_{Ag,Ca}^{pot} = -6.7 \), CaISE pseudo-reference was used. To provide constant potential a constant 10⁻⁵ M Ca(NO₃)₂ background was established in all solutions during the EMF measurements.

<table>
<thead>
<tr>
<th>Ionophores</th>
<th>J</th>
<th>Na X</th>
<th>K I⁺</th>
<th>K III⁺⁺</th>
<th>Ca IV</th>
</tr>
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<tbody>
<tr>
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<td>-</td>
<td>-1.9</td>
<td>-1.8</td>
<td>-6.9</td>
</tr>
<tr>
<td>K⁺</td>
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<td>-3.2</td>
<td>-3.1</td>
<td>-</td>
</tr>
<tr>
<td>Ag⁺</td>
<td></td>
<td>-0.6</td>
<td>-1.7</td>
<td>-1.5</td>
<td>-4.3</td>
</tr>
</tbody>
</table>

*K I ionophore is also known as valinomycin and **K III as BME-44.
The nitrocellulose paper used has a significant water absorption capacity of 8.06 mg H₂O /cm² (1.61 mg H₂O /mg paper) enabling the potential measurement of silver ions in the solution phase entrapped within the paper. For studying the quantitation of silver ions directly in the nitrocellulose, calibrations were performed by wetting the paper strips, sandwiched between the electrodes, with solutions containing different AgNO₃ concentrations but a steady 10⁻⁵ M Ca(NO₃)₂ and 1 % H₂O₂ background. An excess of these solutions was applied through a hole in the upper silicone sheet and was taken by capillary action to the electrodes. The resulting calibration curve was Nernstian, however a significant difference was recognized from calibrations measured with the same electrodes in stirred 100 ml solutions (see Figure 27).

![Figure 27](image_url) Potentiometric calibration curves for silver ion (black) in stirred solution phase and (red) in paper.

The LDL for the paper-based calibration was somewhat better than 10⁻⁵ M, which result lag by 2.5 orders of magnitude behind that of the solution-based detection limit. The slope of the Nernstian part did not change. The exact reason for this behaviour is not clear, most likely the sluggish mass transport and the contingent presence of Ag⁺ complexing sites within the paper affect the paper-based calibration.

For the potentiometric detection of the dot-blot assay the membrane was cut into stripes containing one dot each, the dot facing the AgISE in the measurement setup. In the first step blots with the same amount of IgE but various silver enhancement times were studied (Figure 28). The oxidative dissolution of the silver enhancement within the spots was initiated by applying
30 µl freshly prepared 1 % H₂O₂ solution with 10⁻⁵ M Ca(NO₃)₂ background. The reaction took place relatively fast, resulting in a potential reading peak within 2 minutes. The maximum EMF change of the potential transients was used for quantitative determinations, but it was first corrected with the background signal measured on the blank blot. Potential signal showed linear response with the silver deposition time.

**Figure 28** Amplification effect of the silver enhancement by increasing the time of the reaction. (A) Images of dots with the same amount of IgE after various length of the silver enhancement and (B) their corresponding potential readings.

**Figure 29** Calibration curve for IgE using dot-blot assay with potentiometric detection.

The result of potentiometric dot-blot assay for human IgE is shown in Figure 29. The analytical performance of potentiometric detection was matching that of the reflectance-based assay. Despite of the higher detection limit for the calibration in paper, the lowest detectable amount of IgE was the same, 50 fmol,
as in the reflectance-based detection in the exact same conditions. This suggests that potentiometric detection can be a viable alternative to the conventionally used optical detection. However, the potentiometric technique has still important reserves to be exploited, i.e. if the detection limit of paper-based potentiometric ion detection could be improved to approach that of the solution phase measurements it could even outperform optical measurements.

**Outlook**

Lately microfluidic paper-based analytical devices (μPADs)\(^{[251]}\)\(^{[252]}\) and point-of-care diagnostic devices\(^{[253]}\)\(^{[254]}\) became very popular. Although in a growing number they utilize electrochemical detection methods (electrochemical PADs, ePADs\(^{[255]}\)), still very few are based on potentiometry. The group of Bobacka studied paper-based microfluidic sampling together with potentiometric detection by pressing solid-contact ISEs\(^{[256]}\), solid-contact reference electrodes\(^{[256]}\) and pH glass electrodes\(^{[257]}\) against the paper substrate. In paper-based measurements, similarly to findings mentioned above, they observed 2-3 orders of magnitude higher detection limits than in solution phase for KISEs\(^{[256]}\) and CdISEs\(^{[257]}\) but not for ClISEs\(^{[257]}\) in various different paper types. This suggests that there are interactions between paper and cations, presumably due to the presence of carboxyl and hydroxyl groups. This finding increases the applicability of the potentiometric lipophilic anion measuring scheme.

Another interesting new research direction is the field of paper-based ion-selective electrodes. The impregnation of paper with carbon nanotube inks to make it conductive\(^{[258]}\)\(^{[259]}\) made it possible to build paper-based ion-selective potentiometric sensors\(^{[260]}\). Using MMA-DMA-based membrane even nanomolar detection limits were achieved\(^{[261]}\) and a paper-based solid state reference electrode was developed as well\(^{[262]}\). Utilizing both paper-based ISE and reference electrode a 50 μl volume potentiometric cell was reported to monitor Li\(^+\) levels in whole blood\(^{[262]}\). Another potentiometric ePAD using paper electrodes measures in only 10 μl sample volume\(^{[263]}\). Most recently, using 4 paper-based sensors an electronic tongue was developed for water analysis\(^{[264]}\).
6.1.3. Conclusions

Potentiometric detection methods both for the classic microtiter plate format and for the paper-based immunoassays have proved to be a viable alternative to the conventionally used optical detection. The potentiometric immunoassays provided a sufficiently low detection limit matching that of the optical methods and complying with the requirements of in vitro diagnostics. However, in both cases considerable improvements are expected if the selectivity of the basic sensor is further enhanced, and if the detection limit of paper-based sensing could approach that of solution phase measurements, respectively. The huge advantage of the anion-selective detection of enzymatic products is that it is not affected by contingent adsorption and/or complexation cations suffer by biomolecules or in paper, while the nanoparticle-based potentiometric detection is superior in terms of analysis time. Although the multiplexation capabilities of potentiometric detection can hardly compete with the highly parallel manner in which optical assays are performed in the laboratory, they can easily have a niche for point-of-care diagnostic applications. Owing to simple methodology and low cost, potentiometric immunoassays seem to offer a feasible alternative to develop in vitro diagnostic platforms.
6.2. Development of solid-contact ion-selective electrodes

As described in the previous chapter, for bioassay applications electrodes with high selectivity and low detection limit are required. Obtaining good long-term potential stability and reproducible electrode-to-electrode $E^0$ standard potentials are also an important premise for their use in diagnostic platforms. In this field solid-contact ion-selective electrodes offer advantages in terms of inexpensive, maintenance-free construction, miniaturization and compatibility with microfabrication techniques. In this part of the thesis two possible alternatives will be discussed to achieve ultratrace detection limit, exceptional selectivities and good device-to-device reproducibility, respectively.

6.2.1. Silicon rubber-based solid-contact ISEs

The aim of this work was to prepare solid-contact silver-selective electrodes with low detection limit. As discussed before, the elimination of the inner filling solution can avoid leaching of the primary ion and hence help to lower the detection limit. Possible leakage, however, from the ISM itself still remains. In this respect, to lower the LOD, it is especially important to use membranes characterized by low ion diffusion rates. Low ion diffusion, furthermore, also provide stable potentials at low primary ion concentrations. In this work, for the first time, solid-contact electrodes with silicone rubber-based membranes were studied. This was the first attempt to assess the feasibility of using SR membranes for constructing low LOD ion-selective electrodes. Moreover, silver ionophores have neither been tested in SR matrixes before, although Ag$^+$ is among the most studied ions with PVC-based ISEs for ultratrace analysis. Therefore, a solid comparison base is available for conventional membrane matrices.

As the solid contact of the AgSCISEs a polyaniline nanoparticle dispersion, PANI D1003, was utilized. Apart from conventional PANI materials, the electrically conducting emeraldine salt form of this PANI solution was previously found\cite{129} to have excellent pH stability at pH $\leq 12$. This PANI dispersion allows the easy preparation of SCISEs exhibiting reproducible standard potentials, good potential stability, and no light sensitivity\cite{129}. PANI was drop casted on the electrode surface and allowed to dry overnight before the outer SR-based silver selective membrane was applied on the top of it. To minimize the dissolution of the nanoparticles in the excess of THF, the membrane cocktail was prepared with a rather high viscosity, dry weight 31
wt%. However, it was observed that the PANI layer was slightly dissolved in the outer SR membrane during the drop casting. The possible post-diffusion of the nanoparticles after the curing process was studied by contacting a blank SR membrane with another containing 2 wt% PANI. During 24 h, no changes were observed over the cross-section of the interface between the two membranes, measured by spectroelectrochemical microscopy. This indicates that PANI won’t diffuse into the membrane. Although the minor intermixing of the SC and SR membrane during drop casting is beneficial in obtaining good mechanical strength and adhesion of the SC/SR interface, it affects the potential response of the electrodes. To determine this effect, the analytical performance of the SCISEs were side-by-side assessed with those of CWEs.

As membrane matrix room temperature vulcanising silicon rubber, RTV 3140 was used. Without adding plasticizer to the membrane the ionophore dissolved only partially in it, resulting in poor analytical performance. The solubility of the ionophore was therefore facilitated by adding 5-15 % DOS. The plasticizer content had a strong effect on the electrodes as well.

Table 4 Unbiased selectivity coefficients ($log K_{Ag,j}^{Pot}$) of the SR and plasticized PVC-based CWEs and SCISEs, measured by the separate solution method at 1 mM level. *Typical standard deviations for the selectivities were ≤0.6 and 1.4 units for the CWEs and SCISEs, respectively. **The Ag*-selective SR-based membrane cocktail (25 µl) and the PANI nanoparticle dispersion (3 µl) were mixed before being applied in one step on the electrode surface.

<table>
<thead>
<tr>
<th>J</th>
<th>10% DOS</th>
<th>5% DOS</th>
<th>10% DOS</th>
<th>5% DOS</th>
<th>10% DOS</th>
<th>56% DOS</th>
<th>56% oNPOE</th>
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<td>-8.8</td>
<td>-9.7</td>
<td>-8.2</td>
<td>-11.1</td>
</tr>
</tbody>
</table>

The values were taken from reference[19] for the same ionophore, measured with liquid contact electrodes with PVC membrane plasticized with oNPOE. The values were taken from reference[140] for the same ionophore, measured with solid-contact electrodes with MMA-DMA membrane and POT solid-contact.

First, the unbiased selectivity coefficients were determined for the various AgCWEs and AgSCISEs, as summarized in Table 4. Although previous studies for PVC[19] and MMA-DMA-based[140] membranes using the same silver
ionophore (last two columns in Table 4) reported on excellent selectivities, mostly around $10^{-10}$, the SR-based electrodes, both CWEs and SCISEs, showed significantly better results approaching $10^{-16}$ for divalent and $10^{-14}$ for monovalent ions. These results are outstanding, exceeding any selectivities reported before. Interestingly, the CWEs had somewhat better selectivities than PANI-based SCISEs with the same DOS content in the membrane. This suggests that the nanoparticles slightly intermixed with the outer membrane affect the selectivities. To better understand this effect electrodes with premixed SR cocktail and PANI dispersion, using the same amounts as for the two step drop casting (i.e. 3 µl PANI + 25 µl cocktail), deposited as a single composite layer were prepared. The selectivity coefficients worsened by ca. 3 orders of magnitude. While the slight intermixing during the drop casting cannot be avoided, further mixing of the two layers doesn’t happen, as discussed earlier.

Figure 30 Gradual disappearance of the super-Nernstian response of unconditioned AgISEs with (A) 5 µl and (B) 20 µl of membrane cocktail cast on the electrode surface. The membrane thicknesses were ~50 µm and ~200 µm, respectively. The time instances demark the start of the calibration curve from low to high concentrations.

When unconditioned electrodes (previously not exposed to Ag$^+$) are calibrated from low to high concentration, they exhibit a super-Nernstian potential jump of several hundred mV between $10^{-7}$ and $10^{-5}$ M AgNO$_3$ (Figure 30). This is the so-called Hulanicki effect$^{[12][265]}$, caused by the high primary ion uptake of the membrane. The virtually zero Ag$^+$ surface concentration can increase only when the bulk concentration becomes high enough to compensate and exceed the rate of silver ion uptake. The relatively high concentration at which the super-Nernstian jump occurs suggest that the ion mobility in the membrane is higher than expected. The ion diffusion coefficient in 10 wt% DOS-containing and DOS-free membranes was determined by using the chronopotentiometric
method\textsuperscript{[266]}. The diffusion coefficient was found to be ca. 3 orders of magnitude lower in the DOS-free membranes, i.e. $6.0 \times 10^{-12}$ cm$^2$/s, than in those containing 10\% DOS, i.e. $6.3 \times 10^{-9}$ cm$^2$/s. It means, that while the diffusion coefficient in the plasticized SR is hardly lower than that in plasticized PVC ($\sim 10^{-8}$ cm$^2$/s\textsuperscript{[267]}), the diffusion coefficient in pure SR matches that of PA membranes ($10^{-11} - 10^{-12}$ cm$^2$/s\textsuperscript{[60]}). The gradual disappearance of the super-Nernstian response is highly dependent on the membrane thickness (50 µm thick membrane Figure 30A, 200 µm thick Figure 30B), but for conventional thicknesses (< 250 µm) it establishes within 1.5 days. Therefore the proper conditioning of the membrane is essential.

The conditioned SR-based electrodes were calibrated in $10^{-10} - 10^{-4}$ M AgNO$_3$ solutions under continuous stirring (Figure 31). Both the CWEs and SCISEs provided close to Nernstian slopes (Table 5). The detection limit of both the CWEs and SCISEs was $2 \times 10^{-8}$ M. The relatively high LOD is somewhat unsatisfactory when considering the extraordinary selectivities predicting a static LOD around 1 fM. The higher diffusion coefficient of the DOS-containing SR membrane adversely affect the LOD, but still not enough reason for the almost 4 orders of magnitude difference. A possible explanation could be that the strong silver complexation could cause partial decomposition of the active membrane components leading to the decrease of the silver selectivity. Unfortunately this assumption cannot be tested. However, it is important to note that, as visible in Table 5, the SR-based AgSCISEs and AgCWEs were superior in every aspect to PVC formulated membranes.

Table 5 Slopes, linear ranges and detection limits of the potentiometric calibration curves of SR and plasticized PVC-based AgCWEs and AgISEs.

<table>
<thead>
<tr>
<th>membrane</th>
<th>electrode type</th>
<th>slope [mV/decade]</th>
<th>concentration range [M]</th>
<th>LOD [M]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC</td>
<td>CWE</td>
<td>55.6</td>
<td>$10^{-4}$ to $10^{-6}$</td>
<td>$5 \times 10^{-6}$</td>
</tr>
<tr>
<td></td>
<td>SCISE</td>
<td>46.8</td>
<td>$10^{-4}$ to $10^{-6}$</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>SR</td>
<td>CWE</td>
<td>54.5</td>
<td>$10^{-4}$ to $10^{-7}$</td>
<td>$2 \times 10^{-8}$</td>
</tr>
<tr>
<td></td>
<td>SCISE</td>
<td>54.7</td>
<td>$10^{-4}$ to $10^{-7}$</td>
<td>$2 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

Figure 31 A and B shows the potential traces and the corresponding calibration curves of two identically prepared SCISEs. Most remarkably, the solid-contact electrodes showed excellent potential reproducibility between the electrodes. Similarly good reproducibility of the $E^0$ values was observed for uncoated PANI layers, exhibiting a standard deviation of 3.8 mV. The potential traces of
the SCISE calibration curve (Figure 31B) showed, that the potential in AgNO₃ solutions stabilize within a minute to ≤ 1 mV at concentrations ≥ 10⁻⁷ M, however the response time and the noise becomes considerably larger at lower concentrations. As shown in Figure 31C, another important benefit of PANI nanoparticle-based SC is obviously the reduced potential noise. Coated wire electrodes had much noisier potential, with standard deviation up to 8.4 mV, due to their higher membrane resistance (see below, and Figure 32). The almost noiseless potentials of the SCISEs at higher concentrations show the efficiency of the PANI-based SC as ion-to-electron transducer between the electrically conducting electrode substrate and the ionically conducting SR membrane. The slight intermixing of PANI layer and the upper SR membrane considerably lowers the membrane resistance (see below) and hence is beneficial for lowering the noise of the SCISEs.

**Figure 31** (A) Calibration curves of two identically prepared AgSCISEs conditioned in 1 nM AgNO₃. The corresponding potential traces of (B) these two AgSCISEs and (C) one of the SCISEs and a CWE.
The water uptake of the low temperature vulcanising silicone rubber-based membranes was found by FTIR-ATR spectroscopy to be much lower than that of plasticized PVC and PA membranes\cite{61}. In case of the SR-based AgSCISEs the potentiometric aqueous layer test cannot be implemented to prove that. The extraordinary selectivities of the ionophore means in practice that the primary ion cannot be exchanged in the membrane by an interfering ion to the extent to generate significant transmembrane ion-flux. It makes the reconditioning of the membrane with interfering ions practically impossible, and thus prohibit the use of the potentiometric aqueous layer test to investigate the formation of an aqueous layer beneath the membrane. On the other hand while the aqueous layer test takes ca. 12 h for a conventional plasticized PVC membrane (diffusion coefficient $\sim 10^{-8}$ cm$^2$/s\cite{267}), in case of the SR membrane containing 10 % DOS it would take a few days owing the higher diffusion coefficient ($6 \times 10^{-9}$ cm$^2$/s), and years for the DOS-free silicon rubber membrane ($6 \times 10^{-12}$ cm$^2$/s). However, for the very same reason the silver-selective membrane is not expected to be affected significantly by drifts originating from the corroborated effect of a contingent aqueous layer beneath the ISM and transmembrane ion fluxes.

The influence of dissolved O$_2$ on the potential response was tested by purging N$_2$ gas through a stirred 1 mM AgNO$_3$ solution for 30 min. The potential of all electrodes, including the CWEs, were stable. By varying the ratio of Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ at a total concentration of 2 mM in 1 mM AgNO$_3$ no redox sensitivity were observed either. In good accordance with earlier studies\cite{129},
the electrodes did not show light sensitivity to intense illumination of a 20 W halogen lamp. Impedance measurements showed that the DOS content of the membrane and the slight dissolution of PANI in the upper ISM both have a strong influence on the bulk resistance of the membrane (Figure 32). Uncoated PANI layer had a bulk resistance as low as ~0.15 MΩ (Figure 32A). The resistance of the DOS-free SR membrane was 700 MΩ, while 10 wt% plasticizer lowered it by more than an order of magnitude to 60 MΩ. Owing to the slight intermixing of PANI and the membrane, in case of the SCISEs these values were reduced to 1/20th and 1/3rd of the original, i.e. to 35 MΩ and 20 MΩ, for DOS-free (Figure 32B) and plasticized silicon rubber (Figure 32C), respectively. Due to the inherently much lower membrane resistance of the DOS-containing membranes, the effect is less significant for them. As it was already pointed out, the slight intermixing of the SC and SR layer during the drop casting procedure is advantageous for obtaining electrodes with low noise levels and good mechanical strength of the PANI/SR interface.
6.2.2. Ion-selective electrodes with 3D ordered solid contact

Solid contact materials with high surface area have subsequently high capacitances leading to remarkable long-term potential stabilities of the SCISEs, with potential drifts as low as 11.7-1.3 μV/s (see chapter other solid contact materials). The aim of this work was to explore the feasibility of unifying the benefits of large surface area nanostructures with the well-defined ion-to-electron transduction and well controllable and rapid fabrication that conducting polymers offer. Therefore, 3D ordered PEDOT(PSS) nanostructures were prepared using nanosphere lithography. Another important goal was to prepare electrodes with well-defined/controllable $E^0$ values to achieve good electrode-to-electrode reproducibility. For this purpose the voids created in the 3D polymer film were loaded by an equimolar ratio of the oxidized and reduced form of a redox mediator to control the phase boundary potential at the SC/ISM interface. The properties of both the nanostructured conducting polymer and that of the AgSCISEs with the redox couple infiltrated 3D PEDOT(PSS) solid contacts were investigated.

Figure 33 SEM images of (A) one layer, (B) 3 layers and (C) 30 layers thick PEDOT(PSS) films after removal of PS beads (ø 746 nm) used as template

The 3D ordered PEDOT(PSS) solid contacts were fabricated by nanosphere lithography as shown in Figure 14 (page 43). In order to have an easily accessible inside pore structure the outside, i.e. the uppermost layer should be exposed, which practically means that the film growth has to be stopped when the upper template layer is about half buried in the polymer. To achieve that, the electrosynthesis of the polymer was optimized. First, a single layer of polystyrene beads were deposited on the electrode surface and PEDOT(PSS) was electropolymerized with different amounts of charge. The ideal charge to have film thicknesses on the order of one bead radius was found to be 4.2 mC/cm$^2$ (see Figure 33A). To decide whether for the polymerization of an $n$ layer multilayer the $Q = 4.2 + 8.5 \times (n - 1)$ mC/cm$^2$ assumption can be
extrapolated a layer-by-layer investigation was made in detail. Polymer films with two, three (Figure 33B) and five layer were prepared and subjected to SEM analysis. Although the structures were not defect-free, the samples were found to have a high level of order extending well over the hundred micrometer level, and the diameter of the uppermost pores were in the size of the template spheres. As the film thickness was found to be well-controllable by the amount of charge applied for electropolymerization, 10 and 30 layers (Figure 33C) of PS template were synthesized as well. The final 3D structures exhibited well-connected electrically conducting wall structures made of PEDOT(PSS) as well as open and interconnected pores in all dimensions. The channels interconnecting the voids originate from the contact surface between the template beads. For reference, compact (untemplated) PEDOT(PSS) polymer films were also prepared with the same polymerization charges. These compact layers were about 2.5 times thinner than their 3D ordered counterparts. The different film thicknesses as a function of polymerization charge are compiled in Table 6.

**Table 6** Calculated thicknesses (h) of 3D ordered and compact PEDOT(PSS) films synthesized with different polymerization charges.

<table>
<thead>
<tr>
<th>polymerization charge [mC/cm²]</th>
<th>no. of layers</th>
<th>3D h [µm]</th>
<th>compact h [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>1</td>
<td>0.37</td>
<td>0.15</td>
</tr>
<tr>
<td>12.7</td>
<td>2</td>
<td>1.12</td>
<td>0.44</td>
</tr>
<tr>
<td>21.2</td>
<td>3</td>
<td>1.87</td>
<td>0.74</td>
</tr>
<tr>
<td>38.2</td>
<td>5</td>
<td>3.36</td>
<td>1.33</td>
</tr>
<tr>
<td>80.7</td>
<td>10</td>
<td>7.09</td>
<td>2.80</td>
</tr>
<tr>
<td>250.7</td>
<td>30</td>
<td>22.01</td>
<td>8.71</td>
</tr>
</tbody>
</table>

**Investigation of the 3D PEDOT(PSS) solid contacts**

As mentioned earlier, the aim of nanostructuring the polymer film was to have solid contact layers with high bulk capacitances. The specific capacitance values of the 3D ordered solid contact films were studied by EIS measurements, at \( E_{dc} = 0.25 \) V where the PEDOT(PSS) is in the oxidized and electrically conducting form.

Typical impedance spectra of the GC/PEDOT(PSS) electrodes in 1mM KCl background electrolyte solution are shown in Figure 34. For both the compact and 3D polymer films (A and B, respectively), the impedance plots are
dominated by almost vertical capacitive lines at low frequencies, which is related to the bulk redox capacitance of PEDOT(PSS). The differences between the two types of polymer films are more visible on the Bode plot shown in Figure 34C.

Figure 34 (A-B) Impedance plots and (C) Bode plots for GC/PEDOT(PSS) electrodes with different polymerization charges: (black) 4.2, (green) 21.1, (blue) 80.7 and (red) 250.7 mC/cm² measured in 1 mM KCl. (A and C—solid lines) Electrodes with compact and (B and C—dotted lines) 3D ordered conducting polymer layer (frequency range 10 mHz to 1 kHz).

The equivalent circuit shown in Figure 35, proposed earlier for Pt/PEDOT electrodes,[268] was used to interpret the experimental data. The model was found to give excellent fits not only for the different compact film thicknesses but also for the different 3D ordered PEDOT(PSS) layers. The model is composed of the solution resistance ($R_s$), the bulk capacitance ($C_d$) and the finite-length Warburg diffusion impedance ($T$). The $T$ element is characterized by the diffusion time constant ($\tau_D$), the diffusion pseudocapacitance ($C_D$) and the diffusion resistance ($R_D = \tau_D/C_D$). Both $C_D$ and $C_d$ are related to the polymer bulk, and the total bulk redox capacitance can be calculated as the two bulk capacitances in series ($\frac{1}{C_{tot}} = \frac{1}{C_D} + \frac{1}{C_d}$). The value of $C_D$ is about an order of magnitude higher than that of $C_d$ for the same film thickness, meaning that $C_{tot}$ is mostly determined by $C_d$.

Figure 35 Equivalent electrical circuit used for fitting the EIS data measured in 1 mM KCl, where $R_s =$ solution resistance, $T =$ finite-length Warburg diffusion impedance and $C_d =$ electronic bulk capacitance of the polymer film.
At low frequencies the redox capacitance can as well be roughly estimated from the EIS data by using the following equation valid for a pure capacitor:

$$C = \frac{1}{2\pi f |Z''|}$$  \hfill (15)

The capacitances can be determined by line fitting of the EIS data plotted as $|Z''|$ vs $f^{-1}$ and by calculating the capacitances from the slope of the straight lines. Here, the frequencies of 10, 12.59 and 15.85 mHz were used for the line fittings. Results obtained by this simple method show very good agreement with the capacitance values obtained by fitting the EIS data to the equivalent circuit in Figure 35.

The capacitance of the bare GC electrode was found to be 1.73±0.4 µF. Capacitance values of the compact PEDOT(PSS) film show a very good correspondence with the results from the literature[268]. However, in contrast to the preliminary expectations, the capacitance values obtained by fitting experimental data to the equivalent circuit model or by calculations based on Eq.(15) do not differ considerably for compact and 3D ordered conducting polymer films (Figure 36).

![Figure 36](image)

**Figure 36** Capacitances as a function of polymerization charge and polymer thickness for GC/PEDOT(PSS) electrodes with (black) compact and (red) 3D ordered conducting polymer, measured in 1 mM KCl. The (filled) values obtained by fitting experimental data with the model shown in Figure 35 correspond with (open) capacitances calculated by using Eq.(15) and do not show significant difference between the two types of PEDOT(PSS) layers. The values are also in good agreement with (blue) the literature[268].

This indicates that the bulk redox capacitance of PEDOT(PSS) is determined by the amount of polymer rather than the surface of it, independent of the 3D
porosity. The magnitude of the bulk capacitance of the PEDOT(PSS) film is thus determined primarily by the concentration of charge carriers in the polymer in case of freely mobile doping ions. As the polymer mass is the same for both compact and 3D layers of the same polymerization charge the total bulk capacitance of these two types of GC/PEDOT(PSS) electrodes do not differ.

When PEDOT(PSS) is applied as solid contact in SCISEs, the polymer layer is in contact with the hydrophobic ISM and not with an aqueous phase. Therefore the behavior of PEDOT(PSS) in contact with a lipophilic salt in non-aqueous solution was further investigated. For this purpose EIS measurements were performed in 1 mM ETH 500 in acetonitrile (Figure 37). ETH500 is a lipophilic salt with bulky anion and cation (see Figure 5C on page 15) that unlike K⁺ and Cl⁻ cannot easily penetrate the bulk of PEDOT(PSS).

**Figure 37** Impedance plots for GC/PEDOT(PSS) electrodes with (A) compact and (B) 3D nanostructured polymer film of different polymerization charges: (black) 38.2, (blue) 80.7 and (red) 250.7 mC/cm², respectively, measured in acetonitrile solution of 1 mM ETH500. Frequency range = 30 mHz to 1 kHz.

**Figure 38** Equivalent electrical circuits used for fitting the EIS data of (A) the compact and (B) the 3D ordered PEDOT(PSS) film measured in acetonitrile solution of 1 mM ETH500. (R_solution - solution resistance, C - double layer capacitance of the polymer|electrolyte interface, CPE₁ - constant phase element of the polymer|electrolyte interface, R_charge - charge transfer resistance
between the electrolyte and the polymer film and CPE2 - constant phase element of the bulk polymer)

The equivalent circuit used for fitting the impedance spectra measured in 1 mM ETH500-acetonitrile is shown in Figure 38. The fit of the GC/PEDOT(PSS) electrodes with compact polymer layer contains the solution resistance ($R_s$), the double layer capacitance of the interface ($C$), charge transfer resistance ($R_{ct}$) and the constant phase element of the polymer film ($CPE_2$). A dielectric relaxation is usually modelled with a resistor and a capacitor in parallel to each other ($C/R_{ct}$). This part of the circuit is associated with the electrolyte/polymer interface. In case of the 3D ordered polymer the ideal capacitor is replaced with a CPE, and $CPE_1/R_{ct}$ stands for $C/R_{ct}$. A CPE with a phase value ($\alpha$) of 1.0 resembles an ideal capacitor, whereas a CPE with a phase value < 1 is connected with a distributed 3D interface or diffusion, CPEs with $\alpha = 0.5$ behave as Warburg impedances. In this case, the fitted phase values of 0.50-0.57 suggest that $CPE_1/R_{ct}$ element is associated with finite-length diffusion and that $R_{ct}$ represents the resistance of the electrolyte within the 3D PEDOT(PSS) structure[153].

![Figure 39](image)

**Figure 39** Capacitances as a function of polymerization charge and polymer thickness for GC/PEDOT(PSS) electrodes with (black) compact and (red) 3D nanostructured PEDOT(PSS), measured in acetonitrile solution of 1 mM ETH500. The values are obtained by fitting experimental data to the model in Figure 38.

The $CPE_2$ element corresponds to the polymer bulk. $CPE_2$ has a $Q$ value of 15.4 ± 0.6 $\mu\text{s}^{\alpha}/\Omega$ for compact, and 37.4 – 104.8 $\mu\text{s}^{\alpha}/\Omega$ for 3D polymer, and average phase values of 0.58 and 0.74, respectively (Figure 39). Note, that in contrast to the measurements in aqueous solutions, there is no capacitance
difference between the thinnest and thickest compact layers. This indicates that the bulky ions of ETH500 do not penetrate the PEDOT(PSS) and, as a consequence of this, mainly the outer surface of the polymer film contributes to the capacitance. 3D ordered polymer films show a linear increase of their capacitance ($Q$) as a function of the film thickness, with the thickest 3D films having about 7 times higher capacitance than their compact counterparts. In contact with a non-aqueous solvent and bulky counterions able to penetrate the porous 3D structure of PEDOT(PSS), which is of relevance for the SCISE systems, this shows a clear advantage for the nanostructured PEDOT(PSS) layers.

**Investigation of the AgSCISEs with nanostructured solid contact layer**

To test the analytical performance of the different AgSCISE structures, three identically prepared electrodes were made for each of the 4 following electrode types: (i) without redox couple GC/3D PEDOT(PSS)/ISM, GC/compact PEDOT(PSS)/ISM and (ii) with redox couple GC/3D PEDOT(PSS)/DMFe/ISM, GC/compactPEDOT(PSS)/DMFe/ISM. For comparison CWEs were also prepared by applying the membrane cocktail directly onto the GC electrodes: GC/ISM, GC/redox couple/ISM. Although both PVC and SR-based ion-selective membranes were tested, only the latter will be discussed into detail here. While PVC-based redox couple-free AgSCISEs had good analytical performance, DMFe extracted into the PVC membrane, strongly affecting both the selectivity coefficients and the potentiometric slopes of the respective electrodes. The performance decrease deteriorated further upon storage which correlated with the observed coloration of the membrane. In order to avoid the extraction of the redox couple into the membrane, the much lower diffusivity silicone rubber was used. With the SR-based membrane no coloration effect was observed with the DMFe loaded SCISEs even by longer time, which indicates that the extraction of dimethylferrocene into the ISM is effectively suppressed. There is, however, a minor intermixing of the redox couple and the SR membrane during the drop casting, which cannot be avoided even by using 33 wt% dry weight cocktails.Apparently, even this slight contamination of the ISM can cause some loss of selectivity when compared to previous results discussed in the previous chapter. However, this is by far not as dramatic as in case of PVC membranes and would not hinder the application of the respective SCISEs.
First, the potentiometric response of the different silver-selective electrodes were investigated in the range of $10^{-10} - 10^{-3}$ M AgNO$_3$ solutions, slopes and detection limits of the corresponding calibration curves are summarized in Table 7. The linear range of all the calibration curves was $10^{-3} - 10^{-6}$ M. It’s important to note that the analytical performance of the different electrodes was not influenced significantly either by the structure (i.e. 3D/compact) or thickness of the solid contact, nor by the presence (51.8 ± 0.8 mV / decade) or absence (48.4 ± 0.9 mV / decade) of the redox mediator. DMF loading and polymer thickness only caused a shift in the potential values (Figure 40). The relatively high LOD ($9 \times 10^{-6} - 1 \times 10^{-7}$ M) for all these electrodes is somewhat unsatisfactory, it’s an order of magnitude higher than the results in the previous chapter.

**Table 7** Slopes and detection limits of the potentiometric calibration curves of SR-based CWEs and SCISEs

<table>
<thead>
<tr>
<th>Redox couple</th>
<th>Polymerization charge [mC/cm$^2$]</th>
<th>Slope [mV/decade]</th>
<th>LOD [M]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CWE</td>
<td>compact 3D</td>
<td>compact 3D</td>
</tr>
<tr>
<td>no</td>
<td>21.2</td>
<td>48.3 49.2</td>
<td>8 $\times$ 10$^{-6}$ 1 $\times$ 10$^{-7}$</td>
</tr>
<tr>
<td></td>
<td>250.7</td>
<td>47.4 47.5</td>
<td>9 $\times$ 10$^{-6}$ 9 $\times$ 10$^{-6}$</td>
</tr>
<tr>
<td>yes</td>
<td>CWE</td>
<td>53.1</td>
<td>9 $\times$ 10$^{-6}$</td>
</tr>
<tr>
<td></td>
<td>21.2</td>
<td>51.1 51.7</td>
<td>9 $\times$ 10$^{-6}$ 9 $\times$ 10$^{-6}$</td>
</tr>
<tr>
<td></td>
<td>250.7</td>
<td>51.8 51.4</td>
<td>9 $\times$ 10$^{-6}$ 9 $\times$ 10$^{-6}$</td>
</tr>
</tbody>
</table>
Most importantly, however, the DMFe loading seems to fulfil our expectations of providing better electrode-to-electrode reproducibility of the $E^0$ values. The redox couple in the polymer structure acts as an internal reference standard, controlling the interfacial potential between the SC and the ISM. The improved reproducibility of electrodes with 3D ordered PEDOT(PSS) filled with the redox couple is shown in Figure 40. The effect was more pronounced for electrodes with thicker nanostructured solid contact layers, i.e., the standard deviation of $E^0$ for SCISEs with 3D ordered PEDOT(PSS) polymerized with 21.2 and 250.7 mC/cm$^2$ was ± 5.4 and 3.9 mV, respectively. These values are 8 times smaller than those for the same 3D ordered SCs but without DMFe loading, ± 43.4 and ± 31.4 mV, respectively; but lag behind the extraordinary $E^0$ reproducibility reported by Hu et al (0.7 mV)$^{[154]}$.

Figure 41 Impedance spectra for different electrodes: (black) AgCWE; AgSCISEs with (violet, blue) compact and (pink, red) 3D ordered PEDOT(PSS) layer, with two different polymer thicknesses, 21.2 and 250.7 mC/cm$^2$, respectively. (A) SR-based ISEs with additional redox couple loaded in the SC. The spectra presented in (B) are for PVC-based ISEs with a thin membrane obtained by drop casting only 1/3 of the typical amount of ISM cocktail, and without redox couple in the SC. The impedance spectra were measured in 1 mM AgNO$_3$ (frequency range 100 mHz to 100 kHz, $\Delta E_{ac} = 50$ mV).

To study if the compact and 3D ordered PEDOT(PSS) solid contact works different in AgSCISEs the respective electrodes were investigated by impedance spectroscopy (Figure 41). The high frequency part, i.e. the “semi-circle”, in the spectra of the SR-based electrodes (Figure 41A) is dominated by the bulk membrane resistances of the silicon rubber. Due to the high impedance of the SR the low frequency, i.e. “tail”, part of the spectra, which is related to the ion-to-electron transduction process, is not seen clearly. To better reveal the contribution of the solid contact, SCISEs with a thin layer of PVC-based
membrane obtained by drop casting only 1/3 of the typical amount of ISM cocktail were also investigated (Figure 41B). The high frequency part of the plot, dominated by the bulk resistance of the PVC matrix, was similar for all solid contacts. The low frequency part shows that both compact and 3D PEDOT(PSS) have better performance than CWE, but there is no significant difference between the two structures. 3D ordered solid contact works similar to the compact PEDOT(PSS) layer and they both improve the ion-to-electron transduction process compared to CWE.

The potential stability of the various electrodes was evaluated by chronopotentiometry[108] by applying consecutive current pulses of +1 nA and -1 nA for 60 s each while recording the potential as shown in Figure 42. The measurements were performed in 1 mM AgNO₃ solution. The slope of the E-t curves at longer times gives a direct measure of the potential stability of the electrodes and is related to the low-frequency capacitance of the solid contact ($\Delta E/\Delta t = i/c$).

![Figure 42 Chronopotentiograms (applied current: +1 nA for 60 s and -1 nA for 60s) for the SCISEs with SR-based ISM and redox couple in the intermediate layer; (black) AgCWEs, and AgSCISEs with (violet) compact and (red) 3D SC layer.]

Due to their ill-defined phase boundary potentials CWE electrodes were exhibiting large potential drifts, up to 476 µV/s, with a low-frequency capacitance of 2.1 µF (Table 8). Meanwhile, the potential drifts of SCISEs was considerably lower, especially electrodes with 3D ordered PEDOT(PSS) filled with the redox couple showed good stability. The drift of GC/3D PEDOT(PSS)/DMFe/ISM electrodes was 4.4 times smaller than their DFM-free counterparts (19.8 and 10.6 vs. 87.7, 46.9 µV/s, respectively) and 1.5 times smaller than those with DMFe but compact polymer film (19.8 and 10.6 vs.
28.7, 15.4 µV/s, respectively). The electrodes with the thickest nanostructured PEDOT(PSS) showed the smallest potential drift, 10.6 ± 2.3 µV/s (94.0 µF capacitance).

The potential jump in the chronopotentiometric E-t curves can be used to estimate the total bulk resistance (R) of the membrane (R = E/i) (Table 8). While the presence of the redox couple didn’t influence the membrane resistance of CWEs, it significantly increased that of SCISEs, this effect was more pronounced in electrodes with nanostructured PEDOT(PSS) layers.

Table 8 Potential drifts, related capacitance values and membrane resistances for various SR-based AgSCISEs and CWEs with compact and 3D ordered SC layer. The values in the table were calculated from the chronopotentiometric experiments shown in Figure 42.

<table>
<thead>
<tr>
<th>Redox couple</th>
<th>Polim. charge [mC/cm²]</th>
<th>Potential drift [µV/s]</th>
<th>Capacitance [µF]</th>
<th>Resistance [MΩ]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>compact 3D</td>
<td>compact 3D</td>
<td>compact 3D</td>
</tr>
<tr>
<td>no CWE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.2</td>
<td>476.2</td>
<td>21.2</td>
<td>2.1</td>
<td>32.3</td>
</tr>
<tr>
<td>250.7</td>
<td>17.5 87.7</td>
<td>46.9</td>
<td>11.4</td>
<td>24.0 26.8</td>
</tr>
<tr>
<td>yes CWE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.2</td>
<td>277.8</td>
<td>28.7 19.8</td>
<td>57.2 21.3</td>
<td>27.5 26.4</td>
</tr>
<tr>
<td>250.7</td>
<td>15.4 10.6</td>
<td>19.8</td>
<td>65.0 94.0</td>
<td>86.1 128.4</td>
</tr>
</tbody>
</table>

6.2.3. Conclusions

Two different methods were presented to improve the analytical performance of silver ion-selective solid-contact electrodes. The use of silicon rubber as membrane matrix not only helped to eliminate the water-layer problem but due to the low ion diffusion rate in the membrane effectively helped to lower the detection limit to reach ultratrace LOD of 2 × 10⁻⁸ M. The selectivity of the studied silver-selective SCISEs was outstanding exceeding any selectivities reported before. With an attempt to find methods to create electrodes with good device-to-device reproducibility of the E⁰ standard potentials both the PANI nanoparticle dispersion and the redox mediator loaded in the voids of the 3D ordered nanostructured PEDOT(PSS) solid-contact film proved to be beneficial.
6.3. Molecularly imprinted polymer-based protein assay

Molecularly imprinted polymers for biomacromolecule recognition should have binding sites easily accessible for these large molecules, e.g. confined to their surface (see chapter 4.2.3.1./MIPs). The aim of this work was to create surface-imprinted polymeric nanostructures (surface-imprinted polymers, SIPs) by nanosphere lithography. The idea was to apply protein-modified nanoparticles on the transducer surface and electropolymerize a layer of conducting polymer around them with thicknesses on the order of the bead radius. After removal of the particles not only a 2D arrays of periodic complementary cavities remain in the polymer but the imprints of the protein as well. These protein imprints are selective recognition sites for the target protein on the enlarged surface of the polymer film.

As proof of principle, to imprint proteins via nanosphere lithography for the first time, avidin was used as target molecule. A layer of 750 nm diameter avidin-modified polystyrene particles were deposited on the planar gold surface of a quartz crystal resonator. The protein imprint was created in two dimensionally ordered PEDOT(PSS) film, electropolymerized around the nanoparticle conjugates, after the dissolution of the beads in toluene. The rebinding of avidin into the selective recognition sites of the surface-imprinted polymer surface was measured by QCM.

To determine the effect of the surface imprinting on the binding properties of the polymer, non-specific binding was studied as well by preparing non-imprinted polymer films (NIPs). The NIP films were synthesized in the exact same way as the SIPs but unmodified (not modified with avidin) nanoparticles were used for patterning the PEDOT(PSS). A compact layer of PEDOT(PSS) was prepared as well with the same polymerization charge, but untemplated. The optimization steps were carried out on the NIP films as well.

My part of the work mainly contained the optimization of the polymer layer thickness, as well as preliminary non-specific binding studies including the optimization of the blocking process.

Similarly to the previously described nanosphere lithography work, the polystyrene nanoparticles were simply drop casted from an aqueous suspension, but this time onto the gold surface of a quartz crystal of a QCM resonator. Generating compact hexagonal monolayers of unmodified nanoparticles 750 nm in diameter on a 7.07 mm² glassy carbon electrode surface proved to be straightforward, as discussed before (see images on page 77). Approaching the same result, however, on a 3 times larger (20.5 mm²) gold
surface proved to be more challenging. (Especially later on for the protein-modified particles, the array of the avidin-modified beads was less uniform and compact than those assembled from unmodified particles on the same surface.) The goal was to create a monolayer as compact as possible on the surface. Obviously, a compact layer is desirable as it maximizes the imprinted to non-imprinted ratio and hence reduce the non-specific binding. The voids between the deposited nanoparticles were filled by potentiostatically growing a 2D film of PEDOT(PSS) from aqueous solution. As such, the electropolymerization could be performed in mild conditions compatible with the protein target, unlike most of the conventional MIP generating polymerizations carried out in aprotic organic solvents. The choice of PEDOT(PSS) was furthermore motivated by its inherently high biocompatibility. The polymerization process was easily controllable by monitoring the current. The removal of the beads was carried out by dissolving the polystyrene particles in toluene.

![Figure 43 Microscope image at the same magnification of (A) a monolayer of avidin-modified polystyrene beads (ø = 750 nm) drop casted onto the gold surface of the quartz crystal resonator and (B) partial removal of the beads from the imprinted polymer layer revealing both the beads and their imprints.](image)

To obtain maximum imprinted to non-imprinted surface ratio, based on simple geometrical calculations and assuming uniform growth of the film, a polymer thickness of 395 nm, slightly higher than the half-height of the beads (375 nm) would be ideal. The optimal experimental conditions for the polymerization were determined empirically. Films of different thicknesses were prepared by controlling the electrical charge during the polymerization and examining the patterned polymer layers with atomic force microscopy (AFM) after the dissolution of the beads, as shown in Figure 44. It must be noted, that the depth of the cavities in case of the thicker films cannot be measured directly, due to the geometry of the gradually closing cavities and the tip.
Figure 44 AFM images and relevant line scans showing the surface topography of PEDOT(PSS) films prepared by nanosphere lithography using 750 nm diameter beads. Scans (A-D) are representative of 2.2 µm × 2.2 µm areas of patterned PEDOT(PSS) films prepared using 7.5, 19, 30, and 41 mC/cm² surface charge densities, respectively.

Figure 45 Theoretical correspondence between the average polymer thickness and the applied polymerization charge based on the AFM measurements, assuming uniform growth of the film.
AFM measurements revealed preferential growth of the PEDOT(PSS) film alongside the particles, so the average polymer thicknesses (Figure 45) were calculated based on the exposed diameter of the cavity, measured on several AFM images of different parts of the corresponding layers.

At a charge density of 7.5 mC/cm² small cavities with a depth of ca. 57 nm were formed, that increased at 19 mC/cm² to 195 nm. Higher charge densities of 30 and 41 mC/cm² resulted in a gradual enfolding of the nanoparticles by the grown polymer film, as shown by the decrease in the exposed diameter of the cavity (550 and 410, respectively), and by the polymer thicknesses, 600 and 689 nm, respectively. As Figure 45 shows, even assuming uniform growth of the film the control over the polymer thickness between 250 and 500 nm is the most critical, and due to the preferential growth of the polymer around the particles it becomes more complex. To ensure a better control over the polymer growth and be less affected by preferential growth around the beads, a somewhat lower charge density, 17 mC/cm², was chosen.

The dissolution of polymer beads exposed parts of bare gold on the substrate corresponding to areas where the beads previously touched the surface, causing a significantly lower non-specific binding of avidin to the compact film than to the NIP. To reduce non-specific binding to these spots, the surface was treated with 1 mM HS-TEG prior to the QCM measurements. As Figure 46 shows, surface blocking indeed reduced the avidin binding of the NIP by 28.5±0.8 %.

![Figure 46](image)

*Figure 46* Effect of blocking the gold surface, exposed after removal of the beads, by HS-TEG. The non-specific adsorption of avidin on (red) patterned PEDOT(PSS) films blocked with HS-TEG reduces by 28 % compared to the (black) same surface but without blocking.

The hereunder measurements were done by Júlia Erdőssy (nee Bognár). The SIP film-modified 10 MHz quartz crystal chips were mounted into a flow cell.
After stabilization of the frequency, increasing concentrations of avidin were injected in the carrier buffer and the frequency change was monitored in real-time. The avidin binding of NIPs was recorded in similar conditions but in a separate experiment. Under the optimized experimental conditions, at the highest avidin concentration studied the amount of avidin bound on the imprinted surface was 1.34 µg/cm² while on the NIP surface 0.21 µg/cm² (Figure 47). This accounts for an imprinting factor of 6.5. Although this value is higher than the vast majority of imprinting factors reported, cannot reach that of the surface-imprinted microbands[192]. This is most likely due to the relatively large non-imprinted fraction of the surface as result of the non-compact avidin-modified nanoparticle layer.

![Figure 47](image)

**Figure 47** Binding isotherms of avidin to the (red) imprinted and (black) non-imprinted PEDOT(PSS) patterns, measured by Júlia Erdössy. The data points are the average of measurements performed on three different SIP- and NIP-modified quartz crystals.

**Outlook**

The interest for surface confined molecularly imprinted polymer films is growing recently. The template size-matched polymer film thickness was shown[269] to be optimal for these sensors to achieve the best surface-imprinting performance in terms of binding capacity, binding constant, imprinting factor, and binding selectivity.

Lately, the molecular imprinting approach seemed to be carried forward from bulk imprinting to nanoparticle imprinting[180], core-shell type nanocomposites seem to dominate the field. The magnetic core, either magnetic nanoparticle[269]...
or magnetic carbon nanotube is modified with the target molecule and the imprint is created in the thin porous shell on the surface. Most of protein binding MIPs, however, are based on chemical polymerization. The advantages of electropolymerization, such as its ability to fine-tune the film thickness by controlling the charge consumed during deposition, and to grow the film directly and rapidly at a precise area on the transducer surface is more rarely used.

An interesting approach is the surface imprinted hybrid nanofilms, where the natural binding receptor of the target molecule immobilized on a transducer surface is combined with molecularly imprinted thin polymer films electrosynthesized around the receptor-target conjugate. When rebinding the target protein to these hybrid MIP nanolayers, the strength of the specific receptor binding is coupled with the shape-specific selectivity of the molecular imprints, resulting in one of the highest imprinting factors reported.

6.3.1. Conclusions

Nanosphere lithography seem to offer a versatile technique to create surface-imprinted polymers. Due to the inverse nature of the method, the whole macromolecule is imprinted in the polymer film, it simply can’t be too thin for the recognition or too thick for the protein to access the binding sites. The nanosphere carrier, furthermore, offers utmost flexibility in terms of adjusting the local chemical environment of the macromolecular template. The electrochemical deposition of the conducting polymer from aqueous solution offers an easily controllable, rapid fabrication technique and mild conditions highly suitable for protein imprinting.
7. Summary, thesis points

Two different potentiometric measuring schemes were developed for protein detection, and the possibility to improve the analytical performance of ion-selective electrodes used was explored. A biorecognition element based on a two-dimensionally ordered conducting polymer layer was investigated as well.

The first potentiometric immunoassay scheme was based on measuring anions generated by the enzymatic reaction of the galactosidase label. Anion-selective minielectrodes based on a simple anion-exchanger were utilized to measure in 150 µl sample solution in microplates. The suggested procedure was shown to detect human prostate specific antigen (PSA) in real human serum samples with a sufficiently low detection limit complying with the requirements of in vitro diagnostic PSA assays.

In the second potentiometric detection scheme silver ions were generated by oxidative dissolution from the metallic silver enhancement deposited selectively on gold nanoparticle labels in a paper-based immunoassay. Solid-contact minielectrodes were used to measure silver ion directly in the wet paper matrix by placing the electrodes on the surface of the paper. The model IgE dot-blot assay was shown to have the same minimum detectable amount of analyte with the conventional optical and the proposed potentiometric detection methods. For the molecular recognition aptamer-nanoparticle conjugates were used.

Although the multiplexation capabilities of potentiometric detection can hardly compete with the highly parallel manner in which optical assays are performed in the laboratory, they can easily have a niche for point-of-care diagnostic applications. Owing to simple methodology and low cost, potentiometric immunoassays seem to offer a feasible alternative to develop in vitro diagnostic platforms.

For bioassay applications ion-selective electrodes with high selectivity and low detection limit are required. Obtaining good long-term potential stability and reproducible electrode-to-electrode $E^0$ standard potentials are also an important premise for their use in diagnostic platforms. Two possible alternatives were investigated to achieve ultratrace detection limit, exceptional selectivities and good device-to-device reproducibility, respectively.

Solid-contact silver-selective electrodes were fabricated with silicone rubber-based membrane matrix and conducting polymer film as solid-contact layer for
the first time. The silver-selective electrodes had outstanding selectivities exceeding any selectivities reported before and an ultratrace detection limit of $2 \times 10^{-8}$ M.

Three-dimensionally ordered PEDOT(PSS) conducting polymer, loaded with the lipophilic redox mediator 1,1’-dimethylferrocene (DMFe), as large surface area solid-contact in silver ion-selective electrodes was found the be beneficial in terms of $E^0$ reproducibility and long term stability of the electrodes.

By nanosphere lithography polymeric nanostructures of PEDOT(PSS) conducting polymer were synthesized, and the thickness of the layers was optimized using atomic force microscopy. This polymeric nanostructures can be utilized as a biorecognition layer, surface-imprinted polymer.

The main results of this thesis can be summarized in the following points:

1. I developed a novel potentiometric enzyme-linked immunosorbent assay (ELISA) for the detection of prostate specific antigen in human serum samples. The assay scheme was based on measuring an anionic product of the enzyme label with anion-exchanger based minielectrodes. [Paper I.]

2. I made the proof of principle for potentiometric detection of microfluidic paper-based bioaffinity assays by measuring silver ions directly in the wet paper matrix. The silver ions were generated from dissolution of the metallic silver layer deposited on the gold surface of the aptamer-gold nanoparticle conjugates used as biorecognition element in the human IgE assay. [Paper II.]

3. I prepared solid-contact silver-selective electrodes with outstanding selectivities exceeding any selectivities reported before, good $E^0$ reproducibility and ultratrace detection limit. As ion-to-electron transducer layer I used polyaniline nanoparticles and for the ion-selective membrane matrix I utilized room temperature vulcanizing silicon rubber. [Paper III.]
4. I fabricated two- and three dimensionally ordered polymeric nanostructures of PEDOT(PSS) conducting polymer by nanosphere lithography for the first time. I optimized the thickness of the 2D patterned polymer for subsequent application in surface imprinted polymers as biorecognition element and utilized the 3D ordered PEDOT(PSS) as high surface area ion-to-electron transducer in ion-selective electrodes. [Paper IV. and V.]

5. I found the use of 3D ordered PEDOT(PSS) conducting polymer, loaded with a lipophilic redox mediator as large surface area solid-contact in silver-selective electrodes beneficial in terms of $E^0$ reproducibility and long term potential stability of the electrodes. [Paper IV.]
Declaration

I, the undersigned Júlia Szűcs hereby declare, that this doctoral dissertation is my own work, and I have only used the sources that are referred to. I have clearly indicated all parts that were taken from other sources and quoted literally or in paraphrase.

Nyilatkozat

Alulírott, Szűcs Júlia kijelentem, hogy ezt a doktori értekezést magam készítettem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával megjelöltem.

Budapest, 2015.09.15.

[Signature]

Szűcs Júlia
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Publications I-V
Paper I
Potentiometric enzyme immunoassay using miniaturized anion-selective electrodes for detection

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An enzyme-linked immunosorbent assay (ELISA) for prostate specific antigen (PSA) detection in human serum was developed based on the potentiometric detection of 6,8-difluoro-4-methyllumbrellifene (DiFMU). The assays were carried out in anti-human PSA capture antibody modified microtiter plates (150 μL volume). After incubation in the PSA-containing serum samples, β-galactosidase-labeled PSA tracer antibody was added. The β-galactosidase label catalyzed the hydrolysis of 6,8-difluoro-4-methylumbelliferyl-β-D-galactopyranoside (DiFMUG) and the resulting DiFMU− anion was detected by potentiometric microelectrodes with anion-exchanger membrane. The selectivity of the anion-exchanger electrode is governed by the lipophilicity of the anions in the sample. Since DiFMU− is much more lipophilic (log P = 1.83) than any of the inorganic anions normally present in the working buffers and occurs in its anionic form at the physiological pH (pKw = 4.19), it was chosen as the species to be detected. The potentiometric ELISA-based method detects PSA in serum with a linear concentration range of 0.1–50 ng/mL. These results confirm the applicability of potentiometric detection in diagnostic PSA assays. Owing to simple methodology and low cost, potentiometric immunoassays seem to offer a feasible alternative to the development of in vitro diagnostic platforms.

Introduction

The introduction of enzyme immunoassays in the early 1970s1,2 made it possible to move immunodiagnostics from specialized radioisotope laboratories to general chemistry laboratories, to be applied also by unspecialized users and in field applications. Various detection methodologies were implemented to match the requirements of sensitivity, selectivity, and massively parallel determinations. Optical methods are clearly dominating the field; however, electrochemical detection3 has also been shown to provide benefits in terms of increased sensitivity,4,5 cost-effective mass production, and miniaturization. Very early on, extremely sensitive voltammetric techniques were shown to be the electrochemical methods of choice in enzyme immunoassays. Still, some of the inherent characteristics of voltammetric sensing such as limited selectivity, susceptibility of the electrode to fouling and, unless ultramicroelectrodes are used, strong dependence of the current signal on mass transport conditions, render difficult its use in conventional setups. While most of these problems were solved using a variety of innovative approaches,7 the implementation of more robust detection methodologies would have its niche among immunoenzymes, especially in field applications. Ion-selective potentiometry is not affected by the above-mentioned issues and provides an even simpler measurement method with widely available instrumentation and well-established techniques for miniaturization and low-cost fabrication.8

The latter advantages are well documented by the extensive use of ion-selective sensors in commercial point-of-care and automatic blood-gas analyzers. These electrolyte analyzers are based on potentiometric detection; hence, their compatibility with potentiometric immunoassays could lead to more versatile in vitro diagnostic instruments. Therefore, the limited number of potentiometric immunoassays reported up to now is rather surprising, although research in this field had started in the 1970s, with pioneering contributions by the group of Rechnitz.9–11 Early efforts were mainly focused on using Severinghouse-type potentiometric gas sensors such as ammonia or CO2 electrodes in conjunction with urease-,12–14 asparaginase-,13 adenosine deaminase-,13,14 and chloroperoxidase-labeled15 immunoreagents. Another research direction aimed at developing enzymatic reaction schemes and generating ions detectable with the most established all-solid-state ion sensors such as iodide-16 and fluoride-selective17 electrodes. Horseradish peroxidase (HRP) used as a label of immunoreagents was shown to catalyze the oxidation of iodide to iodine18 and the rupture of C–F bonds while, more recently, alkaline phosphatase (ALP) was reported to catalyze the hydrolysis of C–P bonds,19 inducing changes in the concentration of the relevant anions, I− and F−.

The direct potentiometric detection of a participant in the immunoreaction was realized in the so-called ionophore modulation immunoassay20 and by using polycation-selective electrodes for competitive homogeneous assays.21 In the first case,20 a K+−selective ionophore was covalently linked to the steroidal cardiac drug digoxin, and the presence of digoxin antibodies in the sample was found to alter the emf of the electrode. In the

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second study, the potentiometric response of a synthetic polycation-analyte substrate is suppressed when binding to the antibody occurs. Further variations of direct potentiometric detection were proposed but the commercial unavailability of the reagents, and in some cases, the unidentified response mechanism could impose limitations to their applicability.

Recent improvements in the lower detection limits of polymer membrane electrodes allowing the measurement of sub-femtomolar amounts of analyte motivated researchers to revisit potentiometry as a detection method in immunoassays. Thus, a miniaturized Ag+-selective electrode was successfully used as a transducer for sandwich immunoassays in connection with the capture and silver enhancement of gold nanoparticle tracers. The silver ions released by the oxidative dissolution of silver were detected by an Ag+-selective electrode in close analogy to a previously proposed voltammetric assay. Following the same principle, immunoassays were built on the detection of Cd released from CdSe quantum-dot-labeled tracer antibodies. In these studies, it became apparent that to detect cations in bioassays might be rendered difficult owing to their contingent adsorption and/or complexation by biomolecules decreasing their activity. This might be one of the reasons why even in simple model systems only parts-per-million detection limits could be achieved for mouse IgG detection. Therefore, we here explore the feasibility of designing enzymatic schemes for ELISAs based on the generation and detection of organic anions. In general, ionophores for anions have much poorer selectivity than those for cations which, at first sight, may constitute a serious impediment in developing potentiometric transducers based on anion detection. However, if sufficiently lipophilic anions are generated by an enzymatic reaction, a simple anion-exchanger electrode could be used for their detection. This approach is beneficial also in terms of fabrication costs since it eliminates the need for an expensive ionophore. According to our knowledge, the only use of ion-exchanger electrodes (albeit for organic cations) as transducers has been reported by Umezawa’s group. In their elegant approach, limited exclusively to lipid antigens, tetrathyrammonium ions (TPA+) were trapped in the aqueous inner reservoir of liposomes partly constructed from the lipid antigen. Upon immune lysis by interaction with the specific antibody, the released TPA+ was detected by a cation-exchanger electrode.

It seems that till now little follow-up work was done on most of the early potentiometric immunoassay approaches, which might be due, partly, to the limited availability of commercial reagents needed for unconventional ELISA systems as well as to the poor detection limit and dynamic range of the early potentiometric ion or gas sensors. Therefore, we have focused our work on the three enzyme labels widely used in ELISA, i.e. alkaline phosphatase, horseradish peroxidase, and galactosidase, and commercially available substrates. The feasibility of the potentiometric detection and its optimization was demonstrated by determining the prostate specific antigen (PSA) from serum samples.

**Experimental**

**Reagents**

The membrane components and solvents were tridodecylmethylammonium nitrate (TDMA-NO3), 2-nitrophenyl octyl ether (α-NPOE), bis(2-ethylhexyl) sebacate (DOS), poly(vinyl chloride) (PVC), and tetrahydrofuran (THF) of Selectophore grade from Fluka (Buchs, Switzerland). SuperBlock (TBS) Blocking Buffer Dry Blend, Protein-Free (TBS) Blocking Buffer, and NeutrAvidin were obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). 6,8-Difluoro-4-methylumbelliferyl-β-D-galactopyranoside (DiFMUG), 6,8-difluoro-4-methylumbellifereone (DiFMU) from Invitrogen (Carlsbad, CA, USA), and 4-nitrophenyl-β-D-galactopyranoside (pNPG) from Sigma-Aldrich Inc. (St. Louis, MO, USA).

The capture (anti-human PSA SPRN-1) and tracer (anti-human PSA SPRN-5) antibodies (Ab) were purchased from Medix Biochemica (Kauniainen, Finland). The latter was biotinylated by the Institute of Isotopes Co., Ltd. (Budapest, Hungary), which also provided the prostate specific antigen (PSA) standards, controls, serum samples, and washing solutions. Galactosidase conjugates, β-galactosidase-biotin (GAL-biot) labeled from Escherichia coli and streptavidin-galactosidase (Str-GAL) were from Sigma and Thermo Fisher Scientific, respectively.

**Electrodes and potentiometric setup**

The typical composition of ion-selective membranes used in this study was PVC (40 wt%), plasticizer (60 wt%), and TDMA-NO3 (10 mmol/kg). After dissolving all components in THF, master membranes were cast in glass rings (23 mm). Miniaturized electrodes were fabricated using 10 μL pipette tips. To improve the adhesion of the ion-selective membrane to the inner pipette wall, the pipette tips were first cleaned by repeatedly dipping them into THF and draining out the solvent.

Membrane segments were cut out from the master membrane and dissolved in THF (1 : 13, w/w). The pipette tips were dipped into this membrane solution until the column of the liquid reached 4–5 mm. Then, the pipettes were placed in a holder and the THF was left to evaporate overnight yielding solvent polymeric membranes of ca. 300–500 μm thickness situated at the very end of the pipette tip. The pipette was filled with a solution containing 1 mM phosphate buffer (PB), 1 mM MgSO4, 10 M KCl, and 10 M DiFMU. An inner reference electrode was also prepared from a 10 M pipette tip whose narrower opening was obstructed by a porous plug made from a hydrophobic polypropylene membrane (Celgard®). After filling it with 10 mM KCl and inserting an Ag/AgCl wire, the other opening of the pipette was sealed with Parafilm®. This pipette tip was then placed into the pipette tip containing the sensing membrane and secured with Parafilm®. A small orifice was made in the side of the latter tip to avoid overpressure during the assembly, which could damage the ion-selective membrane. To complete the emf cell, a miniature reference electrode was fabricated exactly as described above for the inner reference electrode. The potentiometric response was measured with a high-input impedance (1012 Ω) 16-channel pH meter (Lawson Labs, Inc., Malvern, PA, USA). Calibrations were made using 150 μL samples confined to the wells of a microtiter plate. A special electrode holder was designed to accommodate the reference electrode and concentrically up to five ion sensors, which could be used simultaneously in a single well (Fig. 1). Since DiFMU concentrations throughout this study never exceeded 1 mM, the ionic strength of all solutions being.
substrate in the working buffer (150 µL) was applied for 30 min. After removing this solution, working buffer also containing Protein-Free blocking buffer was placed in the polystyrene microwells, which were then sealed and incubated at 4 °C overnight. The wells were rinsed with 400 µL aliquots of washing buffer three times, whereupon the surface was blocked using 400 µL of blocking buffer (i.e. either SuperBlock, Protein-Free, or BSA (20 µg/mL) solution). After drying, the modified plates were stored at 4 °C if not used immediately.

The assay started by adding 100 µL of different concentrations of human PSA (hPSA) serum solutions into the wells and incubating for 1 h. Following this step, a solution (100 µL) of biotin-conjugated tracer Ab (5 µg/mL) in the working buffer was injected and incubated for 1 h. After the formation of the sandwich immunocomplex was completed, a solution (100 µL) of streptavidin conjugated β-galactosidase (5 µg/mL) in the working buffer also containing Protein-Free blocking buffer (1%, v/v) was applied for 30 min. After removing this solution and rinsing the wells, freshly prepared 0.5 mM DiFMUG (15 µL) was added and incubated at room temperature for another 30 min. Finally, the enzymatic reaction was stopped with 2 mM of CuSO₄ (15 µL) and the released DiFMU was detected potentiometrically.

**Results and discussion**

A reaction scheme showing the formation of organic anions can be designed for each of the most commonly used enzyme labels in immunoassays, i.e. horseradish peroxidase (HRP), alkaline phosphatase (ALP), and galactosidase (GAL). However, the anions should comply with the following criteria for optimal potentiometric practice:

- the resulting organic anion should be lipophilic enough to be selectively determined by solvent polymeric anion-exchanger-based electrodes, while its solubility in aqueous solutions and that of the substrate from which it originates, should reach about millimolar concentrations;
- the product should be in anionic form, preferably singly charged, at the pH of the measurement, i.e. at the optimal pH of the enzyme catalysis. If a significant fraction of the compound is in neutral form, then, its extraction into the membrane can lead to a non-Nernstian response and a poor detection limit. For most of the organic anions, this translates into having a pKₐ smaller by at least 2 logarithmic units than the working pH;
- for practical usefulness, both the enzyme substrate and the generated organic anion should be commercially available;
- both the substrate and the resulting anion should have low toxicity and chemical stability;
- the enzyme substrate should provide a high turnover rate;
- the organic anion to be detected should preferably be formed directly in a one-step enzymatic reaction.

Typical reaction schemes of the three widely used enzyme labels that generate organic anions are shown in Fig. 2. While HRP provides limited flexibility in terms of anion-generating substrates, there is a plethora of commercially available substrates for ALP and GAL enzymes. Remarkably, the detected products generated by these two hydrolyzing enzymes are practically the same since the artificial substrates differ only in having either an inorganic phosphate group or a β-D-galactopyranoside to be cleaved. The use of ALP was, however, immediately ruled out since the substrate also is an anion and there is little difference in the lipophilicities of the substrate and the product. In addition, the substrate is in large excess with respect to the resulting product. To overcome this problem, alternatively, tris(4-nitrophenyl) phosphate or nitrophenyl phosphates with alkylated, non-ionizable phosphate esters could, in principle, be used as substrates. However, while the former compound is unstable at basic pH, the latter compound class comprehends notorious neurotoxins. Therefore, all arguments seem to advocate the use of β-galactosidase conjugates, and this choice is further enforced by the rather good solubility of the galactopyranoside conjugates in aqueous solution. β-Galactosidase is less popular than HRP and ALP for ELISA, mainly due to the much higher molecular weight (464 kDa). However, on the other hand, it has a series of attractive properties such as a high turnover number, good compatibility with an extremely large range of buffers and stabilizers, and, unlike other commonly used enzyme labels, it is not present in mammalian tissues.

One disadvantage of using GAL as a label is the severe constraint with respect to the pKₐ of the generated organic acid. Unlike ALP, which has an optimal pH range of 9–10 where most of the weak acids are ionized, GAL has its pH optimum between 7 and 8. Therefore, the enzymatically generated product should have a pKₐ of <5–6 and still be lipophilic enough to ensure good selectivity. Screening the many of commercially available artificial substrates based on the above-mentioned criteria basically results in only one galactosidase substrate, i.e. 6,8-difluoro-4-methylumbelliferyl-β-D-galactopyranoside (DiFMUG), which...
yields a hydrolysis product, 6,8-difluoro-4-methylumbelliferone, having a $pK_a$ of 4.9 and a calculated log $P$ of 1.83. However, it should be mentioned that the criterion regarding the $pK_a$ is of importance in continuous kinetic measurements only. If endpoint detection is pursued, then, a much larger number of substrates become available because the enzymatic reaction can be stopped by injecting highly concentrated NaOH. Thus, the endpoint pH of the solution is ca. 10, which allows the use of, for example, nitrophenyl derivatives of $pK_a$ around 7.

The multi-step approach used in heterogeneous immunoassays provides practically ideal conditions for the last step involved, which allows the use of simple anion-exchanger-based membranes for potentiometric detection. The only interference to be expected could arise from the inorganic anions of the buffer solution needed to ensure optimal enzyme activity by adjusting the optimal pH and Mg$^{2+}$ level as well to generate the anionic form of DiFMU. Screening the potentiometric selectivity behavior of some of the commonly encountered inorganic anions and other potential organic anions for detection by the separate solution method at 1 mM level demonstrates that DiFMU, indeed, is the best choice. Not only that the selectivity coefficient improved by roughly a half an order of magnitude with respect to the 4-methylumbelliferone anion ($MU^-$) and $p$-nitrophenolate ($PNP^-$) but also selectivities reaching or exceeding −4 logarithmic units were observed for $SO_4^{2-}$, $Cl^-$ and $OH^-$ (Table 1). Interestingly, little difference was found in the selectivities when using plasticizers of markedly diverse dielectric constants ($\alpha$-NPOE and DOS), except for $OH^-$ where the $\alpha$-NPOE-plasticized.

![Fig. 2 Schemes of enzyme-catalyzed reactions generating ionizable products.](image)

Table 1 Potentiometric selectivity coefficients ($\log K_{ij}^{pot}$) of various membranes for detecting DiFMU (for acronyms, cf. text)$^a$

<table>
<thead>
<tr>
<th>Plasticizer</th>
<th>DOS 20</th>
<th>$\alpha$-NPOE 20</th>
<th>$\alpha$-NPOE 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SO_4^{2-}$</td>
<td>−4.9</td>
<td>−5.5</td>
<td>−5.9</td>
</tr>
<tr>
<td>$OH^-$</td>
<td>−4.0</td>
<td>−5.0</td>
<td>−4.5</td>
</tr>
<tr>
<td>$Cl^-$</td>
<td>−3.6</td>
<td>−4.0</td>
<td>−3.9</td>
</tr>
<tr>
<td>$NO_3^-$</td>
<td>−1.9</td>
<td>−1.8</td>
<td>−1.8</td>
</tr>
<tr>
<td>$MU^+$</td>
<td>−0.6</td>
<td>−0.6</td>
<td>−0.5</td>
</tr>
<tr>
<td>$PNP^+$</td>
<td>−0.5</td>
<td>−0.5</td>
<td>−0.5</td>
</tr>
</tbody>
</table>

$^a$ The potentiometric selectivity coefficients were determined by the separate solution method at 1 mM level. In the case of $MU^+$ and $PNP^+$ the pH was adjusted with concentrated NaOH ($\alpha$ pH 9.0, $\beta$ pH 10.0) to ensure the dominance of the anionic form.
membranes exhibited a significantly better selectivity. A compromise between the selectivity and suitability of the buffer solution in terms of stability and optimal enzyme function resulted in the use of a 1 mM phosphate buffer containing 1 mM MgSO4 at pH 7.7. This solution provided a theoretical detection limit of 0.1 μM DiFMU, which was confirmed by calibration curves obtained in microtiter plates using sample volumes of 150 μL and a reading time of <10 min (Fig. 3).

The practically identical calibration curves (data not shown) recorded in conventional sample volumes (5 mL) and in the microtiter plates suggest that no adsorption or contamination of the samples occurs during measurements, which further enforces the choice of using anionic marker species for potentiometric detection. Since during endpoint-type immunoassays, DiFMU detections are performed after adding a proper stopping reagent, calibrations were done both in the presence of 1 mM NaOH and 2 mM CuSO4. The use of Cu2+, which is a well-known inhibitor of the GAL enzyme, proved to be more advantageous in many respects. Its counter anion is better discriminated than OH⁻, while the addition of NaOH in certain cases led to sub-Nernstian response slopes. In contrast, the addition of Ca²⁺ resulted in a linear super-Nernstian response for DiFMU, which considerably increased the sensitivity of the determination. At the present stage, the mechanism causing this super-Nernstian response is yet unknown; however, it has proved to be reproducible.

Prostate specific antigen (PSA) is a 33 kDa single-chain glycoprotein that is released at very low concentrations in the blood of healthy males, where it occurs in both the free and complexed forms. There is some controversy in the clinical literature regarding PSA screening; however, in clinical practice, a biopsy is requested in the case of elevated PSA levels (>3 ng/mL). The determination of human PSA was performed in a sandwich assay with a capture Ab having an affinity constant of 1.0 × 10¹⁰ L/mol.29 The PSA bound to the capture Ab was then potentiometrically detected by using successive incubation in biotinylated tracer Ab, streptavidin-β-galactosidase conjugate, and, finally, DiFMUG.

All optimization steps in terms of concentrations of the capture antibody, tracer antibody, and streptavidin-galactosidase conjugate were done by using potentiometric DiFMU detection. The generated anion was detected sequentially after the addition of the stopping reagent in each individual well by 2–5 electrodes placed concentrically around the reference electrode (Fig. 1A,B). Thus, the results plotted in Fig. 4 are average values from a set of electrodes and three replicate measurements in different microwells.

![Fig. 3](image-url) Calibration curves for DiFMU⁻: (a) in 1 mM PB with 1 mM MgSO4 at pH 7.7, (b) in the same buffer but with a content of 2 mM CuSO4.

![Fig. 4](image-url) Optimization of the amount of (A) capture antibody (50 ng/mL PSA, 5 μg/mL tracer antibody, 20 μg/mL Str-GAL), (B) tracer antibody (5 μg/mL capture antibody, 50 ng/mL PSA, 20 μg/mL Str-GAL), and (C) streptavidin-galactosidase conjugate (5 μg/mL capture antibody, 50 ng/mL PSA, 5 μg/mL tracer antibody) for hPSA detection in potentiometric ELISA format.
Based on the optimization experiments, the lowest concentration of the bioreagents providing maximal, or close to maximal, response (tracer Ab) was used from here on, i.e. 5 µg/mL for the assay components. The assays were also optimized in terms of reducing non-specific adsorption, and a number of blocking agents (SuperBlock and Protein-Free blocking buffers, as well as 20 µg of BSA/mL in 1 mM PB with 1 mM MgSO4, pH 7.7) were screened. The selection criteria involved both minimizing the potential response for the blank sample (0 ng of PSA/mL) and maximizing the potential range of the assay (defined as the potential difference between a sample of 50 ng of PSA/mL and the blank). While in terms of non-specific adsorption, little difference was observed among the different blockers, the potential range increased spectacularly in the order of Protein-Free Blocking Buffer, SuperBlock Blocking Buffer, and the BSA-containing working buffer. Thus, a two-fold extension of the potential range was gained by switching from Protein-Free Blocking Buffer to the solution of 20 µg of BSA/mL.

The PSA calibrations performed with a human serum background were compared to the conventional optical detection-based ELISA kits using HRP labeling (Fig. 5). In accordance with the logarithmic concentration dependence of the potential, semilog calibration curves proved to be linear in the range of 0.1–50 ng of hPSA/mL. Error propagation calculations (after fitting the experimental data with dose–response function) show that the uncertainty of the optical measurements in terms of concentration in the middle of the measuring range is 28.1% while the potentiometric assay has a relative error of 8.9%. There was no significant difference at the 95% confidence level between the results of the two methods for hPSA-spiked serum samples.

Conclusions

The PSA ELISAs described here using the potentiometric detection of an enzymatically generated anionic marker have proved to be a viable alternative to those based on optical absorbance. The procedure in the present format cannot compete in terms of analysis time with the highly parallel microtiter plate readers using optical detection. However, it is by far superior to the recently published nanoparticle-based potentiometric immunoassays and also provides a sufficiently low detection limit (<0.1 ng/mL), which complies with the requirements of in vitro diagnostic PSA assays. It must be noted that at this stage, the detection limit is determined by the selectivity of the ion sensor. Therefore, considerable improvements in the detection are expected if the selectivity of the basic sensors is further enhanced, which is a topic of current investigations.

Acknowledgements

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References

Paper II
Towards Protein Assays on Paper Platforms with Potentiometric Detection

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Abstract
Microfluidic paper-based assays represent a new approach to address the need for simple and cost-effective medical diagnostics. In an effort to couple paper-based assays with a similarly simple signal transduction, here we explored the feasibility of using potentiometric detection as a new modality to quantify affinity assays performed on a paper platform. As proof of principle a dot-blot model assay based on using IgE aptamer-gold nanoparticle conjugates was developed for the quantitative determination of IgE. The gold nanoparticle conjugates further catalyzed the deposition of silver nanoparticles that by oxidative dissolution generated silver ions. The silver ions were detected directly in the wet paper matrix by using a silver ion selective electrode. The analytical performance of potentiometric dot-blot assay matched that of reflectance-based assays, which suggests that potentiometric detection might represent a viable alternative to the conventionally used optical detection.

Keywords: Paper-based assay, Dot-blot, Ion-selective electrodes, IgE, Aptamer-nanoparticle conjugates

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1 Introduction

While most bioassay and biosensor developments focus on pushing the limits in terms of sensitivity and selectivity [1], on the practical side their application is in a great extent restricted by the cost of the assay. Therefore, recently, there is a clear trend in exploring sensing technologies that are able to address the cost, easiness of use by unskilled operators and compliance with resource limited locations without compromising significantly on the analytical performance. In this line of idea the group of Whitesides initiated the so called microfluidic paper-based assay approach [2], which uses paper as a cheap and conveniently disposable platform to perform various analytical tasks. Performing assays on a paper, by itself, is certainly not a new concept given the plethora of fairly conventional paper-based bioanalytical methods such as dot-blot assays [3], reagent test strips [4], various immunochromatographic methods [5] and in particular lateral flow assays [6]. However, the integration and multiplexation of the paper-based platforms with cheap paper microfluidics opens clearly new prospects for cost effective biodiagnostics for the third world countries and not only [7]. Very simple and cost-effective methodologies were introduced for patterning and fabrication of microfluidic channels [2,8] as well as even of 3D [9] and programmable microfluidics [10]. From a series of analytes demanding relatively simple assay methodologies such as glucose and total protein, the technology evolved to the implementation of ELISAs on paper platforms with analytical performances approaching the conventional microtiter plate format but at incomparably lower costs [11]. Certainly, to preserve the advantages of the cost-effective paper platform the detection technique should be a match in terms of easy methodology and low cost. In fact many of the paper-based assays such as lateral flow assays rely on simple visual detection, which provides a yes-or-no type response. While such qualitative responses are perfectly suitable for some tests, such as the pregnancy test, most diagnostic measurements would profit from quantitative results. The dominance of optical detection in bioaffinity methods applies also for paper-based assays despite of the recent successful implementation of various voltammetric techniques [12–13]. Although quantitative colorimetric measurements can be made in transmission mode [14] most often reflectance detection is employed where the reflected light can be conveniently measured without any special laboratory instrument such as with a flatbed scanner or a digital camera [15]. The later approach has further the advantage that mobile phone cameras can be used which enables telemedicine [16]. Still the limited dynamic range and need for reproducible lighting condition in case of digital image acquisitions may easily impose difficulties in quantitative assays. Therefore, we found worthwhile to explore alternative detection possibilities to address the limitations of optical
methods. In this respect, we propose the use of ion-selective potentiometry, which is an extremely mature and well-established technique in clinical analysis [17–18]. The widespread use of pH measurements and amenability of ion-selective electrodes (ISEs) to miniaturization [19–23] is coupled with simple and cost-effective measurement methodology approaching that of reflectance measurements. Indeed, combining affinity assays with ion-selective potentiometry received considerable interest in the last few years and the proof of principle of several potentiometric detection schemes was reported [24–26]. Recently, we showed that a potentiometric ELISA based on detecting lipophilic anions enzymatically generated with a simple anion-exchanger electrode could outperform for real samples the conventional absorbance-based optical assay [27]. While the multiplexation capabilities of potentiometric detection can hardly match the highly parallel manner in which optical assays are performed in the laboratory they can easily have a niche for point-of-care diagnostic applications.

In this paper we report on the first step towards exploring the use of potentiometric transduction for quantitative paper-based bioassays. For this purpose we have investigated probably the simplest format in which an affinity assay can be performed, i.e., dot-blot assay [28–30], by using a reaction scheme that generates silver ions [25] to be potentiometrically detected directly in the paper. Measuring the ion concentration directly in the paper makes the methodology very much different from previous potentiometric investigations performed in the solution phase, i.e., in the well of a microtiter plate [24–25,27]. In an effort to reduce also the cost of the bioagents as a model system we used human IgE as the target compound and an IgE aptamer [31] conjugated to gold nanoparticle for detection. The main goal of the present paper was to evaluate side-by-side the analytical performance of the newly proposed potentiometric and the conventionally used optical detection. To our best knowledge this is the first report on the feasibility of employing potentiometric detection for paper-based bioassay platforms.

2 Experimental

2.1 Chemicals and Reagents

Chemicals for the preparation and modification of gold nanoparticles, hydrogen tetrachloroaurate(III) (HAuCl₄), trisodium citrate, (1-mercaptoundec-11-yl)tetra(ethylene glycol) (HS-TEG) were purchased from Sigma-Aldrich. Oligonucleotides with terminal thiol functional groups and a tttt spacer [32] were custom synthesized at 200 nmol scale and HPLC purified. The following sequences were used: thiol labeled IgE aptamer (Apt: 5'-gggg cacg ttta tccg tccc tcct agtg gcgt gccc cttt tttt-C6-SH-3'); and homooligonucleotide poly T (T44; 5'-tttt tttt tttt tttt tttt tttt tttt tttt tttttt tttttt tttttttt-C6-SH-3'). The oligonucleotide stock solutions were made with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Human immunoglobulin E (IgE) was purchased from Abcam (Cambridge, UK) and mouse IgG from Sigma-Aldrich. The instant skimmed milk powder used as blocking agent was from Bedeco (Zirc, Hungary). The silver enhancement solution LI Silver was obtained from Nanoprobes (Yaphank NY, USA).

The components for ion selective membrane preparation: potassium ionophore I (valinomycin), potassium ionophore III (2-dodecyl-2-methyl-1,3-propanediyl bis[N-[5'-nitro(benzo-15-crown-5)-4'-yl][carbamate], BME 44), sodium ionophore X (4-tert-butylcalix[4]arene-tetra-acetic acid tetraethyl ester), calcium ionophore IV, sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaTFPB), 2-nitrophenyl octyl ether (oNPOE), polyvinyl chloride (PVC), and tetrahydrofuran (THF), all of Selectophore grade were obtained from Fluka (Buchs, Switzerland). The silver ionophore (CsV321) was synthesized by the Department of Organic Chemistry and Technology at Budapest University of Technology and Economics.

All other reagents such as inorganic compounds and buffer components were of the highest analytical grade from Sigma-Aldrich. Solutions were prepared with 18.2 MΩ cm resistivity deionized water (Millipore, Bedford, MA, USA).

2.2 Preparation of Aptamer-Modified Gold Nanoparticles

Gold nanoparticles (AuNPs) were prepared based on a protocol from Liu and Lu [33] reported to result in 13 nm diameter nanoparticles. Briefly, 10 mL 38.8 mM sodium citrate was added to 100 mL of 1 mM boiling HAuCl₄ solution under stirring. The mixture was allowed to reflux for another 20 min and then, apart from the original protocol, after cooling and before further modification the gold nanoparticle solution was centrifuged at 3000 rcf (relative centrifugal force) for 5 min. The nanoparticle-oligonucleotide conjugates were synthesized by injecting 8 μL of 100 μM thiol labeled IgE aptamer to 4 mL solution of the gold nanoparticle solution. After stirring at room temperature for 20 h, 200 μL of 0.5 mM HS-TEG solution was added and the reagent mixture was stirred for another 2 h. The nanoparticles were separated by centrifugation at 12000 rcf for 20 min and the clear supernatant was removed. The resulting DNA-nanoparticle conjugate was resuspended in PBS-Mg buffer (PBS (pH 7.4, 0.01 M phosphate buffer containing 2.7 mM KCl and 137 mM NaCl) with 1 mM MgCl₂). The aptamer-gold nanoparticle (Apt-AuNP) conjugate was stored at 4°C. The same recipe was used for the synthesis of AuNP modified with HS-TEG (TEG-AuNP) and T44 (T44-AuNP).

2.3 Dot-Blot Assay

Dot-blots assays were performed at room temperature on nitrocellulose paper strips having 0.2 μm pore size (Bio-
Rad, Hertfordshire, UK). Ten µL of IgE containing sample or control solution was spotted with a micropipette onto the surface of the nitrocellulose paper. For calibration purposes IgE in PBS-Mg buffer was applied at different concentrations (0–250 µg/mL). Care was taken to minimize the wetted area, i.e., to better localize the spot by applying the samples slowly, without pressure, letting the solution to move laterally by capillary action. After the strip dried the surface was blocked for 10 min by immersing it into 2.5 mL 5 wt% freshly prepared instant skimmed milk powder solution in PBS buffer. Like all incubation steps, the blocking was carried out in a glass beaker of ca. 5 mL volume placed on an orbital stirring platform operated at a constant 400 rpm. The excess of blocking agent was rinsed with PBS-Mg buffer for 2 min, whereupon 1 mL of the freshly prepared AuNP conjugate was added and allowed to incubate for 30 min. The strip was then thoroughly rinsed with deionized water and dried. The light pink to reddish blots were then scanned by using a commercial flatbed scanner at 1200 dpi resolution (HP ScanJet 3800). To amplify the signal a two component silver enhancement solution was freshly prepared by mixing equal volumes of the initiator and enhancer and applied onto the nitrocellulose paper. The silver enhancement solution is nucleated by the gold nanoparticles in the paper, resulting in the precipitation of metallic silver and formation of a dark brown to black silver deposit within the spot a freshly prepared 30 µL 1% H2O2 solution with 10−5 M AgNO3 and 10−3 M Ca(NO3)2 was applied through a hole in the upper silicon rubber sheet. The solution moved by capillary action to the blot while the silver ion activity was monitored continuously with a high-input impedance (1015 Ω) 16-channel pH meter (Lawson Labs, Inc., Malvern, PA, USA). The maximum EMF change of the potential transients was used for quantitative determinations.

3 Results and Discussions

The dot-blot assay is a fast and simple route to detect, and identify proteins without electrophoretic separation. The sample is applied onto membranes with high binding capacity for proteins (80–100 µg/cm² for the nitrocellulose membranes used in this study) and the presence of the targeted species is perceived through the application of a highly selective reagent and detection of its binding. The principle of the dot-blot assay for IgE is outlined in Figure 2. The IgE containing samples were reacted with IgE aptamer – linked gold nanoparticles that in a subsequent step catalyzed the formation of a silver deposit in the spot. The concentration of silver ions released in the paper by oxidative dissolution was determined. Thus given that the timing of the various steps is rigorously reproducible the concentration of silver ions is proportional with the IgE content of the sample.

Owing to the lack of a capture antibody as well as of the non-specific, and therefore competitive nature of the first binding step, dot-blot assays are generally less sensi-
tive than sandwich or competitive assays. However, for our purpose they offer clearly a simple and effective way to explore the feasibility of using potentiometric detection for paper-based assays. The assay format has further the advantage that all steps and reagents of the optical and potentiometric dot-blot assays are exactly the same prior to detection and thus differences in the analytical performance reflects solely the performance difference of the respective detection techniques.

However, due to little antecedents in using aptamer reagents for dot blot assays, the procedure needed to be optimized in terms of surface blocking and optical detection. The efficiency of the blocking is of utmost importance as it determines, among others, the level of the non-specific adsorption of the Apt-AuNP reagent. The 5% skimmed milk solution in PBS was chosen after thoroughly testing BSA, casein, skim milk and protein-free blocking agents, as this blocking agent gave significantly higher specific vs. non-specific adsorption than all the other reagents tested (data not shown).

A typical image of the strips after optimization of the assay conditions is shown in Figure 3. The effect of the silver enhancement reagent is obvious in terms of lowering the detection limit (row B).

In principle the time of silver deposition can be increased to further lower the detection limit. However, the time is limited by the stability of the silver enhancement solution. In the experimental conditions in which the dot-blot assay was performed after applying the reagent onto the surface we found detectable uniform silver deposition on the background after 20 minutes and deposition in random spots after 17 minutes. Therefore, to reduce non-specific deposition the silver enhancement was performed for 15 minutes in all subsequent assays. The evaluation of the blots in terms of their reflectance allows the quantitation of IgE amounts in the various spots as shown in Figure 4. The smallest detectable amount of IgE by reflectance-based dot-blot assay was 50 fmol, which is a rather good result when compared with a recent report on a fully optimized reflectance-based measurement of thrombin using aptamer-nanoparticle conjugates achieving 14 fmol [29].

Pursuing further with the potentiometric detection we studied first the quantitation of silver ions directly within
the nitrocellulose paper. The nitrocellulose paper has a significant water absorption capacity of 8.06 mg H2O/cm² (1.61 mg H2O/mg). This means that silver ions generated by dissolving the silver deposited during the silver enhancement step are released in the solution phase entrapped within the paper. To determine their concentration a measurement setup was designed in which the paper is sandwiched between a silver and a calcium selective electrode. The later was chosen to avoid contingent Cl⁻ contamination and diffusion potential related uncertainties of conventional Ag/AgCl reference electrodes. The calcium selective electrode was found to provide the highest selectivity against Ag⁺ (logK_{Ca,Ag} = -4.3) among potassium ISEs based on valinomycin (logK_{K,Ag} = -1.7) and BME-44 (logK_{K,Ag} = -1.5) as well as a sodium ionophore X based sodium ISE (logK_{Na,Ag} = -0.6), which were tested in this study. To provide a constant potential to the Ca ISE pseudo-reference a background of 10⁻⁵ M Ca(NO₃)₂ was established in all solutions during EMF measurements.

To explore the feasibility of quantitative Ag⁺ measurements in the nitrocellulose paper, calibrations were performed by using the setup described in Figure 1. Paper strips sandwiched between the Ag and Ca ISEs were wetted through capillary action by an excess of AgNO₃ solutions of different concentrations, all with a 10⁻⁵ M Ca(NO₃)₂ background. We found a significant difference between calibrations performed in stirred solution phase and in the paper using the same reference and indicator electrodes (see Figure 5).

The detection limit for solution-based calibrations was found to approach 10⁻⁸ M while for paper-based calibration was somewhat better than 10⁻⁷ M. The exact reason for this behavior is presently not clear and will form the subject of an independent work, but sluggish mass transfer [34–36] and the contingent presence of Ag⁺ complexing sites within the nitrocellulose paper are likely to affect paper-based calibrations.

The result of the potentiometric dot-blot assay for IgE is shown in Figure 6. While a linear fit seems appropriate to describe the response in the upper concentration range, for fitting the whole concentration range we used the standard 4-parameter logistic model. Despite of the higher detection limit for Ag⁺ detection in the paper than in the solution phase the lowest detectable amount of IgE was 50 fmol that matches closely the performance of reflectance-based detection.

To test the selectivity of the dot-blot assay, IgG and IgE were spotted at 100 μg/mL concentration on the paper and the binding of different AuNP linked reagents...
was investigated. The binding of the nanoparticle conjugates was assessed by potentiometric detection after silver enhancement (15 min) and dissolution of the silver deposit. Figure 7 shows that the nanoparticle linked aptamer recognizes IgE selectively as compared with IgG containing samples. On the other side AuNPs modified with either the thiol labeled tetraethyleneglycol derivative or a homooligonucleotide poly T sequence of the same length as the aptamer show significantly lower binding to IgE containing blots than the aptamer-AuNP probe.

4 Conclusions

Here we showed the proof of concept of potentiometric detection for paper-based bioassay by demonstrating that ions generated in the paper can be detected by ISEs placed on the paper surface. The potentiometric detection provides the same minimum detectable amount of IgE as reflectance-based optical measurements in the exact same conditions. However, the potentiometric detection has still important reserves to be exploited in terms of refining the assay methodology and measuring setup. In particular if the detection limit of paper-based potentiometric ion detection could be improved with ca. 2 orders of magnitude to approach that of solution phase measurements the potentiometric detection is likely to outperform optical measurements.

Acknowledgements

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References

Paper III
Silicone rubber (SR)-based solid-contact ion-selective electrodes (ISEs) have been prepared for the first time with an electrically conducting polymer as the solid-contact (SC) layer. The Ca$^{2+}$- and Ag$^{+}$-selective electrodes were based on the ionophores ETH 1001 and o-xylenebis(N,N-diisobutyl dithiocarbamate), respectively, integrated in room temperature vulcanizing silicone rubber (RTV 3140). The SC consisted of a polyaniline nanoparticle dispersion, which was found to considerably lower the impedance of the SCISEs in comparison to the SR-based coated wire electrodes (CWE). For the CaSCISEs, the bulk membrane resistance decreased from 700 M$\Omega$ (CaCWE) to 35 M$\Omega$. Both the Ca$^{2+}$- and Ag$^{+}$-selective SCISEs exhibited nanomolar detection limits with fast Nernstian responses down to 10$^{-8}$ M. The potential response of the SCISEs was not influenced by light. The selectivities of the CaSCISEs were similar and for the AgSCISE better than their plasticized PVC-based analogues. Thus, SR seems to be a viable alternative to PVC electrodes are based on eliminating, usually by an applied chemical gradient or electrical current, the primary ion leaching from the ion-selective membranes (ISMs), which by contaminating the sample solution at the ISM/solution interface worsen the attainable limit of detection (LOD). Such ion fluxes, induced by the nonideal permselectivity and selectivity of ISMs, are also greatly suppressed by simply using membranes with low ion diffusion rates. Therefore, different types of poly(acrylate) (PA) membranes with approximately 3 orders of magnitude lower ion diffusion rates ($\sim$10$^{-11}$ cm$^2$ s$^{-1}$) than that of plasticized PVC got established as probably the best candidates for preparing low detection limit ISEs.

The focus of ISE research during recent years, however, has been redirected from liquid contact ISEs toward solid-contact ion-selective measurements. The search for alternative membrane materials was therefore motivated by the need to overcome one or more of the above-mentioned shortcomings. Recently, the importance of substituting PVC membranes gained additional attention with the introduction of mass-transport-controlled ISEs and their applications for trace analysis. These electrodes are based on eliminating, usually by an applied chemical gradient or electrical current, the primary ion leaching from the ion-selective membranes (ISMs), which by contaminating the sample solution at the ISM/solution interface worsen the attainable limit of detection (LOD). Such ion fluxes, induced by the nonideal permselectivity and selectivity of ISMs, are also greatly suppressed by simply using membranes with low ion diffusion rates. Therefore, different types of poly(acrylate) (PA) membranes with approximately 3 orders of magnitude lower ion diffusion rates ($\sim$10$^{-11}$ cm$^2$ s$^{-1}$) than that of plasticized PVC got established as probably the best candidates for preparing low detection limit ISEs.

The focus of ISE research during recent years, however, has been redirected from liquid contact ISEs toward solid-
contact ion-selective electrodes (SCISEs) with electrically conducting polymers (CP) as one of the most established solid-contact (SC) materials. It has been shown that the elimination of the liquid contact, which supports the primary ion leaching from the membrane, together with proper conditioning of the ISMs leads to SCISEs with low LOD. In this respect, however, it is especially important to use membranes characterized by low ion diffusion rates, as they provide stable potentials at low primary ion concentrations. Accordingly, the detection limit of SCISEs could be significantly decreased by using PA-based membranes instead of PVC. Thus SCISEs, which in addition to the potential stability and the LOD of the respective membranes instead of PVC. Therefore, SCISEs, which in addition to the potential stability and the LOD of the membrane to the SC, these processes have adverse effects on both the potential stability and the potential LOD of the respective SCISEs.

Besides the rate of ion transport, the water uptake of ISMs is also important with respect to the attainable LOD and potential stability. It was postulated that a higher water uptake of the ISM results in a higher accumulation (concentration) of primary ions in the membrane phase, which may leach out from the ISM to contaminate the ISM/solution interface, and thus to a decreased adhesion of the membrane to the SC. These processes have adverse effects on both the potential stability and the LOD of the respective SCISEs.

Until recently, it was believed that the water uptake of PA membranes was lower than for their plasticized PVC counterparts. This is probably true on short time scales due to the lower diffusion coefficients in PA membranes, but it has been shown with FTIR-ATR measurements that the equilibrium water uptake is in fact much higher for different PA membranes than for plasticized PVC. We have recently shown by FTIR-ATR spectroscopy that the water uptake of ISMs based on low temperature vulcanizing silicone rubber (SR), commercialized as an insulating coating in the electrical industry, is much lower than that of plasticized PVC and PA membranes. In addition, SRs usually have a superior adhesion to different substrates in combination with good mechanical properties and is associated with lower nonspecific adsorption of proteins in biological samples. The use of SRs as a membrane matrix for ISEs was reported already in 1973, since then, various SR-based ISEs have been studied mainly to a minor extent and for a limited range of ions: Na⁺, K⁺, Cl⁻, H₂PO₄⁻, NH₄⁺, Ca²⁺, Mg²⁺, Cu²⁺, Cd²⁺, and Ag⁺.

EXPERIMENTAL SECTION

Chemicals. Room temperature vulcanizing silicone rubber (RTV 3140) was obtained from Dow Corning. Bis(2-ethylhexyl) sebacate (DOS), potassium tetrakis[3,5-bis(trifluoromethyl)phenoxy]borate (KTFPB), sodium tetrakis[3,5-bis(trifluoromethyl)phenoxy]borate (NaTFPB), calcium ionophore I (ETH 1001), copper(II) ionophore I (o-xylenebis[N,N-diisobutyl dithiocarbamate]); and tetrahydrofuran (THF) of Selectohore grade were received from Fluka. The polyaniline dispersion (PANI(D1003)) was obtained from Ormecon GmbH (Cookson Electronics) and had a mean particle size of 8 nm. The size of 90% of the particles was <14 nm, and the conductivity in vacuum was given as 1.8 × 10⁻³ S/cm. The dispersion had a solid content of 9.7 wt % and was stored in a closed glass bottle. The average molecular weight of the PANI dispersion is not provided by the manufacturer.

Preparation of the CaCWEs and CaSCISEs. The SR-based CaCWEs, AgSCISEs, and the calcium- and silver-coated wire electrodes were prepared by drop casting. The SC layers were usually prepared by applying 3 µL of PANI on GC or Au electrodes (polyether ether ketone (PEEK) body). The SC layers were allowed to dry overnight before 25 µL of the outer SR-based Ca²⁺- or Ag⁺-selective membrane solution (dry weight: 31%) was applied on the top of the SC layer. The membrane solution was prepared with a rather high viscosity to minimize excess THF that could dissolve the PANI solid contact, which is soluble in...
Facilitate the solubilization of the silver ionophore (AgSCISEs).

The AgISEs were conditioned in stirred 1 mM AgNO₃ solution for 1 day. The solutions with concentrations >10⁻³ and 10⁻⁴ M levels in the nitrate salt solutions of the primary Ag⁺ ions (I) and interfering cations (J).

**Impedance Measurements.** All impedance spectra in this work were measured within the frequency range of 100 kHz to 10 Mhz in 1 mM CaCl₂ (CaISEs) or 1 mM AgNO₃ (AgISEs) solutions by using an Autolab PGSTAT 12 potentiostat/galvanostat equipped with a FRA2 impedance module. The excitation potential (ΔEₑ) was 100 mV unless otherwise mentioned.

**RESULTS AND DISCUSSION**

**Potentiometric Response and Selectivity of SR-Based ISEs.** In general, if the SC provides proper potential stability at the inner membrane interface, the potentiometric selectivities and calibration properties are solely determined by the ISM.³⁸ We have however observed that the PANI layer was slightly dissolved in the outer Ca²⁺-selective SR membrane during drop casting of the outer SR membrane, which is beneficial in obtaining good mechanical strength and adhesion of the SC/SR interface.³⁷ On the other hand, it is necessary to determine how this intermixing affects the potential response of the CaSCISE. Therefore, the performance of the CaSCISEs and CaCWEs were assessed side-by-side during the calibration and selectivity determinations. First, the electrodes were calibrated in 10⁻¹–10⁻¹¹ M CaCl₂ solutions under continuous stirring (Figure 1). Both the CaSCISE and the CaCWE (membrane thicknesses: ~200–250 μm) provided close to Nernstian slopes (Table 1). The potential traces of the CaSCISE showed that the potentials in CaCl₂ solutions with concentrations >10⁻⁷ M stabilize within ca. 3 min to a value ≤1 mV of the potentials measured after a contact time of 10 min. However, the response time became considerably longer at concentrations <10⁻² M. As shown in Figure 1b, the main benefit of the PANI nanoparticle-based SC is obviously the reduced potential noise.

The much noisier potential of the CaCWE associated with SR membranes, transfer pipettes, and any volumetric glassware that have been shown to adsorb or release ions was avoided.

The potential response curves were recorded in stirred solutions with a 16-channel high impedance (10¹² Ω) voltmeter (Lawson Laboratories Inc., Malvern, PA). A double junction Ag/AgCl reference electrode (RE) with 3 M KCl and 1.0 M KCl in the inner and outer compartments, respectively, was used for the Ca²⁺-measurements while for the Ag⁺-measurements, the outer compartment was filled with 1 M KNO₃. Unbiased selectivity coefficients were determined with ISMs, which had not previously been in contact with their primary ions. The separate solution method at 0.1 M level was used for the CaISEs, while for the AgISEs, the selectivity coefficients were calculated by using the individual potentials extrapolated to an activity of 1 based on a 2-point calibration at 10⁻³ and 10⁻⁴ M levels in the nitrate salt solutions of the primary Ag⁺ ions (I) and interfering cations (J).

**Po**...
Table 1. Slopes and Linear Ranges of the Potentiometric Calibration Curves of SR and Plasticized PVC-Based CWEs and SCISEs

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<tbody>
<tr>
<td>SR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCWE</td>
<td>28.3 (0.997)</td>
<td>10⁻¹ to 10⁻⁸</td>
</tr>
<tr>
<td>CaSCISE</td>
<td>30.6 (0.987)</td>
<td>10⁻¹ to 10⁻⁴</td>
</tr>
<tr>
<td>AgCWE</td>
<td>54.5 (0.998)</td>
<td>10⁻⁴ to 10⁻²</td>
</tr>
<tr>
<td>AgSCISE</td>
<td>54.7 (0.999)</td>
<td>10⁻⁴ to 10⁻²</td>
</tr>
<tr>
<td>PVC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgCWE</td>
<td>55.6 (1.000)</td>
<td>10⁻⁴ to 10⁻⁴</td>
</tr>
<tr>
<td>AgSCISE</td>
<td>46.8 (0.984)</td>
<td>10⁻⁴ to 10⁻⁹</td>
</tr>
</tbody>
</table>

a The value of $r^2$ is given in parentheses.

Also the slight dissolution of the PANI solid-contact layer into the upper SR-based CaISM (during drop casting of the SR membrane) is beneficial for lowering the noise of the CaSCISEs. The intermixing of PANI and the upper SR membrane considerably lowered the resistance of the CaSCISE in comparison to the CaCWE (see Impedance Measurements). The possible postdiffusion of PANI into the SR membrane, i.e., after the curing process, was studied by contacting a blank SR membrane (without PANI) with a SR membrane containing 2 wt % PANI. During 24 h, no changes were observed in the UV–vis spectrum of the contacted membranes, measured by spectreelectrochemical microscopy (SpECM) over a cross-section of the interface between the two membranes. The membranes had cured for 24 h before the measurements. This indicates that neither PANI nanoparticles nor oligomeric fragments will diffuse from the PANI solid contact into the outer SR matrix. The minor intermixing of the SC and the SR membrane is therefore attributed to the slight redissolution of PANI in THF during the casting of the ISM (see also Experimental Section).

The LOD of both the CaSCISE and the CaCWE was ca. 10⁻¹⁹ M. It should be stressed, that the calibrations shown in Figure 1 were done from 0.1 M down to 10⁻¹¹ M CaCl₂ with unconditioned electrodes. However, when the calibrations were done from low to high concentrations with unconditioned electrodes, they exhibited a supernernstian potential jump of ca. 220 mV between 10⁻⁸ M and 10⁻⁷ M CaCl₂ (data not shown). This so-called Hulanicki effect is due to the high uptake rate of primary ions by the ISM that ensures virtually zero concentration of Ca²⁺ in the solution layer in immediate contact with the ISM. The surface concentration increases only when the bulk concentration becomes high enough to establish mass transfer rates that exceed the rate of primary ion uptake by the membrane. The very low concentrations at which the supernernstian jump occurs suggest that the low diffusivity SR membranes will provide a much more robust potential response than their PVC-based analogues. It must be stressed that the purpose of the measurements with the CaSCISE and CaCWE shown in Figure 1 is to determine their initial response characteristics and not to suggest using the electrodes unconditioned. Since the new SCISE construction presented in this paper consists of a low diffusivity membrane in combination with a CP-based solid-contact material, which has been studied only to a minor extent, it is essential to investigate the response of the unconditioned electrodes and the time required to reach a steady-state response (Figure 2).

In order to demonstrate that potentiometric responses can be routinely and reproducible obtained at ultratrace concentration levels, three identically prepared CaSCISEs were conditioned for 24 h in 10⁻³ M CaCl₂ before the measurements. Figure 2 shows the potential traces and the corresponding calibration curves of the respective ISEs. The potentiometric slopes were 28.0 ± 0.5 mV/decade (10⁻³–10⁻⁸ M) and 26.8 ± 0.5 mV/decade (10⁻⁵–10⁻⁹ M). The potential response curves in Figure 2b show that the equilibrium is established rather fast at all Ca²⁺ concentrations and that the electrodes are responsive from 10⁻⁹ M (LOD = 2 × 10⁻⁹ M). Most remarkably, however, the CaSCISEs showed good reproducibility of the $E'$ values, which has been proved to be a major challenge for most SCISEs. Note, similarly good reproducibility of the $E'$ values was observed for uncoated PANI(D1003) membranes exhibiting a standard deviation of less than 3.8 mV within pH 0–12.

There is rather good agreement between the selectivity coefficients of CaSCISEs and CaCWEs, which indicates that the slight intermixing of the PANI nanoparticle dispersion and the ISM does not affect the selectivity of the CaSCISEs (Table 2). In fact, the selectivity coefficients of the CaSCISEs are even slightly better than for the CaCWEs. Since the measuring protocols (timing) were the same for both type of electrodes the small difference in the selectivities seems to be due to the slightly drifting potentials of the CaCWEs upon solution exchange, which in contrast to the stable potential response obtained immediately for the CaSCISEs affects the potential reading.

The values in parentheses for the CaSCISE are taken from Fluka (conventional composition PVC membranes with bis(1-}

Figure 2. Calibration curves of three identically prepared CaSCISEs conditioned in 1 nM CaCl₂ for 24 h (a) and their corresponding potential traces (b). The theoretical responses (25 °C) are denoted with the dashed lines.
Interestingly, a study on ETH 1001-based SR ISEs showed that selective electrodes motivated by the fact that besides the better proton selectivity (log $K_{\text{Ca}^+/\text{H}^+}$) for CaSCISEs prepared in the present study are slightly lower than alkali metals. The selectivity of SR-based CaCWEs and CaSCISEs prepared in the present study.

Typical standard deviations for the selectivities were ±0.2 units for the CaCWEs and CaSCISEs, and 0.6 and 1.4 for the AgCWE and AgSCISE, respectively. The Ag$^+$-selective membrane cocktail (25 µL) and the PANI nanoparticles dispersion (3 µL) were mixed before being applied in one step on the electrode surface.

Table 2. Unbiased Selectivity Coefficients of the SR and Plasticized PVC-Based CWEs and SCISEs

<table>
<thead>
<tr>
<th></th>
<th>AgCWE$^a$</th>
<th>AgSCISE$^a$</th>
<th>AgSCISE$^b$</th>
<th>PVC (I = Ag$^+$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% DOS</td>
<td>5% DOS</td>
<td>10% DOS</td>
<td>5% DOS</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>−2.5</td>
<td>−3.0 (−3.7)</td>
<td>−13.5</td>
<td>−14.9</td>
</tr>
<tr>
<td>K$^+$</td>
<td>−2.6</td>
<td>−3.0 (−3.7)</td>
<td>−13.1</td>
<td>−14.6</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>−3.6</td>
<td>−3.8 (−4.7)</td>
<td>−15.5</td>
<td>−16.6</td>
</tr>
<tr>
<td>H$^+$</td>
<td>−2.9</td>
<td>−3.1 (−2.9)</td>
<td>−12.2</td>
<td>−13.5</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>−</td>
<td>−15.1</td>
<td>−13.2</td>
<td>−12.8</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>−</td>
<td>−12.6</td>
<td>−12.4</td>
<td>−12.6</td>
</tr>
</tbody>
</table>

$^a$ Typical standard deviations for the selectivities were ±0.2 units for the CaCWEs and CaSCISEs, and 0.6 and 1.4 for the AgCWE and AgSCISE, respectively. The Ag$^+$-selective membrane cocktail (25 µL) and the PANI nanoparticle dispersion (3 µL) were mixed before being applied in one step on the electrode surface.

butylpentyl decan-1,10-diyl diglutarate as the plasticizer and liquid contact) while those for the AgSCISE are from ref 42 (oNPOE plasticised PVC membranes and liquid contact).

There is some controversy in the literature regarding the selectivity of ETH 1001-based membranes, as the selectivity coefficients depend very much on the membrane composition. For PVC membranes, the best results in terms of discrimination against alkali ions were obtained for o-nitrophenyl octyl ether (oNPOE) plasticized membranes with selectivity coefficients approaching 10$^{-6}$. However, these membranes exhibited extremely poor proton selectivity (log $K_{\text{Ca}^+/\text{H}^+}$ = −1.8). More lipophilic plasticizers, such as DOS, were found to improve the proton selectivity but in turn lowered the selectivity toward alkali metals. The selectivity of SR-based CaCWEs and CaSCISEs prepared in the present study are slightly lower than for DOS-plasticized PVC membranes, however, with a slightly better proton selectivity (log $K_{\text{Ca}^+/\text{H}^+}$ = −3.1 for CaSCISEs).

Interestingly, a study on ETH 1001-based SR ISEs showed that the electrode had close to Nernstian slopes only when either tetradodecylammonium tetrakis(p-chlorophenyl) borate (ETH 500) or DOS was added into the membrane cocktail while reasonable alkali ion selectivities required both components. The reason for the discrepancy with the present results is not completely clear; one major difference is that the authors used TpCIPB$^-$ as cation-exchanger, which was found by Malinowska et al. to lead to subnerstian responses as opposed to the more lipophilic and stable TFPB$^-$ used in the present study.

Since Ca$^{2+}$-selective electrodes exhibited significantly lower LOD than any previously reported SR-based electrodes, we were also interested to investigate the feasibility of extending the use of SR matrices to other ions. We chose to study Ag$^+$-selective electrodes motivated by the fact that besides the divalent calcium, cadmium, and lead ions, Ag$^+$ is among the most studied monovalent ions with PVC-based ISEs for ultratrace analysis. Therefore, a solid comparison base is available for conventional membrane matrices, while at the same time, silver ionophores have never been tested in SR matrices. Apparently, the best selectivities for most of the tested ions were obtained with o-xylylenedi(4-N,N-diisobutyl dithiocarbamate); ionophore originally introduced as a copper(II) ionophore. In contrast to the ETH 1001-based SR membranes, the dissolution of the silver ionophore in the ISM was facilitated by adding 5–15 wt % DOS.

Previous studies reported monovalent ion-selectivities approaching 10$^{-12}$ for Na$^+$ (Table 2; values in parentheses for the AgSCISEs), but in general, the selectivities exceeded 10$^{-6}$ for most of the studied interfering cations. Even so, the SR-based AgCWEs and AgSCISEs showed significantly better selectivities than reported either for PVC$^{45}$ or methyl methacrylate–decyl methacrylate$^{46}$-based membranes. Interestingly, the AgCWEs had somewhat better selectivities than PANI-based AgSCISEs. This suggests that the PANI nanoparticles present in the SR membrane, as a result of the slight intermixing of the PANI and the SR membrane upon drop casting of the membrane, affect the silver selectivities. This assumption was proved by determining the selectivity of AgISMs prepared by premixing the SR-based cocktail with PANI nanodispersion using the same amounts as for the AgSCISEs, but deposited as a single composite layer (Table 2). Though the selectivity coefficients worsened by ca. 3 orders of magnitude for all tested interferents, they remained in all cases better than −8.5 logarithmic units, which is still outstanding. While the slight intermixing of PANI and the outer SR membrane cannot be avoided (although it was minimized by reducing the excess of THF in the ion-selective cocktail), further mixing of the two layers after curing is very unlikely, as it has been proven earlier that nanoparticles are immobile even in highly plasticized linear PVC chain-based membranes.

Figure 3 shows the changes in the calibration curve of initially unconditioned AgSCISEs (previously not exposed to Ag$^+$) upon repeated calibrations from low to high concentrations. As in the case of the CalISEs, a pronounced supernernstian response is visible, but due to the plasticizer content (and extraordinary

Ag⁺-selectivities) this occurs at higher concentrations, i.e., as high as $10^{-5}$ M, suggesting that the ion mobility in these membranes is much higher than in those based on unplasticized SRs. This has been confirmed by using the recently introduced chronopotentiometric method for the determination of ionophore diffusion coefficients in the membrane. As there is little difference among the diffusion coefficients of different ionophores in identical membrane compositions, and due to the better solubility of the calcium ionophore ETH 1001 in the SR matrix, the ionophore diffusion coefficient was determined with this ionophore in 10 wt % DOS-containing and DOS-free SR membranes. The diffusion coefficient of ETH 1001 was found to be $6 \times 10^{-12} \pm 5 \times 10^{-12}$ cm² s⁻¹ in those containing 10 wt % DOS ($6.3 \times 10^{-10} \pm 5 \times 10^{-10}$ cm² s⁻¹). The results show unambiguously that while the diffusion coefficient of the free ionophore in the SR membranes containing DOS is similar to those found in conventional PVC membranes, the diffusion coefficient in the pure SR matrices approaches those reported for PA membranes. Figure 3 shows the gradual disappearance of the supernernstian response upon repeated calibrations which is highly dependent on the membrane thickness, but for thicknesses of less than 250 µm, the Nernstian response established within 34 h. Subjecting the electrodes to the same conditioning procedure ($10^{-3}$ M AgNO₃; see Experimental Section) as described for the Ca²⁺-selective membranes, the detection limit was $2 \times 10^{-8}$ M with excellent potential reproducibility between the different electrodes (Figure 4). The relatively high LOD is somewhat unsatisfactory when considering the extraordinary selectivities of the AgSCISEs. While certainly the higher diffusivity of the DOS-containing SR membranes adversely affects the LOD, the reason for such a high LOD is not fully clear when considering the diffusion coefficients of the active membrane components leading to the decrease of the Ag⁺-selectivity. Unfortunately, this assumption cannot be tested, as the unbiased selectivity coefficients cannot be remeasured once the AgISMs have been exposed to silver and the conventional selectivity coefficients are inherently inferior to the unbiased selectivity coefficients. It should be pointed out, however, that the SR-based AgCWEs and AgSCISEs were superior in every aspect to PVC formulated membranes (Table 1).

**Potentiometric Aqueous Layer Test.** While a good short-term stability and good potentiometric characteristics were obtained for the SR-based SCISEs, their long-term stability might be influenced by formation of an aqueous layer beneath the membrane. This possibility was investigated by using the so-called potentiometric aqueous layer test introduced by the Pretsch group. The test is based on detecting potential drifts upon changing from a primary ion solution to a highly concentrated interfering ion solution and then back to the primary ion solution. If an aqueous layer is formed beneath the membrane, the iconic

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**Figure 3.** Gradual disappearance of the supernernstian response of unconditioned Ag⁺-selective electrodes with (a) 5 µL and (b) 20 µL of membrane cocktail cast on the electrode surface. The membrane thicknesses were ~50 µm and ~200 µm, respectively. The time instances demark the start of the calibration curve from low to high concentrations.

**Figure 4.** Calibration curves of two identically prepared AgSCISEs conditioned in 1 nM AgNO₃ (a) and their corresponding potential traces (b). The theoretical responses (25 °C) are denoted with the dashed lines.

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composition of the aqueous layer will also be changed by the transmembrane ion fluxes leading to characteristic potential drifts. The extraordinary silver selectivities of the AgSCISEs and AgCWEs make a significant reconditioning of the membranes with interfering ions practically impossible and thus prohibit the use of the aqueous layer test which was therefore performed only with Ca\(^{2+}\)-electrodes. The driftless potential responses shown in Figure 5 indicate the absence of aqueous layers for both CaSCISEs and CaCWEs. This is in good accordance with recently published results of the low water uptake of a 267 \(\mu\)m thick SR-based CaCWE measured by FTIR-ATR for the same membrane composition as in this study.\(^{26}\) The diffusion coefficients of water in the SR-based CaCWE membrane (\(D_1 = 2.2 \times 10^{-7}\) cm\(^2\) s\(^{-1}\); \(D_2 = 1.3 \times 10^{-4}\) cm\(^2\) s\(^{-1}\)) and the plasticized PVC membrane (\(D_1 = 1.4 \times 10^{-7}\) cm\(^2\) s\(^{-1}\); \(D_2 = 1.2 \times 10^{-3}\) cm\(^2\) s\(^{-1}\)) were very similar but the total water uptake at equilibrium (at an infinite time) was \(\sim 3\) times lower for the SR-based CaCWE in comparison to the PVC-based counterpart\(^{4}\) (plasticized with DOS containing 0.8 wt % calcium ionophore IV (ETH 5234) and 0.45 wt % KTFPB). The \(D_1\) was related to the diffusion process of monomeric and dimeric water, and \(D_2\) to clustered bulk water.\(^{26}\)

Oxygen and Light Sensitivity. The influence of dissolved \(O_2\) on the potentiometric responses of thinner spin-coated calcium CWEs and SCISEs (membrane thickness: \(\sim 60\) \(\mu\)m) was tested by purging \(N_2\) gas through a stirred 1 mM CaCl\(_2\) solution for 30 min (data not shown). The potential of all electrodes, including the CWEs, were stable throughout the test which may indicate again the absence of a water layer beneath the SR membranes. As pointed out by Buck, the water permeating through the membrane is a major obstacle for the potential stabilizing processes at solid conductor/membrane interfaces.\(^{30}\)

No redox sensitivity of the CaSCISEs (<3.5 mV; data not shown) were observed in 1 mM CaCl\(_2\) by varying the ratio of Fe(CN)\(_6^{3-}\)/Fe(CN)\(_6^{4-}\) at a total concentration of 2 mM whereas the potential of a Pt electrode changed 346 mV in the used concentration ratio interval. As uncoated PANI films showed also a large potential change of 99 mV,\(^{37}\) the lack of redox sensitivity of the SCISE indicates that PANI does not form an interpenetrating network in the outer silicone rubber matrix, in contrast to the “single-piece electrode” concept.\(^{45}\)

Importantly, no light sensitivity of the potentiometric response was observed for the CaSCISEs (\(n = 3\)) when exposed to intensive illumination by placing a 20 W halogen lamp at a distance of ca.

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**Figure 5.** Aqueous layer test for (a) a CaSCISE and (b) a CaCWE.

**Figure 6.** Impedance spectra measured in 1 mM CaCl\(_2\): (a) CaSCISE (●) and PANI (D1003) (insert); (b) AgSCISE (●) and AgCWE (○). \(f = 100\) kHz to 10 mHz; \(\Delta E = 100\) mV [PANI (D1003): \(\Delta E = 10\) mV].

**Figure 7.** The impedance spectra measured over 4 days in 1 mM CaCl\(_2\): (a) CaSCISE (●) and CaCWE. (●) Unconditioned membrane, (○) measured at day 2, (Δ) day 3, and (x) day 4; \(f = 100\) kHz to 10 mHz; \(\Delta E = 100\) mV.

5 cm for 10 min, which is in good accordance with the observed light insensitivity of PANI(D1003).\(^{34}\) This is remarkable, as one of the major limitations of using CP as solid contacts is their marked sensitivity to light.

**Impedance Measurements.** The PANI solid-contact layer without the outer SR membrane had the lowest bulk resistance (\(R_b\)) (Figure 6a; insert), which increased to \(R_b \approx 35\) M\(\Omega\) for the CaSCISEs (Figure 6a). In comparison, the resistance of an unconditioned CaCWE is \(\sim 900\) M\(\Omega\) (Figure 7b), whereas it is \(\sim 65\) M\(\Omega\) for an unconditioned CaSCISE (Figure 7a). The resistance of both membranes stabilized within 1–2 days. After two days, the resistance of the CaSCISE was 1/20 (\(\sim 35\) M\(\Omega\)) of that of the CaCWE (\(\sim 700\) M\(\Omega\)). The reason for the lower resistance of CaSCISEs is that PANI dissolved slightly in the upper SR-based CaISM. Impedance measurements showed that the bulk resistance of an unconditioned SR membrane decreased by ca. 50% when 5 wt % of PANI was dispersed homogenously into the SR membrane (results not shown here). The mechanism by which PANI lowered the bulk resistance is unclear. However, it is possible that similar to the lipophilic additives in ISMs, the addition of positively charged PANI and its negatively charged counterions to the SR membrane may slightly increase the concentration of cationic and anionic sites in the CaISM, which increases its polarity (dielectric constant) and thus lowers the resistance of the membrane. It was previously reported that the addition of 1–10 wt % PANI to plasticized PVC membranes lowered the bulk...
However, this was not the same type of PANI that was used in this work. As was already pointed out, this is advantageous for obtaining electrodes with low noise levels and good mechanical strength of the PANI/SR interface. The resistance of the AgCWE \( (R_0 = 60 \text{ M}\Omega) \) was ca. 1/12 of the resistance of the CaCWE (Figures 6b and 7b). The reason is that DOS added to the membrane (10 wt %) lowers the resistance of the AgCWE. As in case of the CaSCISEs, the SC layer lowered the resistance of the AgSCISEs \( (R_0 = 20 \text{ M}\Omega) \) for similar reasons. However, due to the inherently much lower membrane resistance of the DOS-containing membranes, the effect is less significant than for the Ca\(^{2+}\)-selective electrodes.

**CONCLUSIONS**

This study shows that CaSCISEs and AgSCISEs with detection limits of \( 2 \times 10^{-9} \text{ M} \) and \( 2 \times 10^{-8} \text{ M} \), respectively, can be prepared with SR as the outer ISM and PANI (D1003) as the SC layer. Both types of electrodes had good potential reproducibility and selectivity, which in case of the Ag\(^+\)-selective membranes exceeds any selectivities reported for monovalent interferents.

Impedance measurements reveal that PANI lowered the resistance of the CaSCISEs \( (R_0 = 35 \text{ M}\Omega) \) and AgSCISEs \( (R_0 = 20 \text{ M}\Omega) \) compared to the CaCWEs \( (R_0 = 700 \text{ M}\Omega) \) and AgCWEs \( (R_0 = 60 \text{ M}\Omega) \), which was shown to be beneficial for preparing SCISEs with low noise levels. The reason for the decreased resistance is the slight dissolution of PANI in the SR membrane. Other major benefits of the SCISEs based on PANI and SR are their good \( E^0 \) reproducibility, light insensitivity of their potential response, and the absence of an aqueous layer beneath the SR membranes.

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Ion-selective electrodes with 3D nanostructured conducting polymer solid contact

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Abstract

Until now both ion-to-electron transducers as well as large surface area nanostructured conducting materials were successfully used as solid contacts for polymer-based ion-selective electrodes. We were interested to explore the combination of these two approaches by fabricating ordered electrically conducting polymer (ECP) nanostructures using 3D nanosphere lithography and electrosynthesis to provide a high surface area and capacitive interface for solid contact ion-selective electrodes (SC-ISEs). Furthermore we also investigated the feasibility of loading the voids created in the polymer film with lipophilic redox mediators to provide the respective ISEs with well-defined/controllable $E_0$ values. For these studies we used poly(3,4-ethylenedioxythiophene)/poly(styrenesulfonate) (PEDOT(PSS)) films with 750 nm diameter interconnected pores as the intermediate layer between a glassy carbon electrode and a Ag$^{+}$-selective polymeric membrane.

Keywords: solid contact ion-selective electrodes, conducting polymers, lipophilic redox couple, PEDOT(PSS), nanosphere lithography

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1. Introduction

While liquid contact ion-selective electrodes (ISEs) have potential stabilities hard to surpass in many routine applications, the liquid contact proved to be limiting in a number of situations. These include high-pressure conditions, e.g., deep-water measurements or high-pressure sterilization, and applications where the electrode orientation is upside down. Very importantly, liquid contacts were found limiting also in terms of mass producing miniaturized ISEs\([1]\) as well as in designing ISEs for ultra-trace analysis. In the latter case the inner filling solution acts as a reservoir of primary ions,\([2]\) and unless properly optimized\([3-5]\) may have a detrimental effect on the lower detection limit of the respective electrodes.

Therefore, solid-contact ion-selective electrodes (SC-ISE) emerged as the most advantageous ISE construction given that the materials used as solid contact fulfill the criteria formulated initially for stable inner phase boundary potential,\([6]\) i.e., (i) reversible transition from ionic conduction, in the ion selective membrane, (ISM) to electronic conduction, (ii) ideally nonpolarizable interface with high exchange current density, which is not influenced by the input current of the measuring amplifier, and (iii) SC materials with stable chemical composition. The group of Pretsch, by introducing the so-called potentiometric aqueous layer test, demonstrated that a thin aqueous layer can form beneath the polymeric ion-selective membrane leading to drifting potential response.\([7]\) Thus, the criteria for well-defined inner phase boundary potential\([8]\) has got complemented with the requirement of hindering the formation of an aqueous layer,\([9-14]\) e.g., by using hydrophobic solid-contact materials and/or ISM matrices.

After several important early approaches to replace liquid contacts,\([15-19]\) the field of SC-ISEs became dominated by electrically conducting polymers (ECPs)\([20-23]\).
that, owing to their mixed ion and electron conduction as well redox capacitance, could interface the electron-conducting substrate electrode and the ion-conducting ISM. A large number of ECPs including poly(pyrrole)[20, 22], poly(aniline) [21], poly(3-octylthiophene) (POT) [24-25] and poly(3,4-ethylenedioxythiophene doped with poly(styrenesulfonate) (PEDOT(PSS))[24] were tested as solid contact materials. In addition to their relatively good performance it was found that simply by proper conditioning lower detection limits, which are about as good as those of the correspondingly optimized liquid-contact electrodes can be achieved for a series of SC-ISEs encompassing highly selective ionophores.[26] This opened up the way to SC-ISEs with subnanomolar detection limits, facilitated also by the use of very low diffusivity membrane matrices, e.g., poly(methyl methacrylate)/poly(decyl methacrylate)[27-28] and silicone rubbers.[29]

More recently, a new line of SC-ISEs were introduced that feature instead of ion-to-electron transducers, materials with high surface areas such as three-dimensionally ordered macroporous (3DOM) carbon,[30] carbon nanotubes,[31] reduced graphene oxide[32] and colloid-imprinted mesoporous carbon (CIM)[33]. It has been shown that the large surface area of these materials results in correspondently large interfacial capacitances,[34] which in the circumstances of extremely low currents allowed by high-input impedance voltmeters prohibit the polarization of the interface. 3DOM and CIM carbon-based SC-ISEs have shown remarkable long-term potential stabilities with the lowest potential drifts reported so far, 11.7 μV/h[30] and 1.3 μV/h[31], respectively. However, in spite of these appealing characteristics the fabrication and handling of such carbonaceous materials is not as simple as drop casting of POT or localized electropolymerization as in case of most ECPs, e.g., the synthesis of CIM carbon SCs may require up to five days. The cleanliness of the carbon materials is also very critical to eliminate aqueous layer formation while redox active surface species may lead to large changes in the standard potential of the respective SC-ISEs.[35] In this work our aim was to explore the feasibility of unifying the benefits of large surface area nanostructures with the well-defined ion-to-electron transduction and rapid fabrication by electropolymerization that ECPs offer. Therefore, we prepared and investigated 3D-ordered ECP nanostructures fabricated by nanosphere lithography[36] onto the surface of glassy carbon (GC) electrodes. As model we electrosynthesised PEDOT(PSS) films with 750 nm diameter interconnected pores as the intermediate layer between a glassy carbon electrode and a Ag⁺ -selective membrane. PEDOT(PSS) films were suggested earlier to prevent the formation of a separate, undesirable water layer underneath of ISM.[37] With a few exceptions the electrode-to-electrode reproducibility of the emf response of solid contact ISEs is not particularly good. Recently it was shown that the reproducibility of E⁰ values can be significantly enhanced beside other, pre- and post-fabrication treatments based approaches,[38-39] by incorporating a hydrophobic redox couple with adjusted ratio of the oxidized and reduced forms in the membrane to adjust the inner phase boundary potential.[40]

To address the need for controlling the phase boundary potential at the interface, in the present work an equimolar ratio of the oxidized and reduced form of the redox couple 1,1’-dimethylferroocene (DMF) was used. Contrary to previous reports this lipophilic redox mediator was not incorporated into the ion-selective membrane, but rather loaded into the voids of the 3D structured polymer. It was expected that the GC/SC phase boundary potential can be adjusted in this way to provide the respective ISEs with well-defined/controlled E⁰ values.

2. Experimental section

2.1. Chemicals

Poly(sodium 4-styrenesulfonate) (NaPSS, MW ~70 kDa), 3,4-ethylenedioxythiophene (EDOT, >97%), 1,1’-dimethylferroocene were purchased from Sigma-Aldrich. Monodisperse polystyrene (PS) nanoparticle suspension with a diameter of 746±2 nm and a dry matter content of 2.6 wt. % was obtained from Polysciences Inc. Room temperature vulcanizing silicone rubber (RTV 3140) was obtained from Dow Corning. High molecular weight poly(vinyl chloride (PVC), bis(2-ethylhexyl)sebacate (DOS), sodium tetrais[bis(triﬂuoromethyl)phenyl]borate (NaTFPB), ionophore o-xylene-bis-(N,N-diisobutylidithiocarbamate), tetradoxalammonium tetrais(4-chlorophenyl)borate (ETH500) and tetrahydrofuran (THF) of Selectrophore grade were received from Fluka. All other chemicals were analytical-reagent grade. Ultrapure deionized water of 18.2 MΩ cm specific resistance was used to prepare all aqueous solutions.

2.2. Preparation of the solid contact layer

Glassy carbon disk working electrodes (0.07 cm²) with Teflon body were used as substrate for template PS bead deposition. The substrate was wet polished with alumina suspension of 1 µm and 0.05 µm particle size followed by rinsing with water and ethanol. The glassy carbon surface was then delimitated with a 470 µm thick silicone ring of 3 mm inner diameter in which 6 µl of the aqueous dispersion of polystyrene nanoparticles (2.6 wt %) was drop cast and left to dry slowly under a controlled relative humidity of 75%. The potentiostatic deposition of PEDOT(PSS) within the voids of the particle array was done at 0.86 V (reference electrode: Ag/AgCl/3M KCl/1M KCl, counter
Infiltration with an equimolar ratio of the oxidized and reduced forms of 1,1'-dimethylferrocene. The ratio of the redox couple in the underlying SC layer. The PVC-based membrane cocktail (dry weight: 33%) consisted of 0.8 wt % (15.4 mmol/kg) o-xylylenebis(N,N-diisobutyl dithiocarbamate), 0.6 wt % (7.1 mmol/kg) NaTFPB, 55.4 wt % DOS, and 43.1 wt % PVC. The silicone rubber-based ISM (dry weight: 33%) contained 88.4 wt % RTV 3140, 0.8 wt % (15.6 mmol/kg) o-xylylenebis(N,N-diisobutyl dithiocarbamate), 0.6 wt% (6.9 mmol/kg) NaTFPB, and 10.2 wt % DOS as plasticizer to facilitate the solubilization of the silver ionophore. After weighing in, the silicone rubber (SR) was dissolved away through several washing cycles in toluene and subsequently in ethanol. While the thinnest film already 1 washing cycle was sufficient (ca. 6 min) the thickest films required up to 5 cycles (60 min). The electrodes were finally rinsed with ethanol and DI water, and dried. Compact PEDOT(PSS) films were prepared the same way (same polymerization charge) but in absence of the PS nanosphere template. The morphology and electrochemical properties of the polymer films were investigated with scanning electron microscopy (SEM) (LEO, Carl Zeiss, Oberkochen, Germany) and electrochemical impedance spectroscopy (EIS), respectively.

In case of solid contacts with incorporated redox couple for E0 adjustment, the conducting polymer films were infiltrated with an equimolar ratio of the oxidized and reduced form of 1,1'-dimethylferrocene. The ratio of the two forms was used adjusted by coulometry, i.e. the reduced form was oxidized by using the theoretical amount of charge. This procedure led to a practical ratio of the two forms of 1.1 as determined by redox potential measurements. The infiltration was made by drop casting, i.e., applying 3 times 10 µl 0.1 M DMeF in acetonitrile onto the SC (1 mmol/kg relative to the ISM applied in the next step). The solutions were allowed to dry after the last drop before 10 µl of the Ag+ selective membrane cocktail was drop cast.

2.3. Preparation of the Ag+ selective membrane

The PVC-based membrane cocktail (dry weight: 33%) of 10 mM EDOT and 25 mM NaPSS as the supporting electrolyte. Prior to polymerization, the monomer solution was allowed to infiltrate the interparticle voids for 20 min to avoid mass transport limitation during the electropolymerization process. During this time the monomer solution was kept under nitrogen to protect it from oxygen. The amount of deposited polymer (thickness of the film) was controlled based on the electrical charge passed during the electropolymerization. After the electropolymerization, the PS template was dissolved away through several washing cycles in toluene and subsequently in ethanol.

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2.4. Electrode fabrication

The following 4 types of Ag+-selective solid contact ISEs (AgSCISEs) were prepared: (i) without redox couple GC/3D PEDOT(PSS)/ISM, GC/compact PEDOT(PSS)/ISM, and (ii) with redox couple GC/3D PEDOT(PSS)/redox couple/ISM, GC/compact PEDOT(PSS)/redox couple/ISM. For comparison, coated-wire electrodes (CWEs) were also prepared by applying the ISM directly onto the GC electrode: GC/ISM, GC/redox couple/ISM. Each different electrode type was prepared in triplicate.

2.5. Potentiometric measurements

The potential responses were recorded with a 16-channel high input resistance (10^12 Ω) voltmeter (Lawson Laboratories Inc., Malvern, PA) at room temperature in stirred solutions. The external reference electrode (RE) consisted of a double-junction Ag/AgCl electrode with 3 M KCl inner electrolyte and 1 M KNO3 bridge electrolyte. Unbiased selectivity coefficients were determined with ISMs, which had not previously been in contact with their primary ions. The selectivity coefficients were calculated by the Fixed Interference Method (FIM) using nitrate salt solution of the primary ion (I) and interfering cations (J).

2.6. Electrochemical impedance measurements

All electrochemical impedance spectra were measured with an Autolab PGSTAT 12 potentiostat/galvanostat equipped with FRA2 impedance module (Metrohm Autolab B.V., Utrecht, The Netherlands). Nova software was used to plot and evaluate the data. The typical frequency range for the measurements was 100 kHz - 30 mHz. The excitation potential (ΔEac) was 5 mV unless otherwise mentioned. For aqueous systems a 1 mM AgNO3 solution was used as the electrolyte with a double junction Ag/AgCl reference electrode with 3 M KCl inner electrolyte and 1 M KNO3 bridge electrolyte. For comparison, coated-wire electrodes (CWEs) were also prepared by applying the ISM directly onto the GC electrode: GC/ISM, GC/redox couple/ISM. Each different electrode type was prepared in triplicate.

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ELECTROANALYSIS

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3. Results and discussion

3.1. Morphology of 3D nanostructured conducting polymer solid contacts

The 3D nanostructured PEDOT(PSS) solid contacts were fabricated both by nanosphere lithography using 750 nm diameter polystyrene beads as template and as untemplated polymer layers. We started from the premise that in order to have a pore structure easily accessible for both the redox mediator and the ISM cocktail, as well as for the solvent to remove the template beads, the polymer film growth should be stopped before the uppermost bead layer is covered, i.e., the upper bead layer should be exposed. The optimization of the procedure was started from a single layer of PS beads deposited on the electrode surface. PEDOT(PSS) was electrosynthesized with different amounts of charge to determine the conditions needed to have film thicknesses on the order of one bead radius. This was found to be 4.2 mC/cm² under the experimental conditions used (Supporting Information, Figure S1). The following question was whether this charge value can be extrapolated to confine the growth of the polymer layer to the thickness of the deposited bead multilayers, i.e., for n layers of PS beads to use \( Q = 4.2 + 8.5 \times (n-1) \) mC/cm² for electropolymerization. This assumption was investigated in detail, i.e., layer-by-layer for the 3D-templated polymer films. Thus polymer films were prepared using two, three and five layers of beads and subjecting them to SEM analysis (Figure 1A). We found that the film growth could be well-controlled by the amount of charge applied for electropolymerization. In all cases the diameters of the uppermost pores matched well that of the template spheres (Figure 1).

![Figure 1](image)

Figure 1. SEM images of (A) 3 layers and (B) 30 layers thick PEDOT(PSS) films after removal of PS beads (Ø 746 nm) used as template.

As the thickness was found to be easily controllable by the polymerization charge, PEDOT(PSS) films templated with 10 and 30 layers of PS beads were synthesized as well (Figure 1B). While the final 3D structures were not defect-free (which is hardly to achieve over such large areas), exhibited well-connected wall structures made of electrically conducting PEDOT(PSS) as well as open and interconnected pores in all dimensions. The channels interconnecting adjacent layers of pores/voids originate in the contact surface between the template bead layers. The thickness of the templated polymer films as a function of polymerization charge and numbers of bead layers are compiled in Table 1. There is a good correlation between the number of bead layers and the thickness of the film that range between 0.4 and 22 µm. In all cases untemplated PEDOT(PSS) polymer films were also prepared for reference. These films were ca. 2.5 times thinner than their 3D structured counterparts polymerized with the same charge (0.2-8.7 µm, Table 1).

Table 1. Calculated thicknesses (h) of 3D structured and compact PEDOT(PSS) films synthesized with different polymerization charges.

<table>
<thead>
<tr>
<th>polymerization charge [mC/cm²]</th>
<th>no. of layers</th>
<th>3D h [µm]</th>
<th>compact h [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>1</td>
<td>0.37</td>
<td>0.15</td>
</tr>
<tr>
<td>12.7</td>
<td>2</td>
<td>1.12</td>
<td>0.44</td>
</tr>
<tr>
<td>21.2</td>
<td>3</td>
<td>1.87</td>
<td>0.74</td>
</tr>
<tr>
<td>38.2</td>
<td>5</td>
<td>3.36</td>
<td>1.33</td>
</tr>
<tr>
<td>80.7</td>
<td>10</td>
<td>7.09</td>
<td>2.80</td>
</tr>
<tr>
<td>250.7</td>
<td>30</td>
<td>22.01</td>
<td>8.71</td>
</tr>
</tbody>
</table>

3.2. Electrochemical investigation of 3D PEDOT(PSS) solid contacts

EIS measurements were found to be the most suitable to determine the specific capacitance values of nanostructured materials. EIS was performed at \( E_{\text{dc}} = 0.25 \) V where PEDOT is in the oxidized and electrically conducting state.

The equivalent circuit shown in Figure 2, proposed earlier for Pt/PEDOT electrodes, was used to interpret the EIS data. The equivalent circuit considers the solution resistance \( R_s \), the bulk capacitance \( C_d \) and the finite-length Warburg diffusion impedance \( \tau \). The \( \tau \) element is characterized by the diffusion time constant \( \tau_d \), the diffusion pseudocapacitance \( C_D \) and the diffusion resistance \( R_D = \tau_d / C_D \). Both \( C_D \) and \( C_d \) are related to the polymer bulk, and the total bulk redox capacitance is given by the two bulk capacitances coupled in series \( 1/C_{\text{tot}} = 1/C_D + 1/C_d \). The model was found to give excellent fits not only for the different compact film thicknesses but also for the different 3D structured PEDOT(PSS) layers.

![Figure 2](image)

Figure 2. Equivalent electrical circuit used for fitting the EIS data measured in 1 mM KCl, where \( R_s \) = solution resistance, \( T \) = finite-length Warburg diffusion impedance and \( C_d \) = electronic bulk capacitance of the polymer film.
Typical impedance spectra of the GC/PEDOT(PSS) electrodes in 1 mM KCl background electrolyte solution are shown in Figure 3. For both the compact and 3D structured polymer films, the impedance plots are dominated by almost vertical capacitive lines at low frequencies, which is related to the bulk redox capacitance of PEDOT(PSS).

The differences between the two types of polymer films are more visible on the Bode plot shown in Figure 4. The high-frequency Z-intercept ($R_s$) is significantly higher for the 3D structured PEDOT(PSS) than for the compact layer of PEDOT(PSS). This may indicate that the aqueous KCl solution does not permeate the pores of the 3D layers resulting in a larger contribution of the electronic resistance of PEDOT(PSS) to $R_s$ when PEDOT(PSS) is in the form of an open 3D structure as compared to the compact film. The value of $C_D$ is several orders of magnitude higher than that of $C_d$ for the same film thickness, meaning that $C_{tot}$ is mostly determined by $C_d$.

Interestingly, the capacitance values obtained by fitting experimental data to the equivalent circuit (Figure 2) do not differ considerably for compact and 3D structured conducting polymer films (see Table 2).

This indicates that the bulk redox capacitance of PEDOT(PSS) is determined by the amount of material (polymerization charge) independent of the 3D porosity. This shows that the reversible oxidation (p-doping) of PEDOT(PSS) is not significantly limited by counterion diffusion when using aqueous KCl as electrolyte, in good agreement with earlier results. The magnitude of the bulk capacitance of the PEDOT(PSS) film is thus determined primarily by the concentration of charge carriers in the polymer in case of freely mobile doping ions. As the polymer mass is the same for both compact and 3D layers of the same polymerization charge the total bulk capacitance of these two types of GC/PEDOT(PSS) electrodes do not differ.

At low frequencies where the EIS data show almost a vertical line the redox capacitance can also be roughly estimated from equation (1) valid for a pure capacitor:

$$C = \frac{1}{2\pi f |Z''|}$$ (1)

The capacitances can thus be determined by line fitting of the EIS data plotted as $|Z''| \text{ vs } f^{-1}$ and by calculating the capacitances from the slope of the straight lines. Here, the frequencies of 10, 12.59 and 15.85 mHz were used for the line fittings. Results obtained by this simple method show very good agreement with the capacitance values obtained by fitting of the EIS data to the equivalent circuit shown in Figure 2 (see Table 2). The capacitance of the bare GC electrode was 1.73 ±0.4 µF.
Table 2. The fitted values of the equivalent circuit elements shown in Figure 2 for GC/PEDOT(PSS) electrodes with different thicknesses of the compact and 3D nanostructured ECP layers, measured in 1 mM KCl. The \( C_{\text{tot}} \) values correspond with capacitances calculated \( (C_{\text{calculated}}) \) by using equation (1) and do not show significant difference between the two types of PEDOT(PSS) layers.

<table>
<thead>
<tr>
<th>Element</th>
<th>4.2 mC/cm(^2) compact</th>
<th>4.2 mC/cm(^2) 3D</th>
<th>21.2 mC/cm(^2) compact</th>
<th>21.2 mC/cm(^2) 3D</th>
<th>80.7 mC/cm(^2) compact</th>
<th>80.7 mC/cm(^2) 3D</th>
<th>250.7 mC/cm(^2) compact</th>
<th>250.7 mC/cm(^2) 3D</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_S/k\Omega )</td>
<td>0.12</td>
<td>0.77</td>
<td>0.11</td>
<td>0.79</td>
<td>0.13</td>
<td>1.13</td>
<td>0.32</td>
<td>1.49</td>
</tr>
<tr>
<td>( R_D/k\Omega )</td>
<td>a</td>
<td>2395.23</td>
<td>2235.82</td>
<td>903.12</td>
<td>1411.94</td>
<td>126.77</td>
<td>284.89</td>
<td>34.24</td>
</tr>
<tr>
<td>( C_D/\mu F )</td>
<td>987.80</td>
<td>459.02</td>
<td>1339.80</td>
<td>856.82</td>
<td>3232.90</td>
<td>3213.65</td>
<td>24600.40</td>
<td>22599.50</td>
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<tr>
<td>( C_{\text{literature}}/\mu F )</td>
<td>22.08</td>
<td>75.66</td>
<td>257.90</td>
<td>733.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) obtained from the T element fitting values of \( Y_0 \) and \( B \) and the relationships \( C_D = Y_0B \) and \( R_D = B/Y_0 \).

\( b \) obtained from polynomial fit on earlier results, measured in 0.1M KCl \[42\].

When PEDOT(PSS) is applied as solid contact in SC-ISEs, the PEDOT(PSS) layer is in contact with the hydrophobic ISM and not with an aqueous phase. Therefore we further investigated the behavior of PEDOT(PSS) in contact with a lipophilic salt in non-aqueous solution. For this purpose EIS measurements of the compact and 3D structured PEDOT(PSS) films were performed in 1 mM ETH 500 in acetonitrile (Figure 5). ETH500 is a lipophilic salt with bulky anion and cation that unlike \( K^+ \) (and \( Cl^- \)) cannot easily penetrate the bulk of PEDOT(PSS). We also expected that the non-aqueous solution may easily fill the template pores of the 3D structured films.

The equivalent circuit used for fitting the impedance spectra of PEDOT(PSS) in 1 mM ETH500-acetonitrile in shown in Figure 6, while the values of the fitting parameters can be found in the Supporting Information. The fit of the GC/PEDOT(PSS) electrode with compact polymer layer contains the solution resistance (\( R_s \)), the double layer capacitance of the interface (\( C \)), charge transfer resistance (\( R_{ct} \)) and the constant phase element of the polymer film (CPE\(_2\)). Electrodes with 3D structured PEDOT(PSS) contain a constant phase element (CPE\(_1\)) instead of the double layer capacitance (\( C \)) in the equivalent circuit. A CPE with a phase (\( \alpha \)) value of 1.0 resembles an ideal capacitor, whereas a CPE with a phase value < 1 is connected with a distributed 3D interface or diffusion.

![Figure 5. Impedance plot for GC/PEDOT(PSS) electrodes with (filled) compact and (open) 3D nanostructured polymer film of different polymerization charges: (■□) 38.2, (●○) 80.7 and (▼▼) 250.7 mC/cm\(^2\) respectively, measured in acetonitrile solution of 1 mM ETH500. Frequency range = 30 mHz to 1 kHz.](image)

![Figure 6. Equivalent electrical circuits used for fitting the EIS data of (A) the compact and (B) the 3D structured PEDOT(PSS) film measured in acetonitrile solution of 1mM ETH500. (R_s - solution resistance, C - double layer capacitance of the polymer|electrolyte interface, CPE\(_1\) - constant phase element of the polymer|electrolyte interface, R_{ct} - charge transfer resistance between the electrolyte and the polymer film and CPE\(_2\) - constant phase element of the bulk polymer)](image)

In this case, the fitted phase values of 0.50-0.57 suggest that CPE\(_2\)/\( R_{ct} \) element is associated with finite-length diffusion and that \( R_{ct} \) represents the resistance of the electrolyte within the 3D PEDOT(PSS) structure\[35\]. The CPE\(_2\) element corresponds to the polymer bulk. CPE\(_2\) has
a Q value of $15.4 \pm 0.6 \mu(s^0/\Omega)$ for compact, and $37.4 - 104.8 \mu(s^0/\Omega)$ for 3D structured polymer, and average phase values of 0.58 and 0.74, respectively (Figure 7). The results show that in contrast to measurements in aqueous solutions, there is no significant capacitance difference between the thinnest and thickest compact PEDOT(PSS) layers. This indicates that the bulky ions of ETH500 do not penetrate the bulk of PEDOT(PSS) and, as a consequence of this, mainly the outer surface of the polymer film contributes to the capacitance. The 3D structured polymer films show a linear increase of their capacitance (Q) as function of the film thickness with the thickest films having ca. 7 times larger capacitance than their compact counterparts. This shows a clear advantage of the 3D structured PEDOT(PSS) layer in contact with a non-aqueous solvent with bulky counterions able to penetrate the porous 3D structured structure of PEDOT(PSS), which is of relevance for the SC-ISE systems.

![Figure 7](image)

Figure 7. Capacitances as a function of polymerization charge and polymer thickness for GC/PEDOT(PSS) electrodes with (●) compact and (■) 3D nanostructured PEDOT(PSS), measured in acetonitrile solution of 1 mM ETH500. The values are obtained by fitting experimental data to the model in Figure 6.

3.3. Analytical performance characteristics of SC-ISEs

First, the potentiometric response of PVC-based ISM electrodes were investigated in the range of $10^{-10}-10^{-3}$ M AgNO₃ solutions (Figure 8). As shown in Table 4 in the absence of the redox couple in the SC, both the AgSCISEs and AgCWE provided close to Nernstian slopes (56.8 ± 0.4 mV / decade). The potential traces of both types of AgSCISEs showed that the potential in AgNO₃ solutions stabilize within a minute and the potential noise is relatively small (<2.1 mV) due to the efficiency of the PEDOT(PSS) solid contact to function as an ion-to-electron transducer between the substrate electrode and ISM. For all electrodes a similar limit of detection of ca. $10^{-6.5}$ M was found without any special conditioning. In case of both compact and 3D structured solid contacts excellent selectivities were obtained in agreement with earlier results[29] (Table 3). The DMeF loading however had a strong negative effect on both selectivity coefficients and potentiometric slopes of the respective freshly-made PVC electrodes. The selectivity coefficients decreased up to ca. seven orders of magnitudes. The response deteriorated further upon storage as determined by recalibrations and correlated with the observed coloration of the PVC-based membrane. The latter effect became more intense with time, indicating the extraction of DMeF into the membrane. The sensitivity loss was especially pronounced in the higher concentration range ($10^{-4}-10^{-3}$ M) with the potentials at this concentrations having a negative drift (Figure 8).

![Figure 8](image)

Figure 8. Calibration curves and (insert) their corresponding potential traces of GC/PEDOT(PSS)/PVC-based ISM electrodes (● and a) without and (▲ and b) with redox couple. The dashed line represents the calibration curve of the latter electrode repeated after ca. a week.
is effectively suppressed. There is however a minor dependent coloration of the SR membrane was observed, characterization. In case of DMeF loaded SCs no time-membranes were cured for 24 h before their potentiometric was drop casted from 33 wt% dry weight cocktail, and the the much lower diffusivity silicone rubber-based ISM. It potential, the plasticized PVC membrane was replaced by the membrane, not to affect the outer phase boundary In order to decrease the extraction of the redox couple into In order to decrease the extraction of the redox couple into the membrane, not to affect the outer phase boundary potential, the plasticized PVC membrane was replaced by the much lower diffusivity silicone rubber-based ISM. It was drop casted from 33 wt% dry weight cocktail, and the membranes were cured for 24 h before their potentiometric characterization. In case of DMeF loaded SCs no time-dependent coloration of the SR membrane was observed, which indicates that the extraction of DMeF into the ISM is effectively suppressed. There is however a minor intermixing of the redox couple and the SR membrane at the casting of the ISM cocktail, but no evidence was found for further uptake of DMeF after the SR membrane cured. Apparently, even this slight contamination of the ISM can cause some loss of selectivity as compared with redox mediator free solid contacts.[34] However, this is by far not as dramatic as in case of PVC membranes (in fact for some of the ions the selectivity is even preserved) and would not hinder the application of the respective SC-ISEs.

<table>
<thead>
<tr>
<th>Redox couple</th>
<th>Polymerization charge</th>
<th>PVC-based ISM</th>
<th>SR-based ISM</th>
<th>PVC-based ISM</th>
<th>SR-based ISM</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>Slope [mV / decade]</td>
<td>LOD [M]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>CWE</td>
<td>56.4</td>
<td>49.5</td>
<td>-6.5</td>
<td>-7.0</td>
</tr>
<tr>
<td></td>
<td>21.2</td>
<td>57.1</td>
<td>48.3 ±0.1</td>
<td>-6.5</td>
<td>-6.5 ±0.0</td>
</tr>
<tr>
<td></td>
<td>250.7</td>
<td>54.5 ±2.2</td>
<td>47.4 ±0.2</td>
<td>-6.5 ±0.0</td>
<td>-6.9 ±0.0</td>
</tr>
<tr>
<td>yes</td>
<td>CWE</td>
<td>51.0</td>
<td>53.1</td>
<td>-6.7</td>
<td>-6.9</td>
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<tr>
<td></td>
<td>21.2</td>
<td>43.9 ±1.3</td>
<td>51.1 ±0.4</td>
<td>-7.0 ±0.2</td>
<td>-6.9 ±0.1</td>
</tr>
<tr>
<td></td>
<td>250.7</td>
<td>43.6 ±5.3</td>
<td>51.8 ±0.4</td>
<td>-6.8 ±0.2</td>
<td>-6.9 ±0.1</td>
</tr>
</tbody>
</table>

Moreover, very importantly, the DMeF loading seem to fulfill our expectations of providing better electrode-to-electrode reproducibility of the E⁰ values. The improved reproducibility of electrodes with 3D structured SC filled with the redox couple is shown in Figure 9. This effect was more pronounced for electrodes with thicker 3D structured SCs, i.e., the standard deviation of E⁰ for ISEs with 3D structured PEDOT(PSS) SCs prepared with 21.2 and 250.7 mC/cm² was ± 5.4 and 3.9 mV, respectively. These values are much better than those for the same 3D structured SCs but without DMeF ± 43.4 and ± 31.4 mV, respectively, but lag behind the E⁰ reproducibility reported by Hu et al (0.7 mV).[33]
Figure 9. (A) Calibration curves and (B) their corresponding potential traces of identically prepared GC/3D PEDOT(PSS)/SR-based SC-ISEs with different polymer thicknesses, (rectangle, solid line) 21.2 mC/cm² and (triangle, dashed line) 250.7 mC/cm² polymerization charge; (a, b) without and (c, d) with filling the nanostructured polymer with redox couple.

The stability of the different SC ISEs was evaluated by chronopotentiometric experiments[44] by applying ± 1 nA on the respective electrodes in 1 mM AgNO₃ solution (see supplementary information for details). The results with both PVC and SR based SC-ISEs (Figure S3) revealed a ca. 1.5 times higher capacitance of the 3D nanostructured as compared with their compact alternatives prepared with the same charge density of 250.7 mC/cm² (PVC: 49.7 vs 33.6 µF, SR: 94.0 vs 65.0 µF, respectively). The SR based SC-ISE with the redox mediator filled 3D nanostructured SC showed the smallest potential drift upon applying the 1 nA current, i.e., 10.9 ± 2.3 µV/s (94.0 µF capacitance) (Supporting Information, Table S2).

5. Conclusions

In this study we demonstrated the feasibility to fabricate 3D nanostructured ECP solid contacts for ISEs. Though at first sight it may seem complicated, the optimized method for preparing the 3D nanostructured PEDOT(PSS) films is relatively easy and fast, i.e., the whole process from template deposition to the ready-to-use 3D polymer could be done within 3 to 5 h depending on the film thickness (layers of PS beads). This is due to the excellent control of the electropolymerization process by the amount of injected charge and the scalability of the layered structure. Such structures are of benefit in terms of providing large capacitance interfaces if the templated interconnected pores are accessible to the contacting media. Interestingly, when in contact with aqueous electrolytes the 3D structured SC offered no benefit in comparison with a compact layer of PEDOT(PSS). However, in case of non-aqueous solution comprising the lipophilic salt ETH500, which is of more relevance for the practical case of hydrophobic ISM covered SCs, the 3D structured PEDOT(PSS) films showed ca. 7 times larger capacitance than their compact counterparts. A further advantage of 3D structured ECP-based solid contacts is the possibility to fill the template voids with a redox couple to stabilize the E₀ value of the respective ISEs. While we were able to show the feasibility of this approach, the choice of the largely available DMeF (as expected) proved not to be optimal as its extraction into the PVC-based ISM led to a dramatic selectivity loss. However, we were able to minimize this problem by using silicone rubber as a low diffusivity membrane material instead of the conventional plasticized PVC.

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References

Paper V
A versatile approach based on nanosphere lithography is proposed to generate surface-imprinted polymers for selective protein recognition. A layer of 750 nm diameter latex bead-protein conjugate is deposited onto the surface of gold-coated quartz crystals followed by the electrosynthesis of a poly(3,4-ethylenedioxythiophene)/poly(styrenesulfonate) (PEDOT/PSS) film with thicknesses on the order of the bead radius. The removal of the polymer bead-protein conjugates, facilitated by using a cleavable protein-nanosphere linkage is shown to result in 2D arrays of periodic complementary size cavities. Here it is demonstrated by nanogravimetric measurements that the imprinting proceeds further at molecular level and the protein (avidin) coating of the beads generates selective recognition sites for avidin on the surface of the PEDOT/PSS film. The binding capacity of such surface-imprinted polymer films is ca. 6.5 times higher than that of films imprinted with unmodified beads. They also exhibit excellent selectivity against analogues of avidin, i.e., extravidin, streptavidin, and neuravidin, the latter being in fact undetectable. This methodology, if coupled with properly oriented conjugation of the macromolecular template to the nanoparticles, offers the possibility of site-directed imprinting.

1. Introduction

Molecular imprinting is a generic method to generate materials with “molecular memory” by performing generally a polymerization of suitable functional monomers in the presence of a target molecule acting as a template. A preassembly of the functional monomers and template, which is then conserved by the polymerization reaction, results in the formation of binding sites for the target in the polymeric matrix. Such molecularly imprinted polymers (MIPs) received much attention due to their applicability for separation, catalysis, sensing and drug delivery. However, molecular imprinting is still facing challenges in terms of selective biomolecular recognition that would fully enable generating synthetic antibodies for analytical applications. Despite of the obvious need for robust selective synthetic receptors/sorbents for biomacromolecules it was reported that less than 2% of the MIP literature involves aspects of macromolecular imprinting. This reflects difficulties in imprinting large, delicate biomolecules and the conceptual changes that this implies.

The bulk synthesis method, with excellent results in generating MIPs for recognition of low-molecular-weight compounds, is hardly applicable to macromolecules due to their hindered mobility in the highly reticulated polymeric networks. Therefore, the essential prerequisite of generating macromolecular imprints clearly should be to create accessible binding sites amenable for free target exchange between the MIP and the sample phase, i.e., to have binding sites confined to the surface of the MIPs. For high binding capacities the surface localization of the binding sites should be combined with fabrication methods leading to high surface area/volume ratios of the respective MIPs. Therefore, generation of polymeric micro- and nanostructures comes as a natural necessity to take full advantage of the surface-imprinted sites. This goal is already achieved by protein imprinting of nanometer thin polymer films, but more sophisticated methods including microcontact printing with protein modified stamps, and using immobilized templates or sacrificial materials were also reported. The stamping approach proved to be especially successful in imprinting cells, viruses and most recently with particles previously imprinted with anti-insulin antibodies to generate insulin binding antibody replicas.

The structural and conformational sensitivity as well as the solubility of biomacromolecules are further reasons why the simple extrapolation of the well-established MIP fabrication technologies for low-molecular-weight compounds is not straightforward. The imprinting of biomacromolecules requires mild conditions and it is generally restricted to aqueous environment. In contrast, low-molecular-weight compounds are typically imprinted in aprotic organic solvents as otherwise...
the hydrogen bonding interactions largely contributing to the affinity of MIPs are suppressed by competition from water molecules. Other intrinsic properties of biomacromolecules such as their surface variability in terms of local charge density and hydrophobicity also contribute to the difficulty in providing proper macromolecular imprints. Due to the latter there is an increased likeness of nonspecific interactions between macromolecules rich in functionality and MIPs, which can easily lead to poor selectivity and cross-reactivity. Epitope imprinting which involves the use of only a fragment of the original macromolecular target for imprinting\(^{[19]}\) was introduced among others as a means to restrict the imprinting to a targeted sequence and by that to reduce cross-reactivity. Further strategies involved close mimicking of the naturally occurring interactions by using monomers with functionalities already known to interact specifically with the target molecule, e.g., boronic acid–diol\(^{[17]}\), metal coordination assisted\(^{[18]}\) and enzyme-inhibitor interactions.\(^{[19]}\)

In principle, surface imprinting is inherently advantageous to minimize non-specific interactions because it reduces the contact area between macromolecular targets and bulk polymeric material. Therefore, previously we introduced new methods based on sacrificial polycarbonate microreactors the inner wall of which was modified with proteins by simple physiosorption to generate surface-imprinted polymer (SIP) micro-\(^{[20]}\) and microparticle.\(^{[21]}\) Here we propose a versatile concept that enables site-directed protein imprinting and has the potential of downsizing to the low nanometer scale.

The new concept is based on the use of nanosphere lithography with protein-polymer bead conjugates for molecular surfaces imprinting as shown in Figure 1.

In contrast to free protein imprinting, the conjugation of the protein target to a solid surface makes possible its oriented immobilization. In this model study we demonstrate this concept by immobilizing avidin (Av) through its biotin binding sites by using a heterofunctional crosslinker, succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-Biotin). The beads were deposited by drop casting onto the surface of gold-coated quartz crystal resonators and the generated inter-bead voids were filled with polymeric material by electropolymerization of 3,4-ethylenedioxythiophene (EDOT) from an aqueous solution comprising also poly(styrenesulfonate) (PSS, MW: 70 kDa). As such, the electropolymerization could be performed in mild condition compatible with the protein target with the polymerization process being readily controllable by monitoring the current. The choice of PEDOT was motivated by its inherently high biocompatibility\(^{[22]}\) due to structure similarity with natural compounds such as melanin\(^{[24]}\) and its electrical conductivity. The duration of the electropolymerization was adjusted to grow PEDOT/PSS layers with thicknesses on the order of the radius of the deposited beads. Dissolving away the beads resulted in complementary cavities the interior of which was expected to bear the molecular imprint of the avidin molecules surrounding the respective beads. Here we show the proof of concept that indeed such recognition sites are generated on the surface of the PEDOT/PSS film by using quartz crystal microbalance (QCM) based nanogravimetric measurements.

2. Results and Discussion

2.1. Deposition of Beads onto the Surface of Gold-Coated Quartz Crystals

While nanosphere lithography is a widely known technique to generate periodic patterns its application is very much focused on fabrication of plasmonic structures\(^{[24]}\) and according to our best knowledge its use for generating molecular imprints of proteins has never been explored. Remarkably, also the self-assembly of protein modified nanospheres on solid surfaces has little antecedents except studies focused on depositing protein-modified beads within arrays of microwells\(^{[25]}\) or planar surfaces\(^{[26]}\) with the aim to increase the local receptor density in bioassay applications. The first step to generate SIPs by nanosphere lithography involved the deposition of Av-modified beads on the planar gold surface of a 10 MHz quartz crystal resonator in as compact layer as possible. Obviously, a compact layer is desirable as it maximizes the imprinted fraction of the polymer surface, however, it should be emphasized that contingent defects in the layer are not as critical for the molecular imprinting process as they are for optical device fabrication.

The Av-conjugated beads were prepared using a cleavable crosslinker (NHS-SS-Biotin) in two successive steps, i.e., first the linker was reacted with the amino surface functionality of the bead and after removing the excess of reagent by centrifugation the Av was linked to the bead through its biotin binding site. The procedure was optimized in terms of the excess of Av required to avoid bead crosslinking. The modification with Av having a pi of ca. 10.5 resulted in a significant shift of the zeta potential of the aliphatic amine modified beads to more positive, i.e., from \(-29.6\) mV to \(+6.7\) mV. Please note that surface pKa of aliphatic amino groups can be as low as 4.4–5.5\(^{[27]}\) compared to 9–10 in bulk solution phase and for comparison carboxylated beads had
a zeta potential of $-73.3$ mV. The use of the cleavable crosslinker was preferred because, unlike beads with COOH and NH$_2$ surface functionality that dissolved practically instantaneously in toluene, the removal of avidin-coated particles by the same procedure was found to leave, in some cases, organic residue on the surface. However, this could be effectively avoided if prior to dissolving away the beads the avidin coating was removed by reducing the disulfide bridge of the crosslinker using 50 mM tris(2-carboxyethyl)phosphine (TCEP). Both the successful modification of the beads with protein and their removal from the polymer film were confirmed by hyperspectral optical microscopy using fluorescently labeled avidin (avidin-FITC) modified polystyrene particles (Figure S1, Supporting Information).

While the surface arrangement of colloidal particles can be performed in a variety of ways, given the inherent fragility of the protein layers we used simple drop casting of an aqueous bead suspension. The assembly of beads under these conditions is based on the cooperative effect of evaporation driven convection and capillary forces. Generating compact hexagonal monolayers of submicron diameter beads bearing carboxylate and amine functionalities proved to be straightforward. However, the arrangement of Av-modified beads required thorough optimization in terms of experimental conditions to result in an optimal bead concentration of 0.13% and deposited amount of 8.98 × 10$^8$ particles cm$^{-2}$. This corresponds to approximately four times the amount required for a compact monolayer to account for the fraction of particles inherently expended to multilayer formation predominantly at the edge of the demarked surface. The compactness of the layers was most significantly improved by subjecting the surface to UV generated ozone cleaning just prior to the drop casting of the bead suspension and using a silicone rubber ring for the localization of the deposited droplet. Even so, the array of Av-modified beads was inherently less uniform and compact than those assembled from unmodified beads. A typical scanning electron micrograph of latex and Av-modified bead layers is shown in Figure 2. Defects in the latex bead monolayers were found to coincide with the occurrence of larger particles present in the commercial bead suspension.

The reason for the deviation of the protein modified beads from a compact hexagonal structure is likely to be caused by the adhesion of these beads to the gold surface, which may promote their free movement and ordering during the evaporation of the solvent.

### 2.2. Synthesis of the Surface-Imprinted PEDOT/PSS Film

The voids between the deposited nanospheres were filled by potentiostatically growing a film of PEDOT/PSS from an aqueous solution of EDOT (10 mM) in the presence of PSS at 0.9 V (vs Ag/AgCl). The polymer layer thickness was optimized to obtain maximum imprinted to non-imprinted surface ratio, which based on simple geometrical calculations and assuming uniform growth of the film corresponds to approximately the half-height of the beads (375 nm). The optimal experimental conditions for the film synthesis were determined empirically. Films of different thicknesses were prepared by controlling the electrical charge injected during the polymerization and examining the patterned polymer layers with atomic force microscopy (AFM) after the dissolution of the beads. As shown in Figure 3, the AFM measurements revealed preferential growth of the PEDOT/PSS film alongside the particles and were also conclusive in terms of local morphology of the generated cavities as a function of the electrical charge used during electropolymerization.

At a charge density of 7.5 mC cm$^{-2}$ small cavities with a mean depth of ca. 65 nm were formed, that increased to 270 nm at 19 mC cm$^{-2}$. Also, the diameter of the cavities increased from 270 nm to ca. 590 nm. Higher charge densities of 30 and 41 mC cm$^{-2}$ resulted in a gradual enfolding of the bead by the grown polymer film, as shown by the decrease in the exposed diameter of the cavity (490 and 260 nm, respectively) as well as its mean depth (460 and 610 nm, respectively). It must be noted that due to the preferential growth of the polymer at the bead surface the thickness value refers to the immediate vicinity of the bead and it is not the average thickness of the film. A somewhat lower charge density, 17 mC cm$^{-2}$, than that would correspond to radius of the beads was chosen as this ensured better control of the polymer thickness, i.e., was less affected by preferential random growth around the bead.

The dissolution of polymer beads exposed parts of bare gold corresponding to areas where the beads touched the surface is noteworthy. To reduce non-specific binding of the protein to these spots the surface was treated with 1 mM (1-mercaptoundec-11-yl)tetra(ethylene glycol) (HS-TEG) for 30 min prior to the QCM measurements (Figure S2, Supporting Information).

### 2.3. Nanogravimetric Measurements of Av Binding to Surface-Imprinted PEDOT/PSS Films

The surface-imprinted polymer film-modified 10 MHz quartz crystal chips were mounted into a flow cell and after the
stabilization of the frequency, increasing concentrations of Av were injected in the carrier buffer and the frequency change was monitored in real-time. To determine the effect of the surface imprinting on the binding properties of the polymer the Av binding to non-imprinted polymer film (NIP) was recorded in similar conditions, but in a separate experiment. The NIP films were synthesized in the exact same way as the MIPs but nanospheres that were not modified with Av were used for patterning the PEDOT/PSS film. Typical QCM sensorgrams are shown in Figure 4.

We examined the effect of surfactant content in the eluent buffer on the suppression of non-specific adsorption. A non-ionic detergent was used for this purpose as ionic detergents were shown to cause complications in protein MIPs. The Av binding to MIP and NIP surfaces was determined at various amounts of Tween-20 added to the Tris-EDTA buffer. Addition of 0.01% Tween-20 to the Tris-EDTA buffer decreased by half the total amount of avidin bound to the NIP (Figure S3, Supporting Information). Interestingly, concomitantly the bound Av quantity increased significantly on the MIP as compared with the detergent-free Tris-EDTA buffer. Therefore, all further experiments were carried out with Tris-EDTA buffer containing 0.01% Tween-20 as carrier solution. Under this condition at the highest avidin concentration studied the amount of avidin bound on the imprinted surfaces was 1.34 μg cm⁻² while on the NIP surface 0.21 μg cm⁻² which accounts for an imprinting factor of 6.5 (Figure 5). This value is higher than the vast majority of imprinting factors reported for acrylic polymers (generally between 1.2 and 4.5). However, we could not reassert our previous result of ~10 obtained with PEDOT/PSS polymer microbands, which is in fact close to the maximum value ever reported for protein MIPs. This is most likely due to the relatively large non-imprinted fraction of the surface as result of the non-compact Av-modified bead layer.

The selectivity of Av-imprinted polymers was determined for three avidin species: ExtrAvidin (EA), NeutrAvidin (NA), and Streptavidin (SA); all of them having a near-neutral isoelectric point (pI) at 6.5, NA: 6.3, SA: 5.5) and various degrees of structural similarity with avidin. ExtrAvidin and NeutrAvidin are deglycosylated forms of avidin, while Streptavidin is a biotin-binding protein found in the bacterium Streptomyces avidinii with only 33% of the amino acid residues identical to those found in Av. The selectivity study further included Lysozyme (Lys), representative for high isoelectric point (pI) proteins and bovine serum albumin (BSA) with widely different pI, but similar molecular weight. The respective proteins were injected at a concentration of 0.1 mg mL⁻¹ and the amount of protein bound to MIP and NIP surfaces was determined by QCM. The surface was regenerated between measurements with 1 M NaCl. The Av-imprinted PEDOT/PSS film showed clearly the greatest affinity for Av, followed in order by Lys, BSA and the avidin-analogue (Figure 6). The significant Lys adsorption is not surprising as it is one of the most surface active proteins that can form multilayer aggregates upon adsorption to charged surfaces. The relatively high amount of adsorbed Lys having a pI of 11.4 suggests that electrostatic interactions play an important role in the recognition process. However, the Lys adsorption is practically the same on both MIP and NIP surfaces and also similar to the Av adsorption on the NIP, which
are inducing both morphological and composition changes in PEDOT/PSS films. \[36\]

3. Conclusions

We have demonstrated that nanosphere lithography can be used to generate surface-imprinted polymers for selective protein recognition. A remarkable selectivity and an imprinting factor close to state of the art were obtained despite of the non-compact nanosphere assembly, which exposes a significant fraction of the non-imprinted surface for interaction. Future work is directed to address this issue as well as the possibility of generating multilayer structures for higher binding capacity surface-imprinted nanostructures. The method proposed has the following potential advantages: (i) the oriented immobilization of the macromolecular template on the nanosphere surface makes feasible site-directed imprinting of the respective macromolecules, (ii) the binding site density on the surface-imprinted polymer can be controlled by adjusting the nanosphere diameter and/or surface density of the protein coating, (iii) the nanosphere carrier offers utmost flexibility in terms of adjusting the local chemical environment of the macromolecular template and imposes less strain on the control of the polymer film thickness as compared with surface-confined direct macromolecular imprinting requiring ultrathin smooth films. \[34\]

4. Experimental Section

**Chemicals and Materials**: Monodisperse polystyrene beads of 750 nm diameter with primary amine (Polybead Amino) and carboxyl (Polybead Carboxylate) surface groups were purchased from Polysciences (Warrington, PA, USA). Avidin, NeutrAvidin and the heterobifunctional crosslinker, succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate, were obtained from Thermo Fisher Scientific (Rockford, IL, USA). The proteins avidin-FITC, Streptavidin, ExtrAvidin, lysozyme (Lys) and bovine serum albumin (BSA) were obtained from Sigma Aldrich (St. Louis, MO, USA). The buffer components: phosphate buffered saline (PBS, pH 7.4, 10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl), Tween containing Tris-EDTA buffer (TET, pH 8.0, 10 mM Tris, 1 mM EDTA, 0.01% Tween 20) as well as the reducing agent tris(2-carboxyethyl)phosphine hydrochloride and (1-mercaptoundec-11-yl) tetra(ethylene glycol) were purchased from Sigma Aldrich. The monomer 3,4-ethylenedioxythiophene and poly(sodium 4-styrenesulfonate) (MW: 70 000 g mol\(^{-1}\)) were from Sigma Aldrich, dimethyl sulfoxide (DMSO) was purchased from ROMIL (Cambridge, UK). Aqueous solutions were prepared with ultrapure deionized water (DI water, 18 MΩ cm, Millipore Corporation, USA).

**Synthesis of Avidin-Polymer Bead Conjugates**: The Polybead Amino stock solution was diluted 10 times with PBS followed by the addition of NHS-SS-Biotin (100 µL, 10 mM) in DMSO. The suspension was incubated under continuous shaking for 60 min and then centrifuged at 16 660g for 10 min. The supernatant was discarded, and the particles were resuspended in PBS (900 µL). This procedure was repeated five times to separate the biotinylated nanoparticle from the free reagent.
Further modification with Av was made by mixing avidin in PBS (100 μL, 10 mg mL⁻¹) with biotinylated bead suspension (900 μL) for 60 min. The beads were centrifuged (at 16 600g for 10 min) and washed five times with DI water, followed by resuspension in DI water (1000 μL) to give a final concentration of ca. 10⁴ particles mL⁻¹.

Preparation and Characterization of Avidin-Imprinted Polymer Film: The surface of the gold electrode (0.205 cm²) on 10 MHz AT-cut gold- upon removal of the microspheres were blocked with HS-TEG (1 mM with ethanol and DI water, and dried. The bare gold surfaces exposed after each protein adsorption.

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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