Inkjet-Printed Electrochemical Devices for Bioelectronics

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To Omy

إلى باسم، أُمي
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In recent years, inkjet printing has received increasing attention from academic and industrial communities as a rapid prototyping and cost-effective tool for manufacturing a wide variety of electronic devices. The printed electronic field has great application in biosensors since printed sensors are cheap, small, portable and flexible. Therefore, substantial efforts have been devoted to the improvement of this powerful technology to render it suitable for microscale fabrication with the prospect of practical applications. The purpose of this research is to create a new alternative platform for developing diagnostic tools with high sensitivity and low cost. In this dissertation, I propose an alternative approach for sensitive detection of disease biomarkers and cardiac action potential using printed technology. This alternative approach is based on three main elements (i) controlled bioreceptor immobilization using photonic immobilization technique, (ii) signal amplification using electrochemical redox cycling, (iii) novel printing methodology.

The first is achieved by combining a rapid and simple printing scheme with the photonic immobilization technique for site-specific immobilization of antibodies. Photonic immobilization, which is easy and fast to perform, is presented on printed chips for the first time, achieving a higher sensitivity and better limit of detection compared to the conventional immobilization approaches. In the first application, I targeted the determination of C-reactive protein (CRP) in the presence of human serum.

The second element, which is signal amplification using electrochemical redox cycling, is realized by repetitive cycling of redox probes between two adjacent electrodes. The sensitivity of a redox cycling sensor depends on the distance between the two electrodes. In other words, a short distance between the electrodes in the micro- or even nanometer regime is required to achieve an effective redox cycling amplification. Therefore, I developed a new scheme for fabricating micro-gap electrodes with in-plane displacement, as well as porous nano-gap electrodes with out-of-plane displacement, using inkjet printing and without prior surface patterning. As a proof of concept, I demonstrated the use of micro-gap redox cycling sensors for the detection of single-stranded DNA (ssDNA) using peptide nucleic acids (PNAs) immobilized on the carbon
microelectrodes. The developed genosensors were then applied to the detection of human immunodeficiency virus-1 (HIV-1) marker sequence encoding of HIV-1 nef gene.

The third element, namely the development of novel printing methodology for high-resolution printing, is accomplished by exploring the different physical, chemical and hydro-dynamical properties of an ink droplet that interacts with the substrate surface. I provide the first demonstration for fabricating microelectrode arrays (MEA) in a rapid prototyping approach based on inkjet printing. The printed MEAs on flexible substrates resulted in a high-resolution outcome with good electrical and outstanding electrochemical characteristics, suitable for cellular recording and stimulation. I cultured HL-1 cells on MEAs printed using gold and carbon inks on flexible substrates. Using the MEAs, the propagating action potentials were recorded across the cellular network with high signal-to-noise ratios. The benefits of having a transparent and flexible printed MEA are well appreciated when it comes to in-vivo applications, such as neuronal implants. With such an application in mind, I printed high resolution MEAs on soft materials such as PDMS, agarose, and gelatin-based substrates including gummy bears. A series of in-vitro extracellular recordings of action potentials were recorded from cardiac HL-1 cells and the results demonstrate that inkjet printing can be used for fabricating functional cell-device interfaces on soft materials in a rapid prototyping approach.

Hereafter, this dissertation contributes significantly to the vision I have for providing healthcare system with sensitive biosensors and soft MEA platforms that avoid expensive fabrication utilities, towards rapid prototyping of ultra-low-cost medical devices.
Zusammenfassung


Im zweiten Teil wurde die Signalverstärkung durch elektrochemisches Redoxcycling realisiert, in dem redoxaktive Moleküle an schnellen, wiederholten Reaktionen mit zwei sich gegenüberliegenden Elektroden teilnehmen. Die Sensitivität eines solchen Redoxcy-

List of Publications

Related publications

The work of this thesis is based on the following publications:

1. Jan Schnitker*, Nouran Adly*, Alexey Yakushenko, Andreas Offenhäusser, and Bernhard Wolfrum. All inkjet-printed, flexible arrays of nanoporous carbon microelectrodes for extracellular Recordings from electrogenic cells. *Advanced Biosystems*, accepted manuscript


5. Bernd Bachmann, Nouran Adly, Jan Schnitker, Alexey Yakushenko, Philipp Rinklin, Andreas Offenhäusser, and Bernhard Wolfrum. All-inkjet printed gold microelectrode arrays for extracellular recording of action potentials. *Flexible and Printed Electronics*, 2017

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Nomenclature

**Acronyms / Abbreviations**

AFM Atomic Force Microscopy  
BSA Bovine Serum Albumin  
CRP C-Reactive Protein  
CV Cyclic Voltammetry  
DLVO Derjaguin–Landau–Verwey–Overbeek  
DNA Deoxyribo Nucleic Acid  
EDX Energy-Dispersive X-Ray Spectroscopy  
Fab Fragment Antigen-Binding  
Fc Fragment Crystallizable  
FIB Focused Ion Beam  
FT-ATR-IR Attenuated Total Reflection Fourier Transform Infrared spectroscopy  
HIV Human Immunodeficiency Virus  
IgG Immunoglobulin G  
IUPAC International Union of Pure and Applied Chemistry  
MEA Microelectrode Array  
MEMS Microelectromechanical Systems  
MPTMS (3-Mercapto-Propyl)Trimethoxysilane
**Nomenclature**

<table>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<tr>
<td>PEN</td>
<td>Polyethylene naphthalate (poly(ethylene 2,6-naphthalate))</td>
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<tr>
<td>PIT</td>
<td>Photonic Immobilization Technique</td>
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<tr>
<td>PNA</td>
<td>Peptide Nucleic Acid</td>
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<tr>
<td>RC</td>
<td>Redox Cycling</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>ssDNA</td>
<td>single-stranded Deoxyribo Nucleic Acid</td>
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Outline of Dissertation

This thesis describes a journey that starts with a simple printed sensor for protein detection and ends with a high-resolution printed chip for extracellular recording of cellular activity. In between, different printing materials (inks and substrates), sensor designs (planar and vertical) and characterizations of these devices in the context of biomedical application are discussed.

Chapter 1 is an overview of fundamental concepts that form the building blocks of printed electrochemical biosensors. It introduces the field of printed electronics, reviews basic aspects of inkjet printing and provides state-of-the-art examples. The chapter also contains essential concepts of electrochemistry and biosensors.

Chapter 2 introduces the results by presenting the outcome of a simple immunosensor printed with combination of gold and polyimide ink. The sensor design represents the first step toward advanced biosensor architecture, yet it presents an unconventional approach to the immobilization of antibodies on gold, namely the photonic immobilization technique (PIT). This immobilization strategy is electrochemically characterized and compared with conventional immobilization methods such as physisorption. The developed immunosensor is then applied to the detection of C-reactive protein (CRP) in serum, showing an excellent detection limit when compared to measurements obtained with conventional immobilization. These results demonstrate a promising ground for further application.

Chapter 3 demonstrates advanced printing in the construction of a sensitive genosensor by introducing a novel printing approach achieving high lateral resolution. This chapter has two main objectives. One is to introduce a new direct printing method for fabricating micro-gap devices. These devices are used for electrochemical redox cycling sensing as a signal amplification strategy. The second objective is to utilize the micro-gap device in a genosensing application. Here, the new redox cycling devices are employed to detect a single-strand DNA marker for the human immunodeficiency virus (HIV) using peptide nucleic acid (PNA) as a biorecognition element immobilized on a carbon electrode.
Chapter 4 extends the results of the redox cycling work. The aim is to create a smaller distance between the two electrodes in order to improve the sensitivity of the redox cycling device. Here, another printing approach alternative to the lateral configuration is explored. The new approach is based on multilayer printing of three-dimensional redox cycling devices with a tunable nanometer gap for electrochemical sensing. The fabrication is achieved by vertically stacking two conductive electrodes made of carbon and gold nanoparticle inks. The two electrodes are electrically separated by a layer of polystyrene nanospheres. This chapter demonstrates that vertical interelectrode spacing down to several hundred nanometers with high precision is feasible using inkjet printing.

Chapter 5 shows for the first time how microelectrode arrays (MEAs) can be fabricated solely by rapid prototyping using inkjet printing technology. In this fabrication, the multilayer printing approach and high-resolution printing are combined into a single chip for extracellular recording from electrogenic cells. The printed MEA chips are fabricated using carbon ink on a flexible polyethylene naphthalate (PEN) substrate. Finally, the printed MEAs are further evaluated for recording extracellular signals from different biological systems such as cardiac myocyte-like HL-1 cells.

Chapter 6 describes the use of softer substrate materials for interfacing cells. In this chapter, MEA chips are printed for the first time in elastomeric polymer polydimethylsiloxane (PDMS), gelatin and agarose gel. Challenges and solutions in MEA fabrication using inkjet printing are further discussed. Finally, cellular recording is successfully measured with a cellular signal comparable to the silicon-based MEA.

To conclude, Chapter 7 provides general conclusions of this work. The chapter also briefly discusses several shortcomings of the overall printing approach and provides additional perspectives that can be applied to future research.
Chapter 1

Fundamentals

Before we dive into the world of printed electronics and electrochemical analysis of biological events, there are some essential concepts we will review in this introductory chapter. This chapter comprises three parts.

Section 1.1 deals with the science of inkjet and how fluids can be jetted into drops, which land on a substrate with a certain velocity and dry or get actively cured to form a functional device. This section focuses explicitly on the rheological properties of fluids for inkjet printing and the impact of a droplet on a substrate.

Section 1.2 focuses on some basics in electrochemistry that are necessary for understanding electrochemical biosensor devices. To this end, we review core concepts in electrochemistry with particular focus on the charge transfer phenomena on the electrode surface. Special attention is devoted to the electrochemical redox cycling technique, which will be used along this thesis as a signal amplification strategy.

Section 1.3 reviews the concept of biosensors as it introduces possible applications of printed electrochemical devices. Therefore we will shortly provide some essential background in biosensors and the necessary parameters in evaluating electrochemical sensors.

1.1 Inkjet printing

Inkjet printing is a promising technology for the fabrication of microelectronic devices and systems. Over the last few decades, printing technology has been receiving an increasing attention from the microfabrication community, as it enables us to develop functional devices in a reasonable time and resolution. As shown in Figure 1.1, it is obvious that printing offers significant reduction in cost, labor and energy. Several research groups have used various printing methods to fabricate sensitive electronic
devices such as transistors, solar cells and light-emitting diodes using exclusively inkjet printing.[11–14] Compared with conventional microfabrication methods, inkjet printing has several advantages including the possibility to integrate new emerging materials in the form of an ink, the ability to easily customize the design of the desired structure in-flight without additional cost, no loss of materials and the scalability to high-volume production. The fascination of this approach for many researchers lies in the fact that no mask is needed during the whole fabrication process, unlike in photo-lithographic methods. This cuts down the cost and allows scientists to explore microfabrication more freely using such an economical research tool. Perhaps the most distinctive advantage of printing, i.e. its low cost, made this technology attractive for bridging the gap between science and technology.

Inkjet printing is a non-contact additive printing technology for depositing inks with a lateral resolution ranging from 10 to 60 µm.[16] Inkjet printing technology can be divided roughly into two broad categories (a) drop on demand and (b) continuous inkjet. Historically, continuous inkjet is one of the oldest inkjet technologies. The idea presented by Lord Kelvin in early 1867 and later commercially produced as medical strip chart recorders by Siemens in 1950. As the name implies, in continuous inkjet
1.1 Inkjet printing

Fig. 1.2 Simplified schematic of a typical piezoelectric inkjet cartridge showing the process of droplet ejection. (a) Under no applied voltage to the piezoelectric element, the ink is resting at the nozzle orifice. (b) Voltage causes the piezoelectric element to bend, leading to a pressure at the reservoir, resulting in a drop formation. The jetted drops land eventually on a moving substrate. (c) The applied voltage waveform is removed.

(CIJ) printers the printhead is continuously releasing liquid ink at very fast speed (64,000 to 165,000 droplets per second) by a high pressure pump. The ink travels from the reservoir chamber into a microscopic nozzle, resulting in a continuous stream of ink droplets. By creating an electrostatic field through an electrode around the drops, the droplet becomes charged. Charged droplets pass through a second electrostatic field and therefore either they are directed to the substrate or deflected away to a collector for recycling. In general, the continuous inkjet method allows for very high speed printing, however, it has its downsides such as less precise drop placement compared to drop on demand printers and all inks have to be suitable for electrostatic charging.[17]

On the other hand, in drop on demand printers (DOD), the ink droplets are generated on request, meaning that the droplet will only be ejected according to the requirement and directly lands on the substrate. Generally speaking, the drops are formed due to a pressure pulse inside the ink chamber. The mechanism of how the pressure is generated can be classified into (i) thermal and (ii) piezoelectric. In thermal mode DOD printers, the drops are formed due to the formation of a bubble inside the ink chamber, resulting in a pressure pulse that pushes the drops through a microscopic
orifice in the nozzle plate. The bubble is generated due to rapid heating of a resistive element up to 400 °C. The heating causes a thin layer of the ink above the resistive component to vaporize rapidly, resulting in a bubble. As the method depends on rapid heating and vaporization, it puts a limitation on the type of inks that can be printed, as they have to withstand a high local temperature without losing their function.

1.1.1 Drop formation in a piezoelectric inkjet printhead

Piezoelectric inkjet printheads use a piezoelectric actuator that contracts and bends upon applying voltage pulses. The ink is separated inside a microfluidic chamber behind each nozzle. The chamber is designed in such a way that it is sensitive to the mechanical pressure generated by the piezoelectric element, as seen in Figure 1.2. Once the pressure pulse is generated, the ink is forced through a narrow orifice, resulting in
ink droplet formation. The opening size of the orifice which is typically 20 to 70 µm in diameter, will determine the volume of the ejected drop. A straightforward approach to decrease the volume of the ejected drop is to decrease the size of the orifice. As another approach, one can consider decreasing the volume of the ejected drop by optimizing other factors influencing the jetting, such as the applied voltage pulse and duration, as reported in literature.[18, 19]

When we look at the liquid-air interface at the orifice during resting state while no voltage is applied, there are several scenarios that contribute to the meniscus shape (see Figure 1.4). The meniscus is held inside the cartridge by applying a slightly negative pressure. An overfill or a convex meniscus would increase the droplet volume upon jetting. A concave meniscus might prevent the ink from being ejected upon applying voltage pulses due to increased resistance. The ink might leak out of the nozzle and accumulate at the nozzle plate, as seen in Figure 1.4c, when the applied negative pressure is inadequate. This would result in wetting of the nozzle plate. It is important to notice that this phenomenon might also occur in the case of a very low surface tension of the used ink or very high surface energy of the nozzle plate. If the applied negative pressure exceeds certain threshold, de-priming of the ink occurs which disturbs the jetting process.

As mentioned earlier, the duration and the magnitude of the applied voltage pulses will determine the volume and shape of the ejected drop. The influence of the applied
Fig. 1.6 Microscopic images of printed diagonal lines with unstable ink-jetting leading to (a) a misplaced drop (b) a drop has not been jetted.

voltage shape by which a piezoelectric printhead generates droplets has been a topic of study for a number of years.[19] Figure 1.3 shows an example of a simple waveform for driving the piezoelectric element for droplet ejection. This waveform is also known as unipolar waveform. It consists of three characteristic phases: (a) rise time $t_r$, (b) dwell time $t_d$ and (c) fall time $t_f$.

Another common waveform is shown in Figure 1.5 which consists of four stages. This waveform begins with a reduced voltage pulse in order to let the fluid inside the nozzle rest back, forming a concave shape, as seen in Figure 1.5 on the right. This phase is commonly referred to as loading work phase. The sudden changes in the applied voltage will create a propagating wave inside the ink reservoir. During phase (a) the fluid goes backward inside the chamber, resulting in the movement of the piezoelectric element upward. In phase (b) the increase in the applied voltage results in pushing the drop outwards, as shown in the ink chamber in the right side of the Figure 1.5. In phases (c) and (d), the contraction of the piezoelectric part is controlled to return to its normal state, while the jet is breaking off. The optimum waveform for some ink formulations has been already established. For example, due to the viscoelastic effects of non-Newtonian fluids such as polymer inks (e.g. polyimide ink), $t_r$ and $t_f$ have to be longer than the dwell time $t_d$. This relatively long time will act as a buffering time for the driving signal to reach the required amplitudes. In this work, the minimum used rise time $t_r$ and fall time $t_f$ were both 3 $\mu$s.

One can get an impression of the effect of the waveform on the quality of the printed results by considering the following example. Figure 1.6 shows an image of printed diagonal lines using the same ink (gold nanoparticle) printed on the same substrate at room temperature. Although the used waveform delivered successfully printed lines,
1.1 Inkjet printing

Fig. 1.7 The influence of the applied voltage pulse on the shape of the ejected and printed drop. (a) Stroboscopic image of an ejected droplet using $t_f = 4 \mu s$. (b) Printed drop array using $t_f = 4 \mu s$. (c) Stroboscopic image of the same ink using shorter $t_f$ of 1 $\mu s$. (d) Printed drop array using $t_f = 1 \mu s$.

the printed patterns suffer from two major errors (indicated by red arrows in Figure 1.6. The first printing error is inaccurate placement of the printed drop (see Figure 1.6a) and thus no continuous electrical path exists along the printed line. Unfortunately this error renders the printed electronic device useless. The second printing error that we can see in Figure 1.6b occurs due to the absence of a drop. Again, this results in noncontinuous electrical path and non-functional electronic device. In both cases the lines were printed with a non-optimal pulse width which should be avoided for accurate landing of the drop on the substrate.

Another experimental example that demonstrates the influence of a slight variation in the duration of the applied voltage is shown in Figure 1.7. Here a uniform drop generation was achieved by setting $t_f = 4 \mu s$ as observed by a stroboscopic camera in Figure 1.7a. This resulted in an array of circular drops as shown in Figure 1.7b. However, when $t_f$ is shorted down to 1 $\mu s$, satellite drop formation takes place due to multiple breaking up of the liquid jet, as shown in Figure 1.7c. This will be reflected in the printed dot array (indicated by red arrows in Figure 1.7d). The disturbing small adjacent dots near the primary dot are known as satellite dots.

The printer used in this work was a piezoelectric DOD printer with a printhead compatible with 1 pL and 10 pL Dimatix cartridges. Each cartridge consists of 16
nozzles with a nozzle spacing of 254 µm. The diameter of the nozzles in the 1 pL and 10 pL are 9 µm and 21 µm respectively. The print cartridge consists of a fluid module and a jetting module. The temperature of the ink inside the cartridge is controlled through a heater located inside the jetting component and can be controlled digitally using a software. The jetting performance of the ink is always affected by the quality of the microelectromechanical systems (MEMS) structure at the bottom of the cartridge. The MEMS structure can be microscopically checked for fracture or deterioration signs from repetitive recycling of the cartridge. In this work, the fluid reservoir components have been replaced by a sealed syringe system. This method appears to carry less ink volume (below 500 µL), which is usually required when working with protein-based inks. The use of syringe system also is an advantage to avoid material incompatibility between the solvents of the ink and the bag inside the fluid component (such as in case of gold nanoparticle ink). The printing direction can be controlled either by the printer head in the x-direction or by the substrate holder which is controlled in the y-direction.

![Fig. 1.8 Schematic showing a liquid jet generated in inkjet printing. Deflection in this figure represents the possible aerodynamic influences during the flight of a drop from the nozzle to the substrate.](image)

### 1.1.2 Surface tension

The ink fluid is maintained at the end of the printhead orifice by surface tension $\gamma$. When the piezoelectric element forms a pressure, if the peak pressure is large enough,
1.1 Inkjet printing

it can overcome the surface tension leading to a liquid jet formation (see Figure 1.8). Surface tension can be defined as a tension formed by cohesive forces in the free surface of a liquid at the boundary with a gas phase. This tension is mainly caused by inter-molecular forces such as hydrogen bonds at the surface of the bulk liquid. Surface tension is often measured by the force that forms the elastic membrane per unit of length; N m\(^{-1}\) (usually expressed as dyn cm\(^{-1}\)). The knowledge of this phenomenon is very important for ink jetting since it describes the liquid behavior between two phases that is the water and gas phase. It also explains the wetting of the substrate by a liquid ink and therefore plays a major role in ink spreading.

As mentioned in the previous section, the ink is held inside the fluidic chamber by external pressure, owing to which a convex meniscus is formed as illustrated in Figure 1.4a. If the applied under-pressure is too high, the nozzles de-priming occurs as shown in Figure 1.4b, which will influence the stability of printing over long period of time. However, if an ink has a relatively low surface tension, the ink will leak out from the nozzle and will tend to wet the nozzle plate as shown in Figure 1.4c. This might dramatically alter the jetting and influence the lifetime of the nozzle as the ink might dry on the nozzle plate and clogging might occur. In order to release a drop from the meniscus, the meniscus is first disturbed by a pressure followed by a jet formation process. Finally, surface tension creates small spherical droplets as seen in Figure 1.8. Typical range of surface tensions of inks for piezoelectric printheads lies in the range of 27 – 35 mN m\(^{-1}\).[16] Generally, if the ink composition is above this range, the ink may not travel through the orifices and the applied voltage would not be sufficient to jet or “push” it out. Additionally, if the surface tension is too high, the ink could not wet the substrate surface when printed. One can overcome this by adjusting the surface tension to the right range by adding surfactants to the final ink solution. The addition of surfactants will disturb the inter-molecular forces on the liquid surface and therefore will reduce surface tension. However, it is important to note that surfactants will reduce the contact angle formed between the printed drop and the substrate. As mentioned earlier, surface tension controls both the jetting performance and the ink spreading on a given substrate.

1.1.3 Viscosity

A second important parameter of the ink, besides surface tension, is viscosity. Viscosity is a parameter to quantify the resistance of a fluid against deformation. Generally speaking, if an ink is too viscous, the ink does not jet easily. On the other hand, if the viscosity is below certain range, the flow of the ink cannot be controlled and
might result in a so-called ‘fluid fishbone’ pattern.\[20\] As shown in Figure 1.8, when the drop is jetted, it breaks into a tail or ligament, depending on the viscosity of the ink and its resistance against the flow. The jet breakup depends mainly on the rheological properties of the ink. The viscosity of the ink determines the motion of the liquid filament (shape, thinning and length), while surface tension is responsible for the spherical shape of the liquid drop. The shear stress is defined as the ratio of the tangential force to the contact area and given by Equation 1.1.\[21\]

\[
\tau = \frac{F}{A} \tag{1.1}
\]

Where \(\tau\) is the stress, \(F\) is the tangential force and \(A\) is the area.

A fluid is called Newtonian if it obeys Newton’s law of viscosity, i.e., a linear approximation is valid for describing the relationship between stress and strain rate for such a fluid, according to the Equation 1.2.\[22\]

\[
\tau = 2 \eta \varepsilon \tag{1.2}
\]

Generally, it is important to know whether the ink is Newtonian or non-Newtonian, since this factor strongly affects the flow characteristics of the ink. If the ink is Newtonian (for example water and water/glycerol), it breaks into a single primary drop and smaller satellite drops.\[23, 24\] On the other hand, if the ink is non-Newtonian, it can form a longer filament with a single primary drop and without satellites.
There are two dimensionless number that are used to characterize the behavior of liquid jets: the Reynolds number and the Weber number. The Reynolds number, \( Re \), describes the ratio between inertial and viscous forces (see Equation 1.3).

\[
Re = \frac{\text{inertial forces}}{\text{viscous forces}} = \frac{\rho v d}{\eta}
\]  

(1.3)

Where \( \rho \), \( v \), \( d \) and \( \eta \) are the ink density, the drop travel velocity, the orifice diameter and the viscosity of the ink, respectively. The Weber number, \( We \), describes the correlation between inertial kinetic energy of the ink and its surface energy, given by Equation 1.4.

\[
We = \frac{\rho v^2 d}{\gamma}
\]  

(1.4)

Here, \( \gamma \) is the surface tension of the ink. Fromm [25] introduced the concept of \( Z \) number in order to predict the jetting performance of inks. \( Z \) number is defined as the ratio between the Reynolds number and the square root of the Weber number, as shown in Equation 1.5.

\[
Z = \frac{Re}{\sqrt{We}} = \frac{\sqrt{d \rho \gamma}}{\eta}
\]  

(1.5)

Note that the \( Z \) number is independent of the velocity. Using the \( Z \) number, Fromm was able to predict the integrity of the jetted drop.[25] Later, using computational fluid dynamics, Reis and Derby identified a suitable range for the \( Z \) number for inkjet printing, which lies between \( 1 < Z < 10 \).[26] Figure 1.9 provides us with a general guideline to formulate an ink suitable for printing. We can see the correlation between Weber and Reynolds numbers on the printability of an ink.[15] The conditions of generating satellites (i.e., smaller drops following the main droplet) which are unfavorable as they reduce the accuracy of printed structures, occur at higher \( Z \) numbers. On the other hand, when \( Z \) number is smaller than 1, the viscous forces dominate and prevent drop jetting.

1.1.4 Post printing treatment: from fluids to functional electronic

In order to achieve conductive or dielectric properties in a printed layer, the printed structure has to be sintered or cured. Both are done after depositing an ink material on a substrate. The sintering process is usually conducted at high temperatures in
order to remove capping agent in metal nanoparticles. Capping agents are commonly used in nanoparticle inks for stabilizing the nanoparticle and keeping the ink dispersed in solution. For example, silver nanoparticle ink, which has high popularity among the printed electronic society, is sintered to remove the organic stabilizers around the silver nanoparticles and to join the metal nanoparticles together to form a continuous and conductive electric path. Sintering is typically accomplished by the application of heat. Recently, other sintering approaches such as photonic sintering, laser sintering, plasma sintering and chemical sintering have been developed to render the sintering process suitable for large scale production on thermosensitive substrates.

Since most of the substrates utilized in flexible electronics are made of polymers, plastic deformation is a major issue that limits the sintering process. On the one hand the sintering process has to be sufficient to cause particles to coalesce. However, on the other hand, the process has to run below the glass transition temperature of the substrate in order to minimize residual stress in the polymer substrate. Not all polymers exhibit the same deformation temperature, thus the sintering step is one of the main factors affecting the choice of the substrate. Kapton® polyimide film-based substrates, polyethylene naphthalate (PEN) and polyethylene terephthalate (PET) are the most widely used substrates in inkjet printing.[28] Figure 1.10 shows the chemical structure of PEN and PET substrate alongside their glass transition temperatures ($T_g$) and melting temperatures ($T_m$).[27] Although the melting temperature differ slightly from one another, the glass transition temperature of PEN is much less than PET owing to its chemical structure in which the phenyl ring in PET is replaced with a naphthalene double ring (see Figure 1.10).
1.1 Inkjet printing

A good example that overcomes the challenge of using thermosensitive substrates while sintering the printed structure is applying short light pulses of XENON lamps. The sintering process here is based on rapidly exposing the printed pattern with a flash of light while the substrate remains relatively cold. This approach is also known as photonic sintering and has several advantages: (1) It has a lower global thermal exposure compared to conventional ovens, which allows the use of highly temperature-sensitive substrates such as paper. (2) It is in a non-contact process that does not require chemical modifications. (3) It takes place in just few milliseconds, making it suitable for roll to roll production of printed electronics.

1.1.5 Limitations of inkjet printing

The previous sections described fundamental aspects in inkjet printing in the context of printed electronic. The following section is intended to make the reader aware of the limitations in inkjet printing. Although inkjet printing holds great promises for low cost flexible electronics and rapid prototyping for microfabrication in particular, one should be aware of the current limitations imposed by the technique. In the following paragraphs, I discuss some of the drawbacks of inkjet printing encountered during the work described here.

Fig. 1.11 Image of the nozzle plate showing (a) two nozzles before jetting and (b) the same nozzles after applying voltage pulses during jetting. Red arrows indicate clear contamination on the nozzle plate.
(a) Challenges for ink development

With new emerging technologies and nanomaterials, inkjet inks are being actively developed and reported in literature. Examples for materials include conductive polymers, nanotubes, semiconducting materials, dielectric inks, proteins or even living cells. However, ink development remains an area where several investigation cycles are usually required. As discussed earlier, the physical properties of the ink will significantly influence the jetting performance. Generally, inkjet inks are limited to a certain viscosity and surface tension range (refer to 1.9). If the rheological properties lie outside this range, the ink has to be re-formulated. This sets a limit for using highly concentrated solutions as the solid loading will alter the viscosity and perhaps stability of the colloidal solution determined by Derjaguin–Landau–Verwey–Overbeek (DLVO) theory. Another example of fluid constraints in inkjet printing, is the case of gold nanoparticle ink. It has been studied and investigated by several groups and in our laboratory as well. It has been suggested that a stable solvent mixture for stabilizing these gold nanoparticle is a mixture of toluene and α-Terpineol. However, due to the high volatility of these solvents it is not proper for inkjet printing even when the right viscosity and surface tension are achieved. In that case, the ink printability time was very short (20 min) as the ink dries in the nozzle and creates clogged.

(b) Variations in drop volume

Drop volume and drop velocity might vary from nozzle to nozzle, especially when a printhead contains several nozzles. For instance, DMP printheads from Fujifilm have 16 nozzles and Samba heads have 2048 nozzles. These variations arise from small differences in the physical dimensions inside the ink channel during manufacturing. One way to overcome this is to apply different waveform pulses for each individual nozzle. Many printheads, including the printhead used in this work, allow for application of individual voltage pulses for each nozzle in order to keep the print velocity and volume similar in all the nozzles. Another possible explanation for drop volume variation is related to the ink formulation itself. If the ink contains large nanoparticles or high molecular weight polymers, this might lead to variations in the breakup time of the ligaments and thus the amount of fluid inside the spherical drop.

(c) Drop placement errors

There are several factors that might change the accuracy of drop placement. The most common one is due to the shape of the orifice. As mentioned in section 1.1.5, the
shape of the orifice might slightly vary from nozzle to nozzle during manufacturing. This variation results in small changes in the jetting angle and can be clearly observed by increasing the distance between the printhead and the substrate. The longer the distance the drop has to travel in the \( z \) direction, the more evident the misplacement will be. Another common reason for a drop to be misplaced is the presence of stacked contamination in the orifice side as seen in Figure 1.11. The contamination around the nozzle outlet is commonly seen when the ink and the printhead are used improperly. For example, consider using a silver nanoparticle ink that exhibit low drying temperature while raising up the temperature of the nozzle plate to 40°C. In this case, the ink might dry and silver nanoparticle will accumulate around the nozzle. The possible solutions to overcome this is to use a new printhead which would eventually increase the production cost.

A third possibility for inaccuracy in drop landing placement is related to the aerodynamic influences during the flight of a drop from the nozzle to the substrate.[40, 41] The aerodynamic effect in inkjet printing has been experimentally observed especially when the drop volume is less than 5 \( \text{pl} \). The air turbulences resulting from a moving substrate or printhead are powerful enough to deflect the jetted drop strongly (see Figure 1.12).[39]
Fundamentals

Fig. 1.13 Transistor circuits with channel length below 5 µm width fabricated using inkjet printer onto a pre-patterned substrate. From [Science, Vol.290 (15 December 2000):2123-2126. DOI: 10.1126/science.290.5499.2123]. Reprinted with permission from AAAS. Ref.[42].

(d) Printing resolution

Unlike photolithography, inkjet printing based microfabrication is a novel field of less than 30 years old which adapts methods used from graphic industry.[43, 44] The method is being actively developed by researchers around the globe towards better printing resolution and quality. However, the resolution is obviously limited by the volume of the ejected drop. Typically, the printed drop diameter is in the range of 10 to 100 µm (based on a drop volume from 0.5 to 500 pL).[44] Nevertheless, it is possible to circumvent this limit to some extent through surface treatment of the substrate. For example, Sirringhaus et al. fabricated all-polymer transistor circuits with channel length down to 5 µm width using inkjet printing.[42] In their approach, PI ink was employed to generate a pattern on a glass substrate before printing PEDOT:PSS drops. The PI ink (see Figure 1.13) created a hydrophobic barrier on the substrate and therefore a small channel could be realized. Nevertheless, a major drawback in pre-patterning of the surface is the introduction of other tools such as shadow mask and electron-beam lithography in the fabrication process. The question that naturally arises here is: if one of the production steps requires a clean-room facility, why not do the whole fabrication within a clean-room?
1.1 Inkjet printing

The volume of the ejected drop through a piezoelectric printhead will always be limited. One approach to overcome the resolution barrier in inkjet printing is to change the mechanism of drop ejection. For instance, on the lab scale, a super inkjet (SIJ) printer is capable of printing patterns down to 1 \( \mu \text{m} \). The jetting mechanism is based on electrospraying technology. The working principle of SIJ depends on applying a pulsed electric field to produce the required force for a drop to be ejected through a nozzle. As the drop volume depends on the strength of the applied electric field, it can generate drop volumes as low as sub-femtoliter. Another example for overcoming the resolution limit in piezoelectric printers is aerosol jet printing which can directly print structures with a 10 \( \mu \text{m} \) resolution.[45]
1.2 Electrochemistry

The impact of electrochemistry in energy can be clearly seen in many technologies including fuel cell and energy storage devices. Today, electrochemistry plays also a major role in investigating and interpreting many chemical, biological and physical processes. Over the past few decades, electrochemical measurements have furnished us with real insight into important biochemical events making it a very useful tool for scientists. One example is the fundamental impact of electrochemistry in revealing cell function and signal transmission across the human body. In this example, electrochemical methods are used to quantify and monitor individual exocytic events.[46] Apart from its powerful role in fundamental research in biology, electrochemistry has emerged as an increasingly important technique for drug-discovery and pharmaceutical research. For instance, analysis of drug metabolism by metabolizing enzymes achieved by electrochemical studies provided us with an important image of drug biotransformation prior to drug administration.[47, 48] Since 1958, when the electroactivity of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was first discovered, the use of electrochemistry in molecular genetics resulted in better understanding of DNA denaturation, renaturation, premelting and mutation. Apart from the impact of electrochemistry in exploring the intrinsic properties of DNA, electrochemical sensing of DNA has emerged as a valuable tool in genetic screening and disease detection. In particular, since the middle of 1990’s, the era of DNA biosensors has begun and the field of nucleic acid research has flourished with the development of electrochemical tools. To conclude, the development of electrochemical science contributed to the understanding of many biological events and helped in the development of medical diagnostics tools. In order to explain a new electrochemical device to serve biological research, it is necessary to first introduce essential principles in electrochemistry.

1.2.1 The electrode-electrolyte interface

In electrochemistry, we primarily deal with phenomena occurring at the electrode-electrolyte interface. When an electrode surface is first exposed to an electrolyte solution, an electrical potential difference is formed at the interface. Without any chemical reactions, the electrode-electrolyte interface can be pictured as an electrical double layer. There are several models that attempt to describe the interface phenomena. In the following sections, the most common three models are presented.
1.2 Electrochemistry

1.2.2 Helmholtz double-layer model

In 1879, Hermann von Helmholtz introduce a simple model for the double layer. In the Helmholtz model, both the metallic electrode and the electrolyte are assumed to be ideal conductors. In other words, all the excess charge is located at the metal-electrolyte interface, creating a double-plate capacitor, featuring a plate-to-plate distance. The specific capacitance in the Helmholtz model, $C_h$, is given by Equation 1.6.

$$C_h = \frac{\epsilon_0 \epsilon_r}{d}$$  \hspace{1cm} (1.6)

Where $\epsilon_0$ denotes the permittivity of free space, $\epsilon_r$ is the relative permittivity of the electrolyte solution and $d$ is the distance between the two plates of the capacitor.

However, the Helmholtz model does not consider the influence of thermal motions of ions, ion concentrations and the applied potential.

1.2.3 Gouy-Chapman model

In 1913, Gouy and Chapman, independently, introduced a model that involves a diffuse double layer rather than a compact rigid layer as in the Helmholtz model. They pointed out that the random thermal motion of the ions prevents them from remaining immobilized on the conductor surface. In their work, the ions are distributed across the diffuse double layer with a potential that decreases exponentially, according to the Boltzmann distribution. The concentration of the ions, $c_i$, is given by Equation 1.7.

$$c_i = c_i^* \exp \left( -\frac{z_i e \psi}{k_B T} \right)$$  \hspace{1cm} (1.7)

Where $z_i$ is the ions signed charge in the number of elementary charges $e$, $c_i^*$ is the concentration of the bulk solution, $\psi$ is the potential of the solution at certain distance, $T$ is the absolute temperature and $k_B$ is the Boltzmann constant. Therefore, the electric potential gradually approaches the bulk solution potential. The specific capacitance in the Gouy-Chapman model, $C_{gc}$, can be calculated from Equation 1.8.[10, 49, 50]

$$C_{gc} = \sqrt{\frac{2 z^2 e^2 c_i^* \epsilon_0 \epsilon_r}{k_B T}} \cosh \left( \frac{z e \psi_0}{k_B T} \right)$$  \hspace{1cm} (1.8)

Here, $\psi_0$ and $z$ denote the electrode potential and the magnitude of the charge on the ions, respectively. This model can only explain systems with low ion concentrations and at low overpotentials.
In 1924, Otto Stern proposed another model that accounts for the finite size of the ions and adsorption. The model basically combines the Helmholtz model with the Gouy-Chapman model. Stern’s model consists of two parts: (i) Stern layer (compact layer of ions) and (ii) Gouy-Chapman layer (diffuse layer). The specific capacitance in Gouy-Chapman-Stern model, $C_{gcs}$, is given by:

$$\frac{1}{C_{gcs}} = \frac{1}{C_h} + \frac{1}{C_{gc}}$$  \hspace{1cm} (1.9)

Stern specified that the ions cannot approach the surface to a distance lower than the ionic radius. Additionally, he considered a distance for a solvation shell, which is formed around the ions. Therefore, Stern layer is a region free from charge adjacent to the electrode surface.

In 1947, Grahame further developed the work of Stern by considering the scenario when the ions lose their hydration shell and penetrate the Stern layer. In his work, also known as Grahame-model, Stern layer is divided into two planes: (1) the inner Helmholtz plane (IHP), which is located at a distance $X_1$ from the electrode surface and consists of molecules and adsorbed ions, and (2) the outer Helmholtz plane (OHP) which contains solvated non-specifically adsorbed ions. As these ions are surrounded by the solvent molecules due to hydration, they are located at a distance of $X_2$. The ions in the OHP region are less concentrated compared to the IHP. Next to this layer comes...
1.2 Electrochemistry

the diffuse double layer. The diffuse double layer has a thickness usually represented by the Debye length, \( \lambda_D \). The region starts from OHP into the solution up to the point where the surface effect is no longer present. Therefore, the Debye length is highly dependent on the concentration of ions in the electrolyte solution. For instance, Debye length for pure water is approximately 1 \( \mu \)m, while Debye length for 1 M KCl dissolved in water is just 0.3 nm.\(^{[51]}\) A schematic representation of the EDL according to the aforementioned models can be seen in Figure 1.14.\(^{[10]}\)

1.2.5 Fick’s laws of diffusion

In the previous section, the equilibrium of ions within an electrolyte in the vicinity of the electrode surface was described. However, to describe the dynamics of ions in a liquid, we can start from the simplistic approach of non-interacting one-dimensional random walk. Imagine a set of discrete allowed positions along the \( x \) axis with a step size of \( \Delta x \) that each can be occupied by an unrestricted number (\( N \)) of particles at a time (\( t \)). Hence we may write \( N = N(x, t) \). After a discrete step in time, \( \Delta t \), all the particles hop into one of the two neighboring sites, with equal probabilities of 0.5 for making a forward or a backward step. Now if we define the diffusion flux, \( J(x, t) \), as the number of particles crossing a unit area, \( A \), of the interface of two neighboring sites per unit of time at a specific position and time (in \( \text{mol m}^{-2} \text{s}^{-1} \)), we can argue that:

\[
J(x, t) = -\frac{1}{A} \frac{1}{\Delta t} \left[ \frac{N(x + \Delta x, t)}{2} - \frac{N(x, t)}{2} \right] \tag{1.10}
\]

Multiplying the numerator and denominator of the Equation 1.10 by \( (\Delta x)^2 \) leads to:

\[
J(x, t) = -\frac{(\Delta x)^2}{2\Delta t} \left[ \frac{N(x + \Delta x, t)}{A (\Delta x)^2} - \frac{N(x, t)}{A (\Delta x)^2} \right] \tag{1.11}
\]

Concentration is defined as the number of particles per unit volume (in \( \text{mol m}^{-3} \)):

\[
c(x, t) = \frac{N(x, t)}{A \Delta x} \tag{1.12}
\]

In addition, in a one-dimensional system, the diffusion constant (\( \text{m}^2 \text{s}^{-1} \)) is defined as:

\[
D = \frac{(\Delta x)^2}{2\Delta t} \tag{1.13}
\]
Equations 1.11, 1.12 and 1.13 lead to:

\[ J(x, t) = -D \left[ \frac{c(x + \Delta x, t)}{\Delta x} - \frac{c(x, t)}{\Delta x} \right] \]  

(1.14)

In the continuum limit, the Equation 1.14 can be written as the following:

\[ J(x, t) = -D \frac{\partial c(x, t)}{\partial x} \]  

(1.15)

The derived Equation 1.15 is the one-dimensional case of the Fick’s first law of diffusion. The multidimensional case of it, with \( J(r, t) \) denoting the diffusion flux vector, could be formulated as follows: \[22\]

\[ J(r, t) = -D \nabla c(r, t) \]  

(1.16)

On the other hand, we know that the change of the number of particles in any volume of the space is only due to inflow and outflow of particles through the boundary of that volume. This is called the *continuity equation* \[22\] which could be written in the following formalism:

\[ \frac{\partial c(r, t)}{\partial t} + \nabla \cdot J(r, t) = 0 \]  

(1.17)

Finally, combining equations 1.16 and 1.17 brings us to the Fick’s second law of diffusion:

\[ \frac{\partial c(r, t)}{\partial t} = D \nabla^2 c(r, t) \]  

(1.18)

Equation 1.18 can be reduced to 1.19, which describes the one-dimensional case.

\[ \frac{\partial c(x, t)}{\partial t} = D \frac{\partial^2 c(x, t)}{\partial x^2} \]  

(1.19)

Obviously in practice we are dealing with multiple dimensions. Nevertheless, the latter equation is applicable when the symmetry of the system allows for reducing it to a one-dimensional case. A planar electrode can be considered as such a system.

In the context of electrochemical reactions, the diffusive flux of an electroactive species can be correlated to the current \( i \) by the following equation:

\[ J(x, t) = \frac{i}{nFA} \]  

(1.20)
Where $F$ is Faraday constant ($F = 96485.3$ C mol$^{-1}$) and $n$ is the number of electrons transferred. Based on Fick’s second law, in 1905 Frederick Gardner Cottrell correlated the time and current when a sufficient potential is applied. Equation 1.21 shows the current equation for a planar electrode.

$$i = \frac{nFAc^* \sqrt{D}}{\sqrt{\pi t}} \quad (1.21)$$

Where $c^*$ is the bulk concentration of the redox molecule. The Cottrell equation is valid when the current of the redox reaction is only governed by the diffusion of the redox probe to the electrode surface.

### 1.2.6 Electrochemical cell

In electrochemical systems, the transport of charge between an electronic conductor (an electrode) and an ionic conductor (an electrolyte) can be measured through different types of electrochemical techniques. If we apply an electric potential to an electrode, the current will travel by the movement of electrons. In electrolyte solution, however, the charges are transferred through the movement of the ions. To construct an electrochemical cell, the electrode has to be sufficiently conductive, inert and stable. Typical electrodes used in electrochemistry are gold, platinum and carbon. The electrode at which the electrochemical reaction occurs is known as the working electrode. There are two main electrochemical cell designs that are commonly used in sensing: (i) three-electrode cells and (ii) two-electrode cells. In case of three-electrode configuration, a counter electrode (also known as an auxiliary electrode) is used as a current sink in order to shunt the excess current, while a reference electrode is used to provide a constant potential. However, if the current density is not large enough to damage the reference electrode, a two-electrode cell configuration can be used. In this case, one electrode is used as both counter and reference electrode.

Both the working electrode and the counter/reference electrode are immersed in an electrolyte solution. The charges are carried by the movement of the ions dissolved in the electrolyte solution. In this thesis, we will encounter aqueous based electrolyte solutions in which ion species such as $H^+$, $Na^+$ and $Cl^-$ are dissolved in water. We can sense an electrochemically active molecule by observing the Faradaic current occurring at the electrode site. To understand the electrochemical reaction, let’s consider the following Equation 1.22.

$$ox + ne^- \xrightleftharpoons[k_i]{k_f} red \quad (1.22)$$
Where \( ox \) is the oxidized species, \( red \) is the reduced species. \( k_f \) and \( k_b \) are the oxidation and reduction rate constants, respectively. The relationship between the concentration of the oxidized, concentration of the reduced and the free energy (\( \Delta G \)) is given as in Equation 1.23.

\[
\Delta G = \Delta G^0 + RT \ln \frac{[ox]}{[red]}
\]

(1.23)

Where \( \Delta G \) is the Gibbs free energy, \( \Delta G^0 \) is the standard Gibbs free energy change [J mol\(^{-1}\)], \( R \) is the gas constant and \( T \) is the temperature in kelvin. Based on that, we can correlate the electrode potential (\( E \)) (also known as open circuit potential) as shown in Equation 1.24.

\[
\Delta G = -nFE
\]

(1.24)

Thus

\[
\Delta G^0 = -nFE^0
\]

(1.25)

Where \( n \) denotes the number of electrons transferred, \( F \) is Faraday constant and \( E^0 \) is the standard potential of the electrochemical cell. This leads us to the Nernst equation which correlates the potential of the cell and concentration of the cell reaction 1.26

\[
E = E^0 + \frac{RT}{nF} \ln \frac{a^*_{ox}}{a^*_{red}}
\]

(1.26)

However, the activity of the redox molecules is affected by the presence of other ions in the solution. Therefore, we can rewrite the Nernst equation using the concentration instead of the activity as in Equation 1.27.

\[
E = E^{0'} + \frac{RT}{nF} \ln \frac{c^*_{ox}}{c^*_{red}}
\]

(1.27)

Where \( E^{0'} \) is the formal potential, \( c^* \) is the bulk concentration of the oxidized or reduced species [mol L\(^{-1}\)] and \( a^* \) is the activity of the oxidized or reduced molecules. The Nernst equation for the redox pair ferricyanide \( Fe^{III}(CN)_6^{3-} \) and ferrocyanide \( Fe^{II}(CN)_6^{4-} \) can be written as shown in Equation 1.28.

\[
E = E^{0'}_{Fe^{II}(CN)_6^{4-}, Fe^{III}(CN)_6^{3-}} + \frac{0.059 V}{1} \log \left( \frac{[Fe^{III}(CN)_6^{3-}]}{[Fe^{II}(CN)_6^{4-}]} \right)
\]

(1.28)

During oxidation reaction, a molecule will lose an electron and become oxidized with more positive charges due to an increase in the oxidation state. If an electrode provides an oxidation potential, that is biased above the redox potential, the molecules in the
surface will lose its electron once it comes close enough to the working electrode. If the chemical process is reversible, upon biasing the electrode to a reducing potential, electron exchange would occur between the electrode surface and the molecule leading to its reduction. The generated current due to an electrochemical reaction is called Faradaic current and is given by Faraday’s Law as in Equation 1.20.

1.2.7 Selected technique: Voltammetry

Up to this point, we have focused solely on general aspects (phenomena and laws) in electrochemistry. In the following section, we will cover voltammetric methods as a common approach for obtaining analytical information about electrode-electrolyte. Special attention is given to cyclic voltammetry in this section because it constitutes the majority of experiments conducted throughout this thesis.

One way to conduct an electrochemical experiment is to use a voltammetric technique where the potential is swept in one way or another. In potential sweep measurements, the current can be recorded upon applying a linear potential sweep at a fixed rate over time. The method is known as linear sweep voltammetry (LSV). Usually, the current is recorded while applying two values for the potential. The first value ($E_1$) is typically below the threshold of the electrochemical reaction to be investigated. While the second value ($E_2$), is chosen at which a chemical reaction (oxidation or reduction) can be driven at the electrode surface. In linear sweep voltammetry, the measurements end at ($E_2$) value. In cyclic voltammetry, once the scan reaches ($E_2$) value, the scan direction is reversed and returns to ($E_1$) value as can be seen in Figure 1.15a.

Cyclic Voltammetry (CV) is a voltammetric technique, meaning that we control the potential (V) and observe the resultant current. It is a highly versatile technique for investigating redox molecules. CV is usually the first technique to be used to understand a new electrochemical system for three reasons. First, the current-voltage curves can provide information about the thermodynamics of redox processes and the kinetics of electron transfer reactions or coupled chemical reactions. Second, it gives us information about the geometrical shape and area of the electrode’s surface. Third, it is easy to perform by changing the applied waveform. The typical voltammogram shape for diffusion controlled electrode reactions with increasing and decreasing current signals giving a characteristic peak can be seen in Figure 1.15b. According to Fick’s first law of diffusion mentioned in Equation 1.16, the current will depend on the concentration gradient at the electrode surface.
The peak height of the voltammogram for a planar diffusion employing an electrochemically-reversible redox species is given by Randles–Sevcik equation:

\[ i_p = 0.4463 \frac{n^2 F^2}{R^2 T^2} A D^{1/2} c^* v^{1/2} \]  

(1.29)

Where the peak current, \( i_p \), depends on the square root of the scan rates \( v \) with a diffusion coefficient \( D \).

\[ i_p \propto v^{1/2} \]  

(1.30)

During voltammetric experiments, the potential scan rate, \( v \), is typically set in the range of a few \( mV s^{-1} \) to a few \( V s^{-1} \) as in Equation 1.30. Figure 1.16 shows the influence of the scan rate on the peak current for a reversible electron transfer process.

One can understand the rate of the mass transport by observing the changes in the shape of the voltammogram. If the mass transfer coefficient, \( K \), is smaller than the electron transfer rate, the peak to peak separation is described by the following equation at 25 °C.[52]

\[ \Delta E_p = E_{pa} - E_{pc} = \frac{59}{n} mV \]  

(1.31)
1.2 Electrochemistry

Fig. 1.16 Relationship between the peak current and the scan rate

\[ \left| \frac{i_{pa}}{i_{pc}} \right| = 1 \] (1.32)

Where \( E_{pa} \) and \( E_{pc} \) are the anodic and cathodic peak potentials as can be seen from Figure 1.15.

In case the mass transfer becomes larger than the electron transfer rate as shown in Figure 1.17 case b-d, the peak separation increases as more energy (overpotential) is needed to drive the reaction. This can take place when the electrochemical reaction is irreversible, as the reverse peak in the backward scan becomes less definite.

In micro- and nanoelectrodes, with spherical diffusion profile, the mass transport towards the electrode surface is typically in a steady state. The voltammogram curve shows a sigmoidal wave as shown in Figure 1.18. In this case, the current is usually referred to as diffusion-limited plateau current or steady-state current. Here, the current can provide us with the radius of the microelectrodes. In order to study the electrode size from the measured current, it is recommended to use a standard redox probe with fast diffusion and rapid heterogeneous electron transfer.

When the mass transport is limited by diffusion, the measured current is proportional to the diffusion coefficient of the redox probe. This is very useful for estimating the diffusion coefficient of redox species.[53–55]

When the size of the electrode becomes smaller than 25 \( \mu \text{m} \), the electrochemical
behavior of the electrode appears not to follow the theoretical expectations based on larger electrodes.[56] These microelectrodes come in a variety of geometrical shapes such as inlaid disks, inlaid rings, inlaid ring-disks, hemispherical and spherical electrodes as shown in Figure 1.19. There are plenty of publications on microelectrode array

![Diagram of normalized current vs. E]

**Fig. 1.17** The effect of the transfer kinetics on the voltammogram shape. Curve (a) shows fast oxidation and reduction at the electrode surface. The peak position shifts (for a quasi-reversible reaction) in curves (b) to (d). The shifts appear to the right with decreasing the rate constant. Reprinted with permission from [53].

![Diagram of typical cyclic voltammogram]

**Fig. 1.18** Typical cyclic voltammogram obtained at microelectrode.
fabrication and electrochemical characterization\cite{57, 58} Table 1.1 summarizes the possible diffusion profiles and the corresponding steady-state current.

![Common microelectrode geometries](image)

**Fig. 1.19** Common microelectrode geometries: A) hemispherical, B) inlaid disk, C) recessed disk. Reproduced with permission from Ref.\cite{59}.

**Table 1.1** Summarizes the steady-state currents for different electrode geometry

<table>
<thead>
<tr>
<th>Electrode type</th>
<th>Steady-state current</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemispherical</td>
<td>$i_{\text{Lim}} = 2\pi n F D c^* r$</td>
<td>[59]</td>
</tr>
<tr>
<td>Inlaid disk</td>
<td>$i_{\text{Lim}} = 4n F D c^* r$</td>
<td>[52]</td>
</tr>
<tr>
<td>Recessed disk</td>
<td>$i_{\text{Lim}} = \frac{2\pi n F D c^* r}{4L + \pi r}$</td>
<td>[60]</td>
</tr>
</tbody>
</table>

In a microelectrode array, it is possible to change from planar to radial diffusion by changing the scan rate. Figure 1.20 demonstrates the voltammogram shape of microelectrode with different scan rate. At a fast scan rate, the peak-shaped voltammogram becomes evident, similar to the planar diffusion. On the other hand, when the scan rate decreases, the voltammogram displays the sigmoidal shape of the radial diffusion. The reason for that is the formation of a stationary diffusion layer as a result of the high diffusion mass transport. Here the current magnitude is independent of the scan rate\cite{61}.

Another critical point when looking at the limiting current in the current voltage curves is observing the effect of concentration polarization of the redox molecule on the current. This will let us know the initial oxidation state of the molecule arriving at the electrode surface. If both oxidized ($\text{ox}$) and reduced ($\text{red}$) molecules are initially
present in the bulk solution, the current potential curve will be similar to the one in Figure 1.21a. Where \( i_{l,a} \) is the anodic limiting current and \( i_{l,c} \) is the cathodic limiting current. When the current is zero, the system is at equilibrium state and \( E = E_{eq} \). Here, the anodic current represents the maximum rate at which the reduced species can travel to the electrode surface for conversion to oxidized molecules. In a system where the reduced species is initially absent i.e. containing only the oxidized molecules, only the cathodic currents will be seen as shown in Figure 1.21b.

### 1.2.8 Redox cycling

Single working-electrode has been commonly used in electrochemistry for a wide variety of application areas such as electrochemical biosensors in which a target analyte is being detected. These electrodes come in different geometrical shapes, arrangements

![Cyclic voltammograms simulated for microelectrode with different scan rates](image)

**Fig. 1.20** Cyclic voltammograms simulated for microelectrode with different scan rates: a) 1 \( mVs^{-1} \), b) 200 \( mVs^{-1} \), c) 2000 \( mVs^{-1} \). Reproduced with permission from Ref.[52].
and designs such as microelectrode arrays. In this system, the electrode is polarized to an electric potential which is sufficient for driving a redox reaction, either oxidation or reduction, but not both. Dual-electrode systems, on the contrary, employ two individually addressable electrodes. One electrode is biased to a reducing potential while the second electrode is biased to a potential where an oxidation reaction occurs. In dual-electrode systems, the result of a reaction taking place at one electrode, commonly referred as the *generator electrode*, is converted back at a second electrode, so-called *collector electrode*. This method is used to amplify the measured current as the molecule is being recycled by traveling back to the generator electrode. This continuous cycling of a redox molecule occurs when a second electrode is placed nearby the first electrode and biased at opposite potential. The concept is based on two closely spaced electrodes biased to a potential above and below the redox potentials of the molecule as shown in Figure 1.22).

Fan and Bard have introduced the idea of a single molecule detection using electrochemical redox cycling.[62, 63] In their work, a nanometer distance was formed between a conductive surface and Pt-Ir tip of electrochemical microscopy. The redox probe was placed between the two conductive surfaces and underwent reparative oxidation and reduction events allowing for a single molecule resolution. The time between oxidation and reduction event, in redox cycling, has to be short. The redox cycling current $i_{rc}$ for a concentration $C$ and in the confined region between the electrodes electrodes is given by Equation 1.33

$$i_{rc} = \frac{z_i e F D A}{h} \, c = \frac{z_i e D}{h^2} \, n$$  

(1.33)
Where $z$ is the number of electrons involved in the redox reaction, $F$ is the Faraday constant, $A$ is the electrode area and $h$ is the distance between the two electrodes. Since the redox cycling principle is used to amplify the measured current, it is necessary to determine the amplification factor. Unfortunately, there are several ways in the literature which describe how to determine this factor. One way to estimate the amplification factor ($AF$) is based on the ratio of currents obtained when using the same electrode in redox-cycling mode, $i_{on}$, and non-redox cycling mode, $i_{off}$, meaning that only one of the working electrodes is active for either oxidation or reduction.

$$AF = \left| \frac{i_{on}}{i_{off}} \right|$$  \hspace{1cm} (1.34)

Another valuable parameter in redox cycling is the collection efficiency $CE$. This parameter provides us with information of the ratio of oxidized and reduced molecules due to redox cycling and can be estimated by Equation 1.35.

$$CE = \frac{i_c}{i_g}$$  \hspace{1cm} (1.35)
Where $i_c$ and $i_g$ are the collector and generator currents, respectively. Obviously, collection efficiency will increase with decreasing distance between the generator and collector electrodes.

1.3 Biosensors

1.3.1 Principle of biosensor

The past decade has witnessed a great expansion in biosensor devices due to its useful applications in detecting and monitoring diseases. For instance, since the introduction of the first home care glucose-meter in 1987, the life quality of diabetic patients has dramatically improved. The idea of sensing glucose level in blood goes back to 1962 when Clark invented the first electrochemical biosensor using the glucose oxidase enzyme.[64] In 1992, The International Union of Pure and Applied Chemistry (IUPAC) has defined a biosensor as “a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals”. [65] The biosensor consists of three main components: a bioreceptor, a transducer, and a signal read out part. Figure 1.23 depicts the main building blocks for a biosensor. A signal is generated when the bioreceptor element captures or recognizes a specific analyte or a target. Biosensors can be classified according to the biological element type or the signal transduction method. The bioreceptor could be tissues, whole cell receptors, enzymes, antibodies or antigens, nucleic acids, aptamers. Generally, biosensors which utilize one of the three latter biorecognition components, are classified as immunosensors, genosensors.

![Fig. 1.23 Illustration of the three main elements in a biosensor.](image)
and aptasensors respectively. Biosensors can also be further classified according to the transduction mode: optical, mechanical, electrical, or electrochemical.

The performance of the biosensor depends strongly on certain factors (see in Figure 1.24). Generally speaking, a biosensor should possess high sensitivity and excellent selectivity. However, such an ideal biosensor device which combines all these features listed in Figure 1.24 still does not exist. Nevertheless, some of these characteristics are essential and have to be taken into account when designing a new biosensor. Below I discuss some of the basic characteristics determining the reliability of biosensors.

![Ideal biosensor characteristics](image)

**1.3.2 Biosensor characteristics**

**Selectivity**

Perhaps proving the selectivity of the biosensor is the most crucial factor determining its usefulness. Selectivity is the ability of the biosensor to identify or discriminate a target analyte in a mixture of other interference molecules. This means that the signal generated by the biosensor has to come only from capturing a target molecule in a solution which contains other admixtures and contaminants. This is usually defined by two factors. First, the binding selectivity of a bioreceptor to its target. For example the antibody-antigen recognition event, or DNA hybridization (recognition) reaction between the single strand DNA (ssDNA) and a complementary ssDNA. The second factor that strongly influences the selectivity is the efficiency in blocking or passivating the transducer surface to prevent non-specific adsorption. This is usually
achieved by creating a repellent surface that prevents any physisorption of unwanted biomolecules. For example, empty area on the transducer surface is typically filled with macromolecules such as bovine serum albumin (BSA) via physical adsorption of the protein. This will ensure that other proteins present in the specimen will not adhere to the surface as it is already being occupied by BSA.

**Sensitivity**

Sensitivity can be defined as the change in the sensor response ($\Delta S$) for a given change in concentration ($\Delta c$). The sensitivity is usually calculated from the slope of the calibration curve in the linear range. The minimum quantity that can be detected is typically referred as the *limit of detection*, (LOD), and can be estimated as the following according to IUPAC convention.[66]

\[
LOD = LoB + 3 \cdot \sigma_{(blank)}
\]  

(1.36)

and

\[
LoB = \overline{B} + 3 \cdot \sigma_{(blank)}
\]  

(1.37)

Where $\overline{B}$ is average blank signal and $LoB$ is the limit of a blank. There are several factors that might effect the sensitivity of the biosensor. These factors include the electrode active area, the mass transport and the thickness of the bioreceptor on the solid surface.

**Stability**

It is important to determine the stability of a biosensor, as this will not only tell us how long the biosensor can be stored until an unreliable signal output is measured, but also it can give a correction factor for an expected drift. For instance, when using a biosensor incorporating an enzyme or an antibody as a biorecognition element, one has to consider the decrease in the sensor signal upon relatively long storage due to enzyme inactivation. To overcome this, a stabilization layer could be added to the biorecognition layer to increase the life expectancy of the sensing element. Most commonly, a stabilization layer such as 10% sucrose solution can be used to protect antibodies when building an immunosensor, while 20% glycerol has shown effective protection for enzyme when developing an enzymatic membrane biosensor.[67]
Fig. 1.25 Schematic illustration of possible orientations of an IgG immobilization leading to (a) fully active antibody, (b) partially active and (c) inactive antibody immobilization.

**Dynamic response**

Another important characteristic in biosensors that has to be analyzed is the dynamic range. The dynamic range of a biosensor can be defined as the concentration window from the maximum unsaturated signal to the minimum detectable quantity or LOD (refer to section 1.3.2 for LOD calculation). This is usually estimated from a calibration curve in which the relationship between a measured analyte’s concentration \( x \) is proportional to the signal \( y \). Depending on the application, the dynamic range requirement varies for different biosensors. For instance, a wide linear range is usually desirable in DNA sensors in order to determine the prognosis of certain diseases such as cancer and HIV. In this case, dynamic response between 30 and 3000 copies of DNA is required for developing an assay.[68, 69]

### 1.3.3 Bioreceptor immobilization techniques

Proper immobilization of the bioreceptor in terms of orientation and density is one of the most critical aspects in determining the success of the detection scheme. The reason for this is the orientation of biomolecules on the solid support will affect the specific binding constants and, thus, the sensitivity of the sensor. In case of immunoassays, antibodies (or antigens) are usually used as a bioreceptor molecules. Here, the ability of an immobilized antibody (Ab) to capture its target antigen (Ag), is used for detecting protein biomarkers. There are several strategies for immobilizing antibodies on the transducer surface: (i) adsorption (ii) covalent immobilization and (iii) non-covalent immobilization. Physical adsorption of antibodies is by far the easiest approach as it requires no modification to the Ab and can achieve high
immobilization densities. However, it suffers from random orientation and denaturation of the antibodies. Moreover, leakage of Ab from the surface, caused by the weak bonding energies, is common, resulting in low reproducibility of the assay.[70]

On the other hand, covalent binding of the antibody to substrate results in a more stable immobilization, thus it can enhance the reproducibility of the assay. Covalent immobilization is achieved via a linkage group such as amine, thiol, carboxyl or hydroxyl. The functional group is typically bonded to a defined linker molecule such as self-assembled monolayers (SAM). However, covalent binding also has disadvantages, including: (a) the need of modification of Abs prior to coupling and (b) lack of control of the orientation of Abs leading to partial or complete blocking the of the active binding site as seen in Figure 1.25 b and c, respectively.

Preserving the biomolecules’ activity and preventing protein denaturation remains a hot topic in research as it directly influences the biosensor performance. In order to avoid protein denaturation, non-covalent immobilization using affinity interactions is usually used. Non-covalent immobilization such as His-tag system, protein G from Streptococcus C40 and protein A from Staphylococcus aureus. are utilized for immobilizing the antibodies to the surface in oriented manner. Protein A and G have a binding affinity to the fragment crystallizable region (Fc region) of the antibody and, thus, ensure a uniform orientation.[71–74]
Chapter 2

Label-free impedimetric immunosensor based on UV-activated antibodies

The following chapter was adapted in part from the following work:

C-reactive protein (CRP) is a plasma biomolecule produced in response to several inflammatory processes. It is a pentameric protein (the monomer weighs about 25 kDa) whose normal level ranges from 1 to 4 µg/mL; however, as a consequence of inflammation or bacterial infection, CRP concentration can rise over 10 µg/mL. For instance, mild inflammation and viral infections typically result in a concentration ranging between 10 and 40 µg/mL, active inflammation and bacterial infection lead to higher levels (40 – 200 µg/mL). CRP concentrations higher than 20 µg/mL have been observed in severe bacterial infections and burns. Moreover, since several studies report that CRP is involved in many pathways, and the level of this protein is related to a wide range of pathologies, traumas and some types of cancer, quantifying this analyte and more specific biomarkers is a crucial task for clinical diagnostics. C-reactive protein is usually quantified using two groups of standard antibody-based assays. Conventional CRP tests are used for the general evaluation of infections, injuries and inflammation processes, and only values above 10 µg/mL are considered clinically relevant. In a second type, high-sensitivity CRP (hsCRP) and cardiac CRP (cCRP) assays provide a detection range between 1 and 10 µg/mL and are used to evaluate the risks of heart attacks and strokes in healthy people. Examples of these clinical laboratory approaches are particle-enhanced immunonephelometry tests, immunoradiometric assays and several enzyme-linked immunosorbent assays (ELISA) methods. These standard and reliable methodologies are extremely sensitive, but the need for expensive equipment and trained personnel strongly limits their use in point-of-care applications. Moreover, in many cases, the knowledge of CRP concentration in blood is not sufficient to perform a correct diagnosis, and other biomarkers should be quantified. These strong motivations underpin the research on easy-to-use and portable detection tools suitable for on-site and multiplex analysis. Over the last 30 years, there has been an increasing interest in improving existing CRP detection technologies and developing novel approaches to overcome the limitations of standard laboratory techniques. In this task, biosensor-based detection methods have gained in importance in view of cost effectiveness, simplicity of use and their extremely high selectivity, which allows one to analyze complex samples such as blood and serum without involving complex and time-consuming pretreatments. For instance, Cheng et al. developed a quantum dot–based immunochromatographic test reporting a limit of detection (LOD) of about 0.25 µg/mL and an exceptionally wide linear response between 0.5 and 10 mg/mL using a one-step sandwich immunoassay. More recently, Lin et al. proposed a low-cost paper-based detection system for
semi-quantitative and rapid determination of CRP level. This technique involves latex-functionalized paper, which is able to interact with CRP in blood, thus driving the flow of the fluid into a channel a few millimeters long. The length of this path is an indicator of CRP concentration; however, this device does not estimate CRP levels lower than 10 µg/mL and is thus a valid tool only for monitoring generic inflammations. Another paper-based approach has been reported by Dong et al., who incorporated microfluidic channels with a smartphone camera, which acts as a detector for a one-step sandwich immunoassay.[90] This method allows for an LOD of about 54 ng/mL but requires the use of gold nanoparticles conjugated antibodies to perform the analysis. Of the many biosensor-based detection methods, electrochemical biosensors gained a dominant role in realizing novel detection approaches to quantify CRP levels because of their small size, ability to perform multiple analyses on the same sample and capability of operating even in turbid media [91]. For instance, Centi et al. have developed an electrochemical assay based on the simultaneous binding of CRP by a Ribonucleic acid (RNA) aptamer immobilized on magnetic particles and a monoclonal antibody coupled with alkaline phosphatase. By applying a magnetic field, the recognition complexes are conveyed onto a carbon-based screen-printed electrode, where the amount of α-naphthol, the product of a reaction catalyzed by the alkaline phosphatase, is detected by using differential pulse voltammetry.[81] The quantification of this molecule allows the CRP concentration to be estimated, thus achieving an LOD of about 200 ng/mL by adopting this complex and laborious procedure. Another RNA aptamer–based approach has been proposed by Qureshi et al., who have effectively detected CRP in a concentration ranging between 100 and 500 pg/mL by using aptamer-functionalized gold interdigitated capacitor arrays. However, this extremely sensitive device has not been tested on a real serum or blood sample.[92] An alternative method based on electrochemical impedance spectroscopy (EIS) has been developed by Bryan et al., who use a complex and time-consuming functionalization procedure to immobilize anti-CRP antibodies onto gold electrodes, thus achieving an LOD of about 176 pM.[93] Another impedimetric approach has been reported by Chen et al., who achieved an effective detection range between 0.1 and 20 ng/mL by using antibody-functionalized three-dimensional ordered macroporous gold film–modified electrodes. However, due to the complexity of the manufacturing and functionalization procedures, trained laboratory personnel and advanced manufacturing technology is required, leading to an increase in production costs and therefore limited use of these devices.[94] Songjaroen et al. report another EIS method based on antibody immobilization onto gold electrodes by a ds-DNA linker, thus achieving a linear response for CRP concentrations ranging
between 3.125 and 25 µg/mL.\[95\] A promising label-free approach has been proposed by Gupta et al., who achieved an LOD of about 11 ng/mL using carbon nanofiber electrodes assembled in a $3 \times 3$ array configuration.\[96\] Despite such high sensitivity, the performance of this device has not been tested in analyzing a real sample.

In this work, we report a simple and reliable label-free EIS-based immunosensor for quantifying CRP in blood serum. We couple an unconventional functionalization approach called the photochemical immobilization technique (PIT) \[97–99\], which is based on the proper anchoring of antibodies upon activation with ultraviolet light (UV) light, with printed gold microelectrode arrays. The gold surface of the electrode was functionalized by using PIT \[100\], a functionalization method based on the immobilization of antibodies onto thiol-reactive surfaces such as gold plates, upon photochemical reduction of disulphide bridges in proteins by UV illumination of nearby aromatic amino acids \[101\]. It is well known that UV radiation strongly affects both the structure and activity of biomolecules, but by studying many different antigen-antibody systems we have found that this photochemical activation of immunoglobulins does not affect their ability to capture the antigen (bb gliadin amylase). In addition, the recent single molecule characterization of UV-activated antibodies by means of atomic force microscopy \[99\] shows that PIT is able to steer the immobilized immunoglobulins into an upright orientation where at least one of the antigen-binding sites of the biomolecule

**Fig. 2.1** Sketch of a typical type-G immunoglobulin (IgG) showing the effect of the photonic activation step. A UV photon is absorbed by the Tryptophan residues and then transferred to the Cys-Cys. As a result, a disulfide bridge is opened, forming free thiol groups, which can covalently bind to the gold electrode.
is well exposed to the environment, thus greatly enhancing the detection efficiency compared to random physisorbed antibodies.

This printed electrode technology allows disposable, cheap and small sensors to be used directly on polymeric flexible substrates, which perfectly fits the requirements for biomedical point-of-care applications. The direct analysis of CRP samples incubated with PIT-functionalized gold electrodes allows this biomarker to be effectively quantified in a concentration range that covers the typical CRP values in blood. The cost effectiveness and simplicity of this approach make our EIS-based immunosensor a valid alternative to conventional laboratory analysis. Moreover, since microelectrode array technology can easily implement multiple and simultaneous analyses of different parameters for the same sample, this methodology could be a powerful tool to realize new detection approaches for wider characterizations of biological fluids, which can be extremely beneficial for clinical diagnostics.

2.2 Materials and methods

2.2.1 Preparation of the gold ink

Gold nanoparticle ink was prepared as reported by Bachmann et al. [4] Briefly, hexanethiol-capped gold nanoparticles were prepared using Brust–Shiffrin synthesis. The prepared gold nanoparticles were dissolved at a concentration of 20 wt% in a 30/70 wt% mixture of toluene and α-terpineol to obtain a jettable viscosity. The solution was filtered through a 0.45 μm filter to obtain the final ink.

2.2.2 Inkjet printing of the electrode array

Figure 2.2 depicts the process flow for the fabrication of the immunosensor. PEN (Q83) was bought from Teijin DuPont Films and was used as a flexible substrate for inkjet printing. An OmniJet 300 inkjet printer, UniJet Co., Republic of Korea, was employed for the device fabrication using either 1 or 10 pL, DMC cartridges from Fujifilm Dimatix Inc., USA. The sintering and curing steps were conducted on a precision hot plate (CT10, Harry Gestigkeit GmbH, Germany). Prior to printing, all inks were sonicated in a sonication bath for 5 min and filtered with a 0.45 μm syringe filter.

After printing the gold layer, the array was exposed to oxygen plasma in a plasma oven (Nano, Diener Electronic, Germany) at a power of 30 W and pressure of 0.2 mbar for 12 s. Subsequently, a dielectric layer of a polyimide-based (PI) ink was purchased (PIN 6400–001, Chisso Coorporation). The ink was filtered via a hydrophobic 0.1 – μm
PTFE. The PI ink was printed with a drop spacing of 25 µm and frequency of 1 kHz. Finally, the passivation ink was cured thermally by heating to 80 °C on a hot plate for 5 min followed by 120 °C for 30 min.

![Diagram](image.png)

**Fig. 2.2** Schematic diagram showing the sequence of fabrication steps.

### 2.2.3 Surface characterization by AFM

Atomic force microscopy (AFM) characterization of printed electrode arrays was performed in tapping mode using a Nanoscope Multimode 8 (Bruker) microscope equipped with a 125 µm piezoelectric scanner and aluminium back–coated Si cantilevers from Bruker (OTESPA-R3) with typical values of $k = 26$ N/m and $f = 0 − 300$ kHz and a tip radius (nom) of 7 nm. The AFM data were processed using NanoScope Analysis 1.50 (Bruker) and SPIP 6.5.2 (Image Metrology).

### 2.2.4 Electrode array preparation

The printed chips were cleaned using a two-step protocol: (i) chemical cleaning followed by (ii) electrochemical cleaning. First, they were cleaned by immersion in an ethanol solution for 15 min followed by rinsing with deionized water (from a Millipore Milli-Q system, 18 Ω·cm$^{-1}$). Next, the electrodes were electrochemically treated by cycling ca. 30 times (scan rate 100 mV/s) between $-0.3$ and $1.6$ V in 0.5 M sulphuric acid. The gold surfaces were considered clean when a stable cyclic voltammetry (CV) plot was recorded, as shown in Figure 2.3. Moreover, in a cyclic voltammetry experiment performed in a ferricyanide Fe$^{III}$(CN)$_6$−3 and ferrocyanide Fe$^{II}$(CN)$_6$−4 in 8 mM. equimolar solution.
2.2 Materials and methods

The arrays were subsequently rinsed in water, dried in a stream of argon and stored in vacuum until used for experiment. Following this cleaning procedure, the sensor surface was ready for functionalization with immunoglobulins.

Fig. 2.3 Cyclic voltammogram in 0.5 M measured in H₂SO₄ for inkjet-printed gold electrode showing the characteristic single sharp cathodic peak due to the reduction of gold oxide at +0.9 V and multiple overlapping anodic peaks at +1.2 – 1.4 V. In H₂SO₄ solution, the hydrogen evolution reaction is observed at −0.1 V vs. Ag/AgCl.

2.2.5 Immunosensor assay

For the electrochemical detection assay, an anti-CRP antibody produced in rabbit (C3527), human CRP (SRP6267), human serum from human male AB plasma (H4522), an anti–mouse polyclonal antibody from goat (M8642), bovine serum albumin (BSA) and IgG from mouse (I5381) were obtained from Sigma–Aldrich.

The activation of the antibody sample (50 µg/mL in 1x PBS) was carried out by irradiating the protein solution for 9 min using a UV lamp emitting in the ultraviolet light in the C spectrum (UV-C) range. Previous experiments investigating the efficiency of different UV sources have shown that these irradiation conditions allow the number of free thiols resulting from this photoreduction mechanism to be maximized [102]. Before working on CRP detection, the functionalization and sensing procedures involving printed electrodes were optimized using an IgG/anti-IgG model system. The whole sensing procedure can be described as follows. First, the protein sample (either
irradiated or non-irradiated antibodies) was drop-casted on the gold sensitive surface, and after 30 min incubation time, the transducer was washed with PBS and the impedance spectrum was recorded. The surface coverage was evaluated by comparing both the CV spectra and the $R_{ct}$ of the electrodes before and after the incubation for 30 min with a BSA solution, which was used to block the remaining free space on the sensor. When the surface was mostly covered by antibodies—which was achieved by incubating the electrode with a protein sample for 45 and 90 min for irradiated and non-irradiated antibodies, respectively—the BSA produced only a slight change in the impedance spectrum. Passivation with BSA was a good indicator for estimating surface coverage; moreover, in these functionalization conditions, because of the bigger number of free thiols produced during the photoreduction process, UV-activated antibodies were more effective in anchoring onto the gold electrode surface compared to non-irradiated proteins. After the passivation phase with BSA and a gentle washing step with PBS, the electrode was incubated with an antigen solution for 30 min followed by a washing step with PBS solution. Then the impedance spectrum was recorded.

### 2.2.6 Electrochemical characterization

Each of the previous steps (cleaning, immobilization and detection) was evaluated electrochemically using both CV and EIS. All the electrochemical measurements were performed using a VSP-300 potentiostat from BioLogic Science Instruments and employing an Ag/AgCl reference electrode (Super Dri-ref SDR 2, World Precision Instruments, USA). Surface characterization using CV was performed for all the chips exploiting the redox pair ferricyanide $\text{Fe}^{\text{III}}(\text{CN})_6^{3-}$ and ferrocyanide $\text{Fe}^{\text{III}}(\text{CN})_6^{4-}$ in 8 mM. The redox-containing solution was prepared by dissolving potassium hexacyanoferrate(III) (Sigma–Aldrich) and potassium hexacyanoferrate(II) trihydrate salts (Sigma–Aldrich) (molar ratio 1:1) in a phosphate-buffered saline (PBS) buffer (pH 7.4, Sigma–Aldrich). The applied potential was cycled between 0 and $+0.6 \text{ V}$ and scanned three times at a rate of $0.1 \text{ V/s}$. For electrochemical detection, impedance measurements were taken in the frequency range of $0.1 – 100 \text{ kHz}$ at a bias potential of 0 and 0.005 V amplitude. The obtained spectras were fitted using a Randles equivalent circuit.
2.3 Results and discussion

2.3.1 Surface characterization of printed gold arrays

Figure 2.5 shows the micrometric characterization of the printed electrode performed using atomic force microscopy. In particular, Figure 3b shows a $120 \times 120 \, \mu m^2$ scan (512 pixels per line, 0.7 Hz scan speed) where three portions of the device can be easily recognized: the PEN plastic substrate, the gold-printed film and the passivation layer. This analysis showed a height of about 150 nm and 2 µm for the metal electrode and the passivating layer, respectively, and an rms-roughness of 13.4 nm for the gold-sensitive surface.

2.3.2 Optimization of the antibody immobilization

The electron-transfer behaviors for the gold-printed immunosensor in preparation processes were evaluated by CV as shown in Figure 2.6. Figure 2.6 shows the typical CV curves recorded during the immobilization by adsorption of the antibodies on the electrode surface. The immobilization of the Abs on the printed gold electrode results in a decrease in peak current of the redox probe. Here, the immobilization of Abs on gold leads to the formation of an insulating film partially blocking the electron-transfer of $Fe(CN)_6^{3-/4}$ to the electrode’s surface. As expected, the addition of BSA and target...
antigen decreased the maximum peak current. When the Abs were irradiated prior to being added to the surface, a significant decrease in the peak current was observed, as shown in Figure 2.6. Additionally, a slight decrease in the current response can be seen when the BSA was added to the surface. This performance was also further explored by EIS measurements and presented by Nyquist plots, as shown in Figure 2.7. The curves were fitted using a modified Randles circuit as an equivalent circuit (see Figure 2.7). The circuit consisted of the electrolyte resistance ($R_e$), charge transfer
2.3 Results and discussion

Fig. 2.6 CV of bare gold electrode (black), antibody physisorption (red), BSA passivation (blue) and response to antigen (purple) (15 µg/m). The measurements were taken in the presence of Fe(CN)$_6^{3-/4}$ as the redox probe with a scan rate of 100 mV/s using an IgG/anti-IgG model system.

resistance ($R_{ct}$), the interface capacitance represented by the electric double layer ($C_{dl}$) and a diffusion element represented by Warburg impedance ($Z_W$). In this work, the semicircle diameter, which represents the electron transfer resistance, was used for comparison. The data obtained using the Irr-Ab-based immunosensor demonstrate the increasing value of $R_{ct}$ as the immunosensor is being modified. On the other hand, Figure 2.8 shows the electrochemical stability of the printed sensors during the incubation times. Here, the sensors were immersed in PBS solution for 10, 20 and 40 min followed by EIS measurements. The results show a clear stability of the electrochemical behaviour during the assay time (40 min). This result proves that the changes in the measurements obtained in Figure 2.7 is due to the immobilization of the biorecognition elements and the capturing of the CRP protein.

The immobilization of Irr-Ab onto the printed gold surface was investigated by evaluating the optimal incubation period in order to maximize the capture of the target antigen. Figure 2.9 shows the change in current ($\Delta i$) resulting from the anodic peak current at 0.25 V before immobilization and after addition of Irr-Ab. As can be seen, the change in current increased gradually with an increase in the incubation time of Irr-Ab and reached the plateau region at about 30 min. The optimal time for Irr-Ab to be immobilized on the surface was found to be 30 min.
Label-free impedimetric immunosensor based on UV-activated antibodies

Fig. 2.7 Typical Nyquist plots obtained from the different stages of the modification of the electrochemical immunosensor for the detection of 15 µg/mL of IgG using immobilized irradiated Abs. The inset shows the impedance plot recorded for a bare electrode (black), UV-activated antibodies (red) and BSA as backfiller (blue). An equivalent circuit was used to model the impedance data ($R_e$: electrolyte resistance; $R_{ct}$: charge transfer resistance; $Z_w$: Warburg impedance; $C_{dl}$: double layer capacitance).

Fig. 2.8 Nyquist plots of impedance experiment obtained by incubating the sensor in PBS. Working solution: $\text{Fe(CN)}_6^{3-/4}$ equimolar solution $2(1 + 1)$ mM in PBS buffer pH 7.4.
2.3 Results and discussion

2.3.3 Quantitative detection of CRP using printed gold arrays

To evaluate the analytical performance and the reliability of the immobilization strategy, we performed impedimetric detection of CRP by recording the change in charge transfer resistance ($R_{ct}$) of the device before and after addition of the target antigen. Subsequently, the immunosensor format was tested using different CRP concentrations prepared in PBS and further in human serum in order to demonstrate its realistic application in clinically relevant samples. Figure 2.10 demonstrates the electrochemical impedance immunosensor performance as a function of the CRP concentration in PBS. Furthermore, we compared the response of the impedimetric immunosensor to irradiated (PIT) and nonirradiated antibodies, as shown in Figure 2.10a. We can see significant improvement in the $R_{CT}$ values when the electrode surface was modified with Irr-Ab, indicating an excellent degree of sensitivity for the detection of CRP using the PIT technique.

As Figure 2.10b indicates, the linear relationship with the target concentration is in the range of 0.5 to 10 nM with a sensitivity of 3.8 MΩ \times mL/µg. The LOD and limit of quantification (LOQ) were estimated to be 0.4 µg/mL and 0.68 µg/mL, respectively.

Finally, in order to prove the feasibility of the presented anchoring approach for label-free detection of CRP in complex biological matrices, we performed several experiments in diluted human serum. Figure 2.11 indicates the obtained results of

**Fig. 2.9** Change in current after immobilization of irradiated antibodies as function of time. n = 3

![Change in current after immobilization](image)
CRP detection in serum. Compared with the data obtained in PBS (Figure 2.10, black curve), the charge transfer resistance in serum was slightly increased, even in the absence of target CRP. This slight deviation is attributed to the unavoidable interference from non-specific binding events, similar to the non-target CRP results.
2.4 Conclusions

In conclusion, we have demonstrated a label-free detection of CRP using a printed electrode array. The immunosensor employs photochemically immobilized antibodies as a transducer element for capturing the target CRP antigen. This is a robust strategy for antibody immobilization, offering the ability to realize disposable, cheap and fast biosensors for point-of-care applications. The selectivity of the assay was subsequently evaluated via detection of CRP showing the lowest cross-reactivity with a non-target solution compared to target antigen.
Chapter 3

Printed Microgap Electrodes for Biosensing Application

The following chapter was adapted from an article published in Advanced Biosystems journal, entitled 'Flexible microgap electrodes by direct inkjet printing for Biosensing Application' DOI: 10.1002/adbi.201600016. The chapter was reproduced with permission from Nouran Adly, Lingyan Feng, Kay J. Krause, Dirk Mayer, Alexey Yakushenko, Andreas Offenhäusser, and Bernhard Wolfrum.[2]
3.1 Overview

A rapid fabrication method of microgap electrodes using inkjet printing is described. In this approach, the lateral spacing between two printed electrode lines is precisely controlled down to 1 \( \mu \text{m} \) without any surface modification or substrate patterning. The strong confinement, well below typical resolution of inkjet printing, relies on complete solvent evaporation between the printing of adjacent electrode structures, which is achieved by controlling the printing speed and temperature profiles. The feasibility of this method is demonstrated by writing electrode structures with two distinct inks, based on carbon and silver nanoparticles, with comparable results. As an application proof-of-principle, arrays of microgap electrodes are fabricated using a carbon nanoparticle ink for electrochemical detection based on redox-cycling, a technique in which the sensitivity of the device depends on the distance between the two electrodes. The redox-cycling amplification of electrochemical signals is demonstrated and it is shown that the printed microgap device can be used as an electrochemical biosensor for the determination of human immunodeficiency virus (HIV)-related single-stranded DNA. This work presents a promising new approach for fabricating low-cost and label-free redox-cycling biosensors using all-inkjet-printed electrodes.

3.2 Introduction

Over the past decades, there has been a rising interest in developing sensitive analytical tools for resource-limited settings.[103–105] In this context, certain features such as selectivity, sensitivity, and fabrication cost are especially important in order to apply these assays for disease diagnosis and monitoring. Multiple assays have been developed to detect specific biomarkers with a particular focus on electrochemical approaches by measuring an oxidation and/or reduction reaction of redox-active molecules.[106, 107] Electrochemical biosensors have attracted industrial attention as they offer a simple and accurate measurement platform using inexpensive portable instrumentation.[108–110] Furthermore, recent advances in electrochemical methods have created innovative routes for amplifying the signal of biorecognition events.[111–114] One such method is redox-cycling,[62, 115–120] which is capable of resolving signals from single molecules.[63, 121, 122] The principle of redox-cycling amplification is based on repeating oxidation and reduction events of redox-active molecules between two closely spaced and independently biased electrodes. Redox-cycling, therefore, strongly depends on the time a molecule needs to travel from one electrode to the
3.2 Introduction

other. High amplification currents are achieved if molecules rapidly shuttle between the oxidizing and reducing electrodes. As most experiments are conducted in solutions with supporting electrolytes, the molecular transport between the electrodes is typically dominated by diffusion. Consequently, a short distance between the electrodes in the micro- or even nanometer regime is required to achieve efficient redox-cycling amplification.

Several methods have been developed for the fabrication of redox-cycling sensors in different geometrical configurations aiming for a distance in the nanometer range between the two electrodes.[5] Lemay and co-workers recently reported nanoscale redox-cycling devices, which were able to detect fluctuations of single molecules within a nanofluidic channel.[121–123] However, these devices are typically fabricated in clean-room facilities using either optical or e-beam lithography, prohibiting rapid prototyping of low-cost biosensors. Further approaches relying on vertical arrangement of two independently addressable electrode layers include nanocavity and nanoporous redox-cycling devices.[124–127] Nevertheless, most fabrication methods aim to integrate the electrodes within the same plane, which avoids problems with multilayer electrode fabrication.

An inherent challenge for the fabrication of in-plane redox-cycling devices is the requirement of closely spaced electrode arrangements. In general, optical lithography is a powerful parallel fabrication technique, capable of producing high-resolution electrode structures. However, it relies on the fabrication of masks and is therefore not suitable for applications, which require repetitive refinement in the sensor design or layout. A flexible route to obtain small gaps between two planar electrodes is the application of focused ion beam (FIB) etching to cut through an electrode layer on a glass substrate.[128, 129] Although submicron interelectrode trenches are reliably fabricated with this method, FIB is an expensive and time-consuming fabrication tool. A low-cost approach for the fabrication of redox-cycling devices with small electrode distances was reported by Marken and co-workers using chemical etching.[130–132] In this case, the two electrodes were assembled using a sacrificial layer resin followed by etching in a piranha solution. The method succeeded in developing small junctions in a controlled manner within the micrometer range between two electrodes. Recently, the same group used a gold electrodeposition process on photolithographically fabricated platinum electrodes in order to reduce the gap between the two microelectrodes and the process succeeded in reducing the gap down to 4 µm.[133] These microgap devices have proven their value for analyzing a variety of biomolecules such as dopamine in the presence of
ascorbate as an interfering agent, utilizing redox-cycling based selectivity.\[130] Yet, the fabrication procedure limits its potential use for mass fabrication.

On the other hand, printing methods have the potential for high-throughput as well as low-cost fabrication of electrochemical redox-cycling sensors. The Sirringhaus and co-workers showed successful separation between two electrodes down to 500 nm using advanced inkjet printing technology.\[134] Their method relied on prepatterned silicon substrates with hydrophobic lines of fluorinated self-assembled monolayer, which were defined by electron-beam lithography. Very recently, Li et al. succeeded in fabricating metal oxide thin-film transistors with short channel length down to 3.5 \( \mu \)m using an inkjet printer.\[135] Nevertheless, substrate patterning using hydrophobic ink with coffee ring effect was necessary in order to define source and drain areas. In this work, we demonstrate the fabrication of micron-scale redox-cycling sensors by direct inkjet printing. We show that with a defined adjustment of printing conditions, interelectrode distances down to 1 \( \mu \)m between two carbon electrodes are feasible without prior modification of the substrate.

As a proof of concept, we demonstrate the use of microgap redox-cycling sensors for the detection of single-stranded DNA (ssDNA) using peptide nucleic acids (PNAs) immobilized on the carbon microelectrodes. HIV-1 marker sequences encoding for HIV-1 nef gene were chosen as they are clinically overexpressed at the early stage of the disease among HIV patients.\[136–139] We believe, the results presented herein will serve to further inspire the research of inkjet printing technology for sensitive low-cost and large-area diagnostic applications.

3.3 Materials and methods

3.3.1 Materials

All peptide nucleic acid (PNA) molecules and DNA strands were purchased from Eurogentec company, Germany. The sequences are as follows:

1. PNA receptor: 5′-AAG CTA CTG GA-Lys (Pyrene)-3′
2. Target DNA: 5′-TCC AGT AGC TT-3′
3. Non-target DNA: 5′-CAC ATC AAT CCA-3′

PBS-Tween and 1,1-ferrocene dimethanol were bought from Sigma-Aldrich. Nano-silver ink (DGP 40LT-15C) was purchased from Advanced Nano Products, Co., Ltd,
3.3 Materials and methods

South Korea. PEN-PQA1M was bought from Teijin DuPont Films and was used as a flexible substrate for inkjet printing. An OmniJet 300 inkjet printer, UniJet Co., Republic of Korea was employed for the device fabrication using either 1 or 10 pL DMC cartridges from Fujifilm Dimatix Inc., USA. The sintering and curing steps were conducted on a precision hot plate (CT10, Harry Gestigkeit GmbH, Germany).

3.3.2 Methods

Carbon ink formulation

1 g of carbon black (Orion Carbons) in 5 g of a 50/50 wt% mixture of ethylene glycol and water were milled with 100 µm yttrium zirconium beads at 1100 rpm for 1 hour in a Pulverisette 7 ball mill (Fritsch, Germany). The milled mass was diluted with further 10 g of ethylene glycol/water mixture to adjust the viscosity with vigorous stirring. The solution was filtered through a 0.45 µm filter to obtain the final ink.

Printing conditions

The temperature of the substrate holder was set to 65 and 25 °C during printing silver and carbon ink respectively, unless otherwise specified. Drop spacing was 28 µm for the silver ink and 25 µm for the carbon ink.

Microgap electrodes fabrication

The electrodes were directly fabricated on flexible PQA1M substrate without prior modification. Initially, silver ink was printed forming the outer electrode contact pads and feedlines. A silver ink was chosen to keep the resistance low and avoid a significant ohmic drop at the feedlines. Printing was performed at a substrate temperature of 65 °C, therefore the ink was completely dried on the substrate. Afterwards, the carbon electrodes were patterned partially connected to the silver lines. Finally, PI passivation ink was printed to cover all the electrodes apart from two regions: one of those regions represents the active electrode areas, while the other defines the contact pads for electrical connection with the potentiostat through FPC connectors.

Electrode modification and detection assay

Carbon electrodes were immersed in vigorously stirred solution of ethanol for 5 minutes followed by MilliQ. Next, for surface immobilization we used 11 mer peptide PNA molecules. Immobilization of PNA was achieved by adding 20 µL of 0.25 µM PNA to
cover the whole chip area and allowed to incubate for five minutes. After incubation the chip was rinsed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween). Subsequently, 20 µL of PBS-Tween solution was added for five minutes to prevent non-specific binding. Finally, the sensor was exposed to 20 µL of ssDNA solution in hybridization buffer [1.2 M NaCl and 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ buffer (pH 7)] for 30 minutes before starting the measurement. Thermal denaturation of PNA-DNA duplexes was performed by heating PBS buffer to 95 °C for 10 min, followed by three washing cycles using PBS-Tween solution. Control experiments prepared in triplicate were performed with electrodes modified with PNA only and exposed to PBS without ssDNA target. Additional control experiments were performed by adding ssDNA to electrodes that have not been modified with PNA.

**Electrochemical characterizations**

All electrochemical measurements were carried out using CHI Instruments (CHI1030B, CH Instruments Inc., USA). Experiments were conducted in a supporting electrolyte of phosphate buffered saline (1x) using 0.5 mM ferrocene dimethanol as a redox tracer unless stated otherwise. The signals were recorded vs an Ag/AgCl reference electrode (Super Dri-ref SDR 2, World Precision Instruments, USA).

Open circuit potential measurements were performed in PBS buffer using a Pt counter electrode and a Ag/AgCl reference electrode in a 3-electrodes setup with the printed carbon electrodes operating as working electrode. The potential was recorded over 1 hour and 50 mins.

### 3.4 Results and discussion

#### 3.4.1 Printing Process

In order to develop a process for printing closely spaced interdigitated electrodes for biosensing applications, we investigate the effect of time delay on line structures by printing two lines close to each other without merging. By carefully selecting drop spacing, print velocity, and substrate temperature, it is possible to print lines with a distance of 1 µm. In order to print narrow stable lines with even edges, the spacing between the drops within the line has to be optimized.[140–142] For the fabrication of closely spaced parallel lines, it is important to print structures free from bulging effects, to avoid merging of two adjacent lines at the bulging points. Additionally, the starting point of the first printed line has to be completely dry before starting the printing of
Fig. 3.1 Example SEM images of high resolution inkjet-printed lines (left) and zoom-in on the gap between two printed lines (right). The structures showing isolated lines were printed with carbon nanoparticle ink at a substrate holder temperature of $65^\circ C$, scale bars for the left and right images represent 100 and 5 $\mu m$, respectively.

the second line. In other words, in the case of unidirectional printing, i.e., when the print head returns to the origin of the axis it has just printed along, the time required to print a whole line and return to the axis origin, $t$, has to be longer than the drying time, $t^*$. This condition directly leads to Equation 3.1, which introduces the critical printing velocity, $v^*$, as a function of the length of the line, $L$, and the drying time $t^*$. Keeping the printing velocity, $v$, below $v^*$ guarantees that the first line has sufficiently dried before starting the printing process of the second line.

$$v^* = \frac{2L}{t^*} > v$$  \hspace{1cm} (3.1)

In order to avoid slow fabrication processes at low critical velocities, it is desirable to reduce the drying time $t^*$ during the printing process. One parameter, which directly influences the drying process is the substrate temperature.[143] By increasing the temperature of the substrate holder, it is possible to print at a higher velocity without individual lines merging. Figure 3.1 shows the results of carbon lines printed in parallel on a flexible PQA1M substrate. The structures were printed at a speed of 84 mms$^{-1}$, a drop pitch (center-to-center drop distance) of 28 $\mu m$, and a substrate holder temperature of $T_{\text{substrate holder}} = 65^\circ C$. The figure shows that the line separation exhibits a wave-like shape caused by the pattern of the individual droplets. The closest distance between the droplet edge of two adjacent lines was $1.4 \pm 0.2 \mu m$ ($n = 8$).

No short circuits between individual lines were observed for printed microgap arrays as demonstrated in Figure 3.2. When the temperature of the substrate holder during the printing process was set to $T_{\text{substrate holder}} = 25^\circ C$, the probability ($P_{\text{merging}}$), defined
as the number of merging pixels per length, increased to 0.16 mm$^{-1}$ (see Figure 3.3). The elevated temperature of the substrate holder enhanced the solvent evaporation prior to the printing of the subsequent line, effectively fixing the drop on its initial location. While in principle, elevated temperatures improve the results for printing closely spaced structures, care has to be taken to avoid in-homogeneous patterns caused by the coin stacking effect.[140] For example, a substrate holder temperature of 75 °C resulted in uneven height distribution within the line due to the complete drying of individual drops (see Figure 3.4). As mentioned previously, another approach to ensure that Equation 3.1 holds true is to decrease the printing velocity.

**Fig. 3.2** Inkjet printing steps for the fabrication of the redox-cycling sensors starting from the substrate (i), the deposition of the carbon ink (ii), and the deposition of the polyimide passivation (iii). On the right side, false color microscopic images of the fabrication sequence of the printed sensors are shown including a) the bare PEN substrate, b) the printed carbon electrodes, and c) the sensor including the passivation layer. Scale bars represent 200 µm in all images.
3.4 Results and discussion

**Fig. 3.3** Example of printed lines (left) with a microgap separation using carbon nanoparticle ink and a magnified SEM image (right) of two printed lines with the substrate holder set to a temperature of 25 °C leading to a connection between the lines. The scale bars in the left and right image represent 100 µm and 5 µm, respectively.

**Fig. 3.4** Microscopic differential interference contrast image of printed carbon nanoparticle ink exhibiting the stacked coin effect at elevated temperature $T_{\text{substrate holder}} = 75$ °C. Scale bar: 50 µm.

Lines with spacing down to 1.5 µm could be reliably printed at a printing velocity of 2 mm · s$^{-1}$ ($T_{\text{substrate holder}}$ set to 25 °C) (data not shown). However, to comply with the need for high-throughput production, we restricted the sensor development to printing velocities of 84 mm · s$^{-1}$ for the fabrication of redox-cycling devices. The printing method could be expanded to other systems such as silver nanoparticle inks. Figure 3.5 shows a scanning electron microscopy (SEM) image of printed silver lines using the same parameters as described above (substrate holder temperature $T_{\text{substrate holder}} = 65$ °C and a printing velocity of 84 mm · s$^{-1}$). Note that the drying time will vary based on
the exact ink composition. Hence, it has to be determined prior to printing individually for each ink system.

![SEM image of printed silver nanoparticle ink forming a microgap electrode.](image)

**Fig. 3.5** SEM image of the printed silver nanoparticle ink forming a microgap electrode. Scale bar: 10 µm.

**Fig. 3.6** a) Typical CV results of inkjet-printed microgap electrodes. Measurements were performed in a 0.25 × 10^{-3} M ferrocene dimethanol solution prepared in PBS by sweeping the center electrode (black curve) from 0.0 to 0.6 V at a scan rate of 20 mVs^{-1} while holding the potential of the outer electrode (red curve) at 0.0 V. The dotted lines show the average current of the forward and backward sweep. b) Limiting currents obtained from CV during redox-cycling as a function of the Fc(MeOH)$_2$ concentration for the center (black line) and the outer electrode (red line). All measurements were performed using Ag/AgCl reference electrode. Error bars show standard deviation, n = 5.
3.4 Results and discussion

Table 3.1 Summary of electrochemical data obtained and calculated from voltammograms during redox-cycling and nonredox-cycling state.

<table>
<thead>
<tr>
<th></th>
<th>Center (oxidizing) electrode</th>
<th>Outer (reducing) electrode</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC on state</td>
<td>15 ± 0.3</td>
<td>12 ± 0.2</td>
</tr>
<tr>
<td>RC off state</td>
<td>3.1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

3.4.2 Device Fabrication and Characterization

We have fabricated interdigitated electrodes with a two finger design surrounding a center electrode. In this configuration, depending on the applied potentials, the outer electrodes can collect molecules that are either reduced or oxidized at the center electrode. Our printed sensor chips consisted of 40 electrodes with a gap of 3.0±0.1 μm between the two lines (n = 20). Figure 3.2 shows images that were obtained after printing (panel a) the carbon redox-cycling electrodes and (panel b) the passivation layer using polyimide (PI) ink for defining the active area of the electrodes. The center electrodes had a width of 25.6 ± 1.0 μm and a length of 75 ± 2 μm, while the outer electrodes had two arms with a width of 27 ± 1 μm and a length of 96 ± 8 μm. To electrochemically characterize the electrodes, we performed cyclic voltammetry (CV) using ferrocene dimethanol as a redox-active tracer with a redox potential around +0.25 V versus Ag/AgCl. In these experiments, all outer electrodes were biased to a fixed potential (0.0 V vs Ag/AgCl), while the middle electrodes were swept repetitively back and forth from 0.0 to 0.6 V. As all experiments are performed with supporting salt, the Debye length (≈ 1 nm) is much shorter than the electrode distance and the electric field does not penetrate significantly into the solution. Figure 3.6 demonstrates the measurements obtained from the printed redox-cycling chip. The CV curve in Figure 3.6a shows a typical redox-cycling curve obtained with a printed redox-cycling sensor at a scan rate of 20 mVs⁻¹. The black curve depicts the current obtained at the center electrode, while the red curve shows the current of the reducing electrode. We see that at an initial potential of 0.0 V versus Ag/AgCl, both electrodes exhibit a reductive current caused by the reduction of molecules from the bulk solution. The center electrode displays an additional capacitive component due to the applied potential sweep (the dotted line shows the average of forward and backward sweep). At potentials above 0.25 V, we observed an oxidation current at the center electrode, which originated from the oxidation of molecules from bulk solution as well as redox-cycling, i.e., the oxidation of molecules that have been previously reduced at the outer electrodes. The influence of redox-cycling can additionally be seen in the increase of the reduction current at the outer electrodes.
Printed Microgap Electrodes for Biosensing Application

Fig. 3.7 (a) Typical cyclic voltammogram of an inkjet-printed microgap electrode in non-redox cycling mode. Measurements were performed in a 0.25 mM ferrocene dimethanol solution prepared in PBS by sweeping the center electrode from 0.0 V to 0.5 V at a scan rate of 20 mVs$^{-1}$ while the outer electrode is floating. Measurements were performed using a Ag/AgCl reference electrode. (b) Open circuit potentials of printed carbon microelectrodes in PBS solution vs. an Ag/AgCl reference electrode.

We investigated the concentration dependence of the sensor response in redox-cycling mode by measuring the current ($V_{\text{outer}} = 0.0 \text{ V}, V_{\text{center}} = 0.45 \text{ V}$), see Figure 3.6b. Using a linear fit, we could calculate the sensitivities of the sensors in redox-cycling mode (the values represent the absolute current, which consists of the redox-cycling current and the contribution of the oxidized and reduced molecules in the bulk solution). The results are summarized in Table 3.1. A typical CV in non-redox cycling mode is shown in Figure 3.7a.

Furthermore, we compared the slope obtained from measuring the same electrodes without redox-cycling, i.e., the outer and the center electrodes being swept in the same direction without providing a fixed potential. As expected, the current was significantly enhanced during redox-cycling (by a factor of 4.8 for the center electrode). The steady-state current in both cases showed an amplification factor as high as 9.1. Here, the amplification factor is defined as the ratio of the current measured during redox-cycling for the sweeping electrode to the current obtained when redox-cycling was off, i.e., both electrodes were swept simultaneously. In addition, we observed a high production yield (number of functional microgap sensors) as no short circuits were detected for 240 electrodes printed under previously mentioned conditions. We further characterized the stability of the printed electrodes over time (see Figure 3.7b). The carbon electrode potential was monitored in an open circuit potential mode against
an Ag/AgCl reference electrode. After an initial equilibration phase of 40 min, the potential shift is less than 80 µV min⁻¹.

### 3.4.3 ssDNA Detection Based on Redox-Cycling Device

#### Proof-of-principle of DNA detection in microgap Sensors

To explore the use of printed microgap devices for biosensing applications, we utilized the fabricated electrodes for HIV gene detection as a model system. One 11-mer HIV-1 marker sequence was chosen as a target DNA; its complementary PNA was immobilized on the carbon electrode as a capture probe via a pyrene group linked to the 3′ end. The pyrene rings subsequently formed π–π interaction with the carbon surface as reported previously.[144–146] The added lysine group between the PNA and the pyrene group acted as a spacer arm and enhanced the solubility of the PNA probe. Furthermore, Tween 20 was added to limit the nonspecific adsorption on the carbon surface.[147, 148]

Figure 3.8a shows typical CV curves before and after immobilization of PNA addition as measured in the ferrocene dimethanol solution. After the immobilization of PNA (curve b), the redox-cycling current decreased due to surface blockage. A similar effect occurred with the addition of ssDNA complementary probe solution (curve c), which implies that the effect of PNA–DNA hybridization could be clearly observed. In order to confirm the DNA/PNA hybridization, we further washed the surface with a dehybridization buffer. The observed current values after the addition of dehybridization buffer at 95 °C (curve d) showed a recovery of the redox-cycling current to the prehybridization state with an 88% intensity recovery compared to the current value before hybridization (curve b), confirming the successful immobilization of the hybridization probes.

#### Influence of the hybridization probe concentration on the hybridization efficiency

In order to evaluate the effect of the surface probe density on the overall hybridization signal, the sensor response for several capture probe concentrations ranging from $0.05 \times 10^{-6}$ to $1.00 \times 10^{-6}$ M was measured (Figure 3.8a). The change in current values ($\Delta i$) resulting from the anodic current at 0.4 V before immobilization and after target addition was plotted versus several PNA concentrations. In this experiment, the surface probe concentration was varied and two different concentrations of complementary target ssDNA ($10 \times 10^{-9}$ and $1000 \times 10^{-9}$ M) were chosen. Responses for the capture
Fig. 3.8 Electrochemical characterization of redox-cycling microgap sensors using ferrocene dimethanol solution of printed carbon electrodes with PNA for ssDNA detection. (a) Current for i) bare carbon electrode (black curve); ii) PNA immobilization on carbon surface (red curve); iii) addition of $1 \times 10^{-6}$M ssDNA complementary target and showing hybridization with PNA surface probe (blue curve); iv) thermal denaturation of PNA–DNA duplex at 95 °C (green curve). (b) Maximum current obtained with varying PNA concentrations measured after hybridization with $10 \times 10^{-9}$ M complementary sequence (black curve) and $1000 \times 10^{-9}$ M complementary sequence (red curve). (c) Cyclic voltammogram obtained using (i) a bare electrode and (ii) after four washing cycle with PBS-Tween at a scan rate of 20 mV·s$^{-1}$. (d) Current changes obtained for $1 \times 10^{-6}$ M ssDNA target versus different control experiments following the same incubation and washing steps: control (i): no PNA, control (ii): no ssDNA target, control (iii): no PNA and no ssDNA, control (iv): noncomplementary ssDNA. Error bars show standard deviation, $n = 3$. 

Printed Microgap Electrodes for Biosensing Application
probe concentration between $250 \times 10^{-9}$ and $500 \times 10^{-9}$ M are very similar at a target concentration of $10 \times 10^{-9}$ M. However, the same concentrations show a significant difference when a $1000 \times 10^{-9}$ M target concentration was used. This observation suggests that a higher capture probe concentration does not offer favorable conditions for the hybridization events to occur, probably due to steric limitation. This observation is consistent with previously reported work.[149, 150] Concentrations below $250 \times 10^{-9}$ M showed the highest hybridization signal and is thus considered the optimal concentration for further immobilization.

**Selectivity study of the redox-cycling sensors**

We conducted a series of control experiments to evaluate the specificity of the redox-cycling sensors. The complementary ssDNA target at a concentration of $1000 \times 10^{-9}$M and the hybridization buffer lacking target ssDNA were chosen as positive and negative controls, respectively. In another control experiment, a non-complementary ssDNA was added to the PNA modified electrode to demonstrate the selectivity of the device. Binding of ssDNA target to non-modified surfaces containing no hybridization probe was also conducted for investigating specificity. In order to rule out the possibility of major current changes originating from instability of the printed device, the electrodes were incubated in phosphate buffered saline (PBS) buffer without PNA followed by washing and incubation in PBS buffer without ssDNA. The CV curves were recorded before and after washing (see Figure 3.8c in the Supporting Information) and the current changes ($\Delta i$) were calculated for three electrodes.

The current changes for all five scenarios are represented in Figure 3.8d. There was a significant increase in the current values due to complementary target strand addition to PNA modified electrodes as compared to the non-complementary DNA, which demonstrates the device’s specificity. Finally, we observe some residual interference of unmodified electrodes exposed to ssDNA and samples exposed to hybridization buffer only, probably caused by nonspecific adsorption of molecules.

**Calibration of redox-cycling sensors for ssDNA detection**

Under previously optimized conditions, calibration experiments on PNA modified electrodes with previously optimized conditions using a range of ssDNA concentrations $0.001 \times 10^{-9} - 1000 \times 10^{-9}$ M are shown in Figure 3.9. The calibration curve reveals a sigmoidal trend with a dynamic range between $1 \times 10^{-9}$ and $100 \times 10^{-9}$ M ($r^2 = 0.98$). The observed sensitivity and limit of detection were $0.4$ nA nm$^{-1}$ and $1 \times 10^{-9}$ M, respectively.
Fig. 3.9 Calibration curve obtained for sensors prepared by immobilizing $0.25 \times 10^{-6}$M PNA surface probe versus ssDNA complementary target. Error bars show standard deviation, $n = 3$.

At lower concentrations of the target DNA, the influence of nonspecific adsorption begins to appear as shown in Figure 3.8d, effectively masking current changes from specific binding events. At higher target concentrations, the sensor surface becomes saturated, resulting in a low and nonlinear increase in the current. The results demonstrate the applicability of the printed microgap device for the detection of target ssDNA using an electrochemical amplification method. We believe that improvement in terms of immobilization and hybridization schemes, in particular focusing on geometric aspect and the reduction of nonspecific adsorption, will eventually increase the sensitivity and lower the detection threshold. The performance of the sensor might be further improved by selecting a negatively charged redox tracer that electrostatically interacts with the capture probe.

3.5 Conclusions

In summary, a new scheme for fabricating microgap electrodes using inkjet printing without prior surface patterning is described. We have shown the applicability of this method for the fabrication of redox-cycling sensor arrays. Our studies demonstrate the potential use of printed redox-cycling devices for DNA sensing. It is expected that the same detection principle can be applied to other systems for monitoring bioaffinity. Finally, the presented method creates unprecedented opportunities not only for the
fabrication of redox-cycling devices but also for fabricating other sensing devices such as printed field-effect transistors that require precise control of the channel length between source and drain electrodes.
Chapter 4

Three-dimensional inkjet-printed redox cycling sensor

The following chapter was partially adapted from an article written for RCS Advance, entitled "Three-dimensional inkjet-printed redox cycling Sensor" DOI: 10.1039/C6RA27170G. The chapter was reproduced with permission from Nouran Adly, Bernd Bachmann, Kay J. Krause, Alexey Yakushenko, Andreas Offenhäusser, and Bernhard Wolfrum.[151]
4.1 Overview

Multilayer inkjet printing is emerging as a robust platform for fabricating flexible electronic devices over a large area. Here, we report a straightforward, scalable and inexpensive method for printing multilayer three-dimensional nanoporous redox cycling devices with a tunable nanometer gap for electrochemical sensing. The fabrication of the electrochemical redox cycling device is based on vertical stacking of two conductive electrodes made of carbon and gold nanoparticle inks. In this configuration, the two electrodes are parallel to each other and electrically separated by a layer of polystyrene nanospheres. As the top and the bottom electrodes are biased to, respectively, oxidizing and reducing potentials, repetitive cycling of redox molecules between them generates a large current amplification. We show that a vertical interelectrode spacing down to several hundred nanometers with high precision using inkjet printing is possible. The printed sensors demonstrate excellent performance in electrochemical sensing of ferrocene dimethanol as a redox-active probe. A collection efficiency of nearly 100% and current amplification up to 30-fold could be obtained. Our method provides a low cost and versatile means for sensitive electrochemical measurements eliminating the need for sophisticated fabrication methods, which could prove useful for sensitive point-of-care diagnostics devices.

4.2 Introduction

Redox cycling at the nanoscale is a highly sensitive method for electrochemical detection of redox-active analytes.[116, 117, 152–156] The method relies on amplification of Faradic currents due to recurrent oxidation and reduction of redox-active molecules between two closely spaced and independently biased electrodes. The amplified current provides quantitative information on the concentration of the redox-active molecules allowing direct detection of analytes at low concentrations.[157, 158] The distance between the two electrodes plays a significant role here, as the amplified current inversely scales with the interelectrode distance. Recently, the Lemay group has demonstrated single-molecule resolution, the ultimate level of detection, using microfabricated nanofluidic redox cycling devices.[121, 122, 158] Since the introduction of redox cycling devices in electrochemistry,[115] a variety of electrode configurations has been studied. Apart from probe-based techniques, such as scanning electrochemical microscopy,[62, 159] most investigations have been performed using interdigitated electrodes (IDE) with a narrow interelectrode distance.[154, 160–164] However, the minimal distance between
the anode and the cathode is limited by the lateral resolution of the fabrication process. Thus, advanced lithographic techniques are required to fabricate efficient redox cycling devices at the nanoscale using IDE configuration. An alternative to the lateral configuration is a multi-layer vertical electrode arrangement. Here, two electrodes are aligned on top of each other and are separated by a thin sacrificial layer, which is etched away in a post-processing step to form a nanogap. In this configuration, the thickness of the sacrificial layer will determine the distance between the two electrodes. Conceptually, this design has the advantage of allowing the realization of a defined gap between the electrodes down to the nanometer range.[165, 166] Another interesting approach is redox cycling in nanoporous devices.[124, 126, 127, 167–171] These sensors are based on metal/insulator/metal stack with pores created on the top electrode and the insulator layer allowing the diffusion of analyte solution to the bottom electrode through the pores offering a high temporal resolution for electrokinetic studies. Yet, all of the aforementioned designs utilize rather expensive state-of-the-art microfabrication techniques. If redox cycling devices are going to be applied in areas requiring low cost of production, such as point-of-care testing, alternative fabrication methods have to be considered. Recently, Park et al.[172] have demonstrated a bench-top fabrication of a redox cycling sensor of vertically stacked electrodes using 0.54 to 8 µm beads as a spacer. However, their method involves several substrate treatment steps and most importantly a manual assembly of two separated electrode plates, which is a very tedious and time-consuming, and therefore, an unscalable procedure.

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**Fig. 4.1** The principle of redox cycling in a nanoporous device: reduced species (blue circles) is first oxidized at the top electrode (red circles) and then diffuses through the pores of the polystyrene layer to be re-reduced at the bottom electrode (blue circles). Thereafter, the process is repeated until the molecule escapes the device.
On the other hand, additive manufacturing methods such as inkjet printing offer a practical solution to cost and scalability problems. [173, 174] Although lateral resolution in ink-jet printing is limited by the smallest drop size to approximately $10 - 20 \, \mu m$, [173–175] if Z resolution of inkjet printing is exploited, vertical interelectrode spacing down to several hundred nanometers is feasible. Here, for the first time, we present an array of fully printed three-dimensional redox cycling sensors using multiple functional inks. Neither substrate pre-treatment nor post-possessing beyond sintering were used during fabrication since the inks inherently possessed all the required functionality. We used inkjet printing for fabricating a vertically stacked redox cycling device using carbon and gold nanoparticle inks. Each printed chip has material costs of less than 5 cents (see Table 4.1).

Table 4.1 Cost calculation for a single printed chip of 40 electrodes.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cost (Euro)</th>
<th>Cost per chip (Cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>2.5 per A6 sheet</td>
<td>2.1</td>
</tr>
<tr>
<td>Silver ink</td>
<td>14 per 1 mL</td>
<td>0.37</td>
</tr>
<tr>
<td>Carbon ink</td>
<td>10 per 1 mL</td>
<td>0.26</td>
</tr>
<tr>
<td>PI ink</td>
<td>30 per 1 mL</td>
<td>0.8</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>3.6</td>
</tr>
</tbody>
</table>

At the same time, we combined a novel nanoporous material made of layers of polystyrene nanospheres for electrical isolation between the two electrodes. As schematically illustrated in Figure 4.1, the redox cycling device consists of a porous top carbon electrode, which allows the transport of the electrolyte solution to the bottom electrode. The bottom electrode (made either of gold or carbon nanoparticle inks) is separated by a nanoporous layer, thereby allowing the diffusion of redox molecules between the two electrodes.

The entire device was passivated with a dielectric ink as described in the experimental section, leaving only the sensor area (three layer system described above) exposed to the electrolyte. We performed a proof-of-principle demonstration of a redox cycling device using a gold nanoparticle ink as a bottom electrode material. Further experiments were carried out using carbon bottom and top electrodes instead of gold for three key reasons: (a) carbon is a cheaper alternative to noble metals; (b) the use of carbon in electrochemistry has been and continues to be of great interest across a wide range of applications due to its wider potential window compared to gold (see Figure 4.2); (c) the use of carbon-based materials in cleanroom technology still poses a
technical challenge, although carbon electrodes are ubiquitous in cutting-edge platforms such as supercapacitors, fuel cells, and bioelectronics.[175]

![Graph showing normalized current values for CV measurements of printed carbon (black line) and gold electrodes (red line) in 0.1 M PBS.](image)

**Fig. 4.2** Normalized current values for CV measurements of printed carbon (black line) and gold electrodes (red line) in 0.1 M PBS.

The results presented here involve the use of printed microelectrodes (ME) as the sensing electrode. As opposed to macroelectrodes, ME feature a three-dimensional diffusion field, which leads to a steady-state current, and faster electron transfer due to smaller size and lower capacitance values, which in turn leads to enhanced sensitivity.[176, 177] Microelectrode array systems have been reported in literature for the single-molecule detection and single-molecule electrocatalysis owing to their inherent advantages.[178–180]

### 4.3 Materials and methods

#### 4.3.1 Materials

Nano-silver ink (DGP 40LT-15C) was bought from Advanced Nano Products, Co., Ltd, South Korea. PEN foil (Optfine® PQA1M) from Teijin DuPont Films was used as a flexible substrate for inkjet printing. All ink jetting experiments were performed using an OmniJet 300 inkjet printer, UniJet Co., Republic of Korea, with 1 or 10 pL DMC cartridges from Fujifilm Dimatix Inc., USA. The sintering and curing steps were conducted on a precision hot plate (CT10, Harry Gestigkeit GmbH, Germany). Prior
to printing, all inks were sonicated in a sonication bath for 5 minutes and filtered with 0.45µm syringe filter.

4.3.2 Preparation of the carbon ink
See section 3.3.2, chapter 3.

4.3.3 Preparation of the gold ink
The gold nanoparticle ink in this work was prepared as mentioned in section 2.2.1, chapter 2.

4.3.4 Fabrication of fully printed redox cycling sensors arrays
The overall process flow for the fabrication of the redox cycling sensor is illustrated in Fig. 1a. First, silver nanoparticle ink was printed with a drop spacing of 22 µm, frequency of 2 kHz and a substrate temperature of 50 °C. Next, carbon nanoparticle ink was patterned on top of the silver ink using a 22 µm drop spacing, a frequency of 1 kHz and a substrate temperature of 25 °C. Next, the printed arrays were sintered at 120 °C for 30 minutes. After printing the carbon and the silver arrays, tuning the surface energy of the PEN substrate (PQA1M) for printing a dielectric layer of polyimide ink was carried out by exposing the whole substrate to oxygen plasma in a plasma oven (Nano, Diener Electronic, Germany) at a power of 30 W and pressure of 0.2 mbar for 12 seconds. Subsequently, the electrode insulation was created by printing the polyimide polymer with a drop spacing of 25 µm and frequency of 5 kHz. Upon printing, the passivation ink was cured thermally by heating to 80 °C on a hot plate for 5 minutes followed by 120 °C for 30 minutes.

4.3.5 Patterning of a nanoporous layer using polystyrene
The colloidal ink of polystyrene nanospheres consisted of 40 wt% polystyrene nanospheres (mean diameter of 120 nm), 50 wt% dipropylene glycol and 10 wt% deionized water (from a Millipore Milli-Q system, 18 Ω·cm⁻¹). Next, the colloidal ink was sonicated in an ultrasonic bath for 15 minutes. Later, the nanoparticle solution was deposited on top of the previously printed carbon electrode using a 10 pL cartridge while the substrate temperature was set to 60 °C. In order to vary the thickness of the nanoporous layer, several printing passes using the polystyrene ink were performed.
4.3 Materials and methods

4.3.6 Top electrode fabrication

The carbon top electrode was aligned and deposited on top of the printed nonporous layer as shown in the schematic in Fig. 1b. In order to mechanically stabilize the top electrode, each sample was flash-annealed by a photonic curing system (PulseForge 1200, NovaCentrix, USA) with six 500 µs long pulses of 200 V (fluence of 0.296 J · cm⁻² as measured using an integrated bolometer) with a flashing frequency of 1 Hz.

4.3.7 Characterization

SEM images were taken with a Nova Nano (FEI, USA) scanning electron microscope (SEM) with an accelerating voltage of 5 kV. Cross-sectional images and FIB cuts were performed by a Helios Nanolab 600i apparatus (FEI, USA). After depositing a protective 500 nm platinum layer, the milling and the polishing were carried on using an ion voltage of 30 kV and current of 80 pA. The printed nanoporous layer thickness was also measured using a surface profiler (DEKTAK 3030).

4.3.8 Chip design layout

The multi-electrode microchip consisted of 50 silver/carbon electrodes/feedlines (inner diameter: 35 ± 4 µm; center to center spacing: 205 ± 5 µm). The size of the printed chip as shown in Fig. 2a was 20 mm × 30 mm. It could be inserted into a 50-pin FPC connector with top contact flip-lock type, the contact pads have a 0.5 mm pitch. Each chip consists 50 feedlines. The width and length of each bottom carbon electrode were 45 µm and 60 µm, respectively, the top electrodes were arranged above the bottom electrode having a width of ∼ 220 µm and a length of ∼ 145 µm.

4.3.9 Electrochemical measurements of the printed sensors

All electrochemical measurements were carried out using CHI Instruments (CHI1030B, CH Instruments Inc., USA) and employing an Ag/AgCl reference electrode (Super Dri-ref SDR 2, World Precision Instruments, USA). Measurements of redox-active molecules were done by preparing a solution of 1,1-ferrocene dimethanol (Sigma-Aldrich) in PBS (pH 7.4). Redox cycling was carried out by fixing the bottom electrodes at a constant potential (0.0 V). The top electrode was swept from 0.0 V to 0.6 V at a scan rate of 20 mV · s⁻¹. Therefore, the redox molecules underwent repetitive oxidation and reduction via continuous diffusion from one electrode to another before eventually escaping the device and diffusing away into the bulk solution through the porous top
electrode. The reducing and oxidizing currents were measured independently. Stability study was done by placing the whole chip in a 250 mL beaker filled with 100 with PBS solution and applying stirring conditions by rotating a 2.5 mm magnetic stirrer bar at 400 rpm.

### 4.3.10 Electrical characterization

The resistance of the printed test structures was measured after sintering and flash-curing using a multimeter (Voltcraft Plus VC 960, Conrad, Germany). The calculated resistivity of printed silver and carbon layers was found to be $6.4 \times 10^{-8} \Omega \cdot m$ and $1.05 \times 10^{-2} \Omega \cdot m$, respectively.

### 4.4 Results and discussion

The printing process is shown in Figure 4.3. Silver nanoparticle ink was first deposited on a polyethylene naphthalate (PEN) substrate forming the feedlines for electrical connections followed by either a gold or carbon ink deposition for the active bottom electrode. Both inks were prepared as described in the experimental section.

![Schematic illustration of the fabrication flow of the redox cycling sensor.](image-url)

**Fig. 4.3** Schematic illustration of the fabrication flow of the redox cycling sensor.
4.4 Results and discussion

Afterward, a dielectric polyimide ink was used to passivate the electrodes and define an electrode opening as shown on the microscopic image of the fabrication sequence in Figure 4.4. As a next step, a polystyrene nanosphere ink was printed onto the bottom electrode. This layer was crucial in providing mechanical stability to the next deposited layer. Upon drying of the polystyrene nanosphere layer, a carbon top electrode was printed on top of it. Finally, the entire sensor was cured using photonic sintering to sinter the upper carbon layer without damaging the polystyrene nanospheres.

An additional potential advantage of using a polystyrene nanosphere ink is the possibility to later utilize carboxyl or amine functionalization to bind a biorecognition element such as DNA or protein for sensing applications. Another feature of this approach, namely using a nanoporous dielectric layer, is the elimination of the sacrificial layer and with it of the post-etching step, which is typical in the fabrication of two superimposed electrodes. Figure 4.5 shows a cross-sectional view obtained from Focused Ion Beam (FIB) milling of the device. Approximately 280 nm separation between the top and the bottom electrodes can be observed as three layers of polystyrene nanospheres were printed.

This demonstrates the ability to print a multilayer stack of four different material inks with high precision. Figure 4.6 demonstrates successful utilization of the fabricated device for sensing of ferrocene dimethanol as a redox-active probe.

Here, the top anode is providing the oxidizing potential to the redox molecules as it is being swept from negative to positive potentials, while the bottom cathode collects and reduces all the molecules that have been previously oxidized at the top anode and
diffused to the cathode. This is evident from the collection efficiency that is close to 100% in our device. The collection efficiency $CE$ is defined by the ratio of the current for reduced species at the bottom electrode divided by the current for the oxidation of reduced species at the top electrode, see Equation 4.1.[180, 181]

$$CE = \frac{i_{\text{bottom}}}{i_{\text{top}}}$$  \hspace{1cm} (4.1)

A camera image of the fabricated redox cycling chip is shown in Figure 4.7a. Each chip consists of 25 sensors (50 feedlines) printed using silver and carbon inks on PEN substrates. The width and length of each bottom carbon electrode were 45 µm and 60 µm, respectively, as seen in Figure 4.7b. The top electrodes were arranged above the bottom electrode having a width of $\sim 220$ µm and a length of $\sim 145$ µm. The repeatability of the process in lateral resolution is in the range of 4%. Figure 4.7c shows the nanoporous layer of as-printed polystyrene nanospheres. The surface morphology and the porosity of the printed carbon electrode are evident from the high magnification SEM image in Figure 4.7d.

The thickness of the polystyrene film critically depends on the number of printed layers. Although a small distance of 200 nm between the top and the bottom electrodes was possible as seen in Figure 4.7c, we printed arrays of redox cycling sensors with a 1200 nm distance. We did that in order to increase the yield of functional devices by avoiding possible failures due to short circuits and to ensure higher mechanical
4.4 Results and discussion

Fig. 4.6 The measured cyclic voltammograms recorded at the carbon top electrode (black curve) and at the bottom gold electrode (red curve) with 500 µM Fc(MeOH)$_2$. The potential of the bottom electrode was held at 0.0 V while the top electrode was swept from 0.0 V to 0.6 V at a scan rate of 20 mV·s$^{-1}$.

stability of the top carbon electrode. This larger distance between the two electrodes was achieved by printing 12 layers of polystyrene nanospheres.

Consequently, this interelectrode distance $d$ defines the amplification. It should be noted that the redox cycling current scales with $1/d$ for vertical gap devices.[178] In the case of a nanoporous gap, additional obstacles to the diffusion of redox molecules are introduced. Therefore, we further characterized the sensors as shown in the electrochemical results in Figure 4.8 using an all-carbon printed device. To test the sensitivity of our devices, we constructed an electrochemical calibration curve with varying concentrations of ferrocene dimethanol as shown in part (a) and (b) of Figure 4.8. Figure 4.8b shows the dependence of the oxidation current at the top electrode on the concentration of the redox molecules. The response was linear ($R^2 = 0.98$) over a range of 250 µM to 1 µM for the top electrode, as shown in Figure 4.8b.

As mentioned earlier, obstacles due to the presence of the polystyrene nanospheres between the oxidizing and the reducing electrodes will affect the amplified current, which is expected to be smaller than in a hollow gap device of the same size. The redox cycling current $I$ can be calculated by Equation 4.2 and 4.3.[182]

$$N_{\text{molecule}} = \frac{D e}{h^2}$$ (4.2)
\[ N_{\text{molecule}} = N_A V c^* \] (4.3)

where \( N_A \) is the Avogadro’s number, \( V \) is the free volume between the two electrodes, \( c^* \) is the bulk concentration of ferrocene dimethanol, \( D \) is the diffusion coefficient of ferrocene dimethanol, \( e \) is elementary charge and \( h \) is the distance between the two electrodes. The free volume can be calculated from the active area of 45 \( \mu m \times 60 \mu m \), where top and bottom electrodes overlap, and a 1200 nm spacing between the two electrodes.

Assuming a ferrocene dimethanol concentration of 250 \( \mu M \) with a diffusion coefficient of \( 6.7 \times 10^{-10} m^2 s^{-1} \) this would result in a redox cycling current of 36.4 nA. This value is only valid for a free volume between the electrodes. Here, the free volume is reduced by the spacing layer of polystyrene nanospheres. Additionally, the actual length of the diffusive pathway will increase due to the hindered diffusion within

Fig. 4.7 Large scale inkjet printing of vertically stacked redox cycling sensors: (a) optical image of inkjet-printed redox cycling device on a flexible substrate. (b) Microscopic image of a printed carbon electrode (c) and (d) SEM images of printed polystyrene nanospheres and carbon, respectively.
the nanoporous layer. If we now, for example, assume a hexagonal dense packing of polystyrene nanospheres, this would reduce the free volume by 74% and elongate the pathway by at least 20% resulting in a suppressed redox cycling current of 6.6 nA. The measured current (Figure 4.8b) during redox cycling mode was 2.9 nA, which is of the same order of magnitude as the expected current calculated by these very basic assumptions. We attributed the deviation to the difference between the geometrical area and the active electrochemical area of the electrodes.

![Fig. 4.8](image)

**Fig. 4.8** (a) Redox cycling current measured for the top electrode at different concentrations of ferrocene dimethanol; the scan rate at the bottom electrode was 20 mV·s⁻¹. (b) Limiting currents obtained from voltammograms of ferrocene dimethanol at the top electrode as a function of concentration. Error bars show standard deviation of three cycles.

Overall, the printed device showed an enhancement in the overall current values of the mostly reduced ferrocene dimethanol redox species, which was about 30 times higher with the redox cycling mode on compared to the mode when the top anode was inactive. We found that the sensitivities of the top and the bottom electrodes were $3.1 \times 10^3 \text{A}(\text{m}^2\text{M})^{-1}$ and $5.9 \times 10^3 \text{A}(\text{m}^2\text{M})^{-1}$, respectively. We were able to distinguish concentrations down to 1 nM, however, the linear slope of these values differed from the one for the higher concentration range. Further investigation is required to explore these discrepancies.

In order to demonstrate the stability of the sensor during long electrochemical measurements, we performed a cyclability test by continuously cycling the top electrode while biasing the bottom electrode. The redox cycling current was measured for a random device over thirty cycles at a slow scan rate of 20 mV s⁻¹ in a 500 µM ferrocene
Three-dimensional inkjet-printed redox cycling sensor

Fig. 4.9 Overlay of CV plots showing cycling stability of printed redox cycling sensors over 30 cycle. Inset showing the first cycle (black curve) as compared to the 30th cycle (red curve).

dimethanol solution in PBS. As seen in Figure 4.9, the current remained almost constant for around 30 cycles, after which degradation started. This relative long-term stability shows a potential for a broad range of applications, which require prolonged or multiple measurements. Furthermore, the chemo-mechanical stability of the vertically stacked electrodes was also evaluated based on the change in redox cycling current in a vigorously stirred PBS solution. We found a negligible change in the current before and after immersing the chip in PBS as shown in Figure 4.10

4.5 Conclusions

In summary, we report a straightforward, scalable and inexpensive method for fabricating three-dimensional nanoporous redox cycling devices with a tunable nanometer gap for electrochemical redox cycling sensing. The separation between the two electrodes is controlled primarily by the number of the polystyrene nanosphere layers. Subsequently, different gap sizes down to hundred nanometers and up to several micrometers can be realized. In contrast to the present state-of-the-art, no cleanroom facilities are required to fabricate such a highly sensitive sensing device. This method enables the fabrication of multiple devices on large-area substrates and completely eliminates the post-processing steps apart from curing. We are currently extending the method to incorporate a biorecognition element into the nanoporous layer directly via a biomodified polystyrene nanosphere ink. In addition, we believe that three-dimensional inkjet printing of two
4.5 Conclusions

**Fig. 4.10** Electrochemical performance of printed redox cycling sensor before washing (black) and after vigorous washing in PBS (red) using 500 μM FeMe(OH)$_2$ on a) top electrode while b) bottom electrode.

Conductive electrodes with a tunable nanometer to micrometer separation could also be applied beyond electrochemical sensing, for example for high-energy storage devices such as supercapacitors [183–190].
Chapter 5

Printed microelectrode array: part I

The following chapter was adapted in part from the following work:

- Bernd Bachmann, Nouran Adly, Jan Schnitker, Alexey Yakushenko, Philipp Rinklin, Andreas Offenhäusser and Bernhard Wolfrum, "All-inkjet printed gold microelectrode arrays for extracellular recording of action potentials" [4]

- Jan Schnitker*, Nouran Adly*, Alexey Yakushenko, Andreas Offenhäusser, and Bernhard Wolfrum, *Equal contribution 'All inkjet-printed, flexible arrays of nanoporous carbon microelectrodes for extracellular recordings from electrogenic cells', Advanced Biosystems, accepted manuscript .
5.1 Overview

As we saw in chapter 3 and chapter 4, sensitive electrochemical devices could be fabricated using inkjet printing with possible applications in DNA and protein sensing. In the following chapter, I will use similar printing method to fabricate microelectrode arrays (MEAs) which can be used to study effects on the single-cell or cell-network level. This chapter will begin by looking at the current state of art in chip-based technologies for neuroscience and cellular bioelectronics. After that, I will present the results obtained by printing MEA using two functional inks, namely carbon and gold nanoparticle ink. At the end of this chapter, I will compare the extracellular recording obtained using these two inks.

5.2 Introduction

Chip-based technologies for neuroscience and cellular bioelectronics aim to better understand cellular signaling processes in organs as complex as the human brain and to provide new generations of neuroprosthetics to restore lost functionality. Such devices and implants must bridge the biological requirements of fragile tissue with the electrical and electrochemical needs for a high-quality bi-directional communication between cell and electrode. Thus, the task to connect living cells with engineered biohybrid devices is very challenging and often driven by progress in material science and technology. Up to now, this challenge was tackled with silicon technology, dominating the fabrication methodology for bio- and neuroelectronics. Among the most established bioelectronic devices are planar microelectrodes for in-vitro experiments that in use since several decades ago and manufactured in costly cleanroom environments.[191, 192] The technologically more advanced introduction of field effect transistors (FET) were based on the big success of silicon technology.[193] CMOS-technology gradually increased complexity while reducing feature size, which led to devices with needle-like electrodes that feature an integrated signal processor such as the “Michigan” arrays [194, 195] and “Utah” arrays [196–198] for chronic neuronal implants. The higher integration densities require a very challenging and expensive manufacturing process on the other hand with long and costly ‘design-to-fabrication’ cycles based on static optical masks or electron beam writing. Although the lateral resolution can reach down to the nanometer-regime, it requires even more technological effort and highly trained personnel. Consequently, silicon technology becomes increasingly cost-prohibitive as a fundamental research technology and limits the wide use of chip-based investigations. Ultimately, even if
these resources are available, silicon technology still struggles to bridge rigid solid-state electronics with the soft nature of cellular, neuronal tissue due to its biophysical mismatch.[199] Many efforts have been made to lower the mismatch and hence improve the coupling between bioelectronic chips and cells such as CNT-based retinal implants,[200] graphene electrodes,[201] graphene transistors,[202] PEDOT/graphite-based electrodes,[203] stretchable microelectrodes,[204] highly conformable polymer electrodes,[205] organic transistors,[206] large-scale in-vitro implants, implantable CMOS-devices [207, 208] and even an electronic dura mater.[209] In order to tackle this challenge we suggest a different paradigm based on printed electronics, which proved to be a versatile tool for bioelectronic applications.[210–212]. Our all-printed approach addresses all of the aforementioned limitations, such as costs, fabrication time, personnel, rapid prototyping capabilities and flexible materials. Printing leverages a mask-less, rapid prototyping approach on flexible substrates with outstanding fabrication speeds and a wide material portfolio ranging from inorganic to organic materials, dielectrics, and even biologically active materials.[213] Our approach utilizes inkjet printing to manufacture microelectrode arrays of gold or carbon in a rapid prototype fashion based on fully digital design and fabrication. Inkjet printing allowed us to reach a structural resolution in the few micrometer range, which is comparable to conventional cleanroom-fabrication. Inkjet printing enabled us to achieve ‘design-to-fabrication’ cycles in the order of hours and costs of less than $3$ cent per chip. Consequently, the printing process itself is fully automated requiring only manual intervention to change an ink within a few minutes.

The first part (section 5.4) of this study focuses on the use of gold nanoparticle ink. Several groups have already reported inkjet-printed gold electrodes, sensors, and printed MEAs.[214–219] However, previous examples either suffer from a relatively low resolution or involve additional clean-room fabrication steps. Roberts et al. fabricated inkjet-printed PEDOT:PSS conductive-polymer electrode arrays with $28 \times 4 \times 4$ mm$^2$ electrodes, which they used for in vivo electromyography and electrocardiography measurements.[210] Khan et al. recently presented an inkjet-printed microelectrode array fabricated using a gold nanoparticle ink.[212] In their work, inkjet-printed gold lines were covered by a spin-coated polymer. Subsequent etching steps yielded 31 flexible free-standing electrode flaps with a minimum feature size of 64 $\mu$m and an electrode pitch of 2 – 7 mm. The same groups developed a MEA comprising 55 electrodes with a diameter of 500 $\mu$m and an electrode spacing of 2.54 mm, which was applied for impedance measurements to detect pressure ulcers in a rat model in vivo.[220] Here, we present the fabrication of low-temperature sintered gold MEAs
for cell recordings using solely inkjet technology. Two different MEA designs were printed using a self-made gold ink. The first layout contains 28 electrodes with an electrode pitch of 400 µm. The second layout exhibits an improved resolution of the MEA containing 64 electrodes with an electrode pitch of 200 µm.

The second part (section 5.5) of this chapter presents different MEA design (line MEA) and focuses more on overcoming main challenges in printing as discussed in chapter 1.1.5. In this part I will focus on a very promising material for cellular recording, which is nanoporous carbon material. Carbon was proposed as a promising candidate for next-generation neuro-devices and has been studied in detail in terms of cell-chip coupling with living cells.[221–223] Printed nanoporous carbon showed excellent electrochemical and material properties which are suitable for a neuroelectronic devices. The applicability of our approach is demonstrated by growing electrically active, cardiomyocyte-like cells on printed microelectrode arrays (MEA) of nanoporous carbon and recorded action potential extracellularly with very high signal-to-noise ratios paving the way towards cell-chip communication.

5.3 Materials and methods

5.3.1 Inks preparation

The gold nanoparticle ink in this work was synthesized as mentioned in section 2.2.1, chapter 2. The carbon ink was prepared as reported in section 3.3.2, chapter 3.

5.4 Printed gold microelectrode arrays

5.4.1 Characterization of gold printed patterns

Prior to printing, the drop formation of both inks, the gold ink and the polymer ink was characterized. In Figure 5.1, stroboscopic image series of an ejected drop of the gold nanoparticle-based (a) and polyimide (PI)-based (b) ink are shown. For both inks, stable drops could be generated.

The resolution of printed gold structures mainly depends on the amount of deposited material as well as on the substrate-ink interactions. In particular, the choice of substrate can significantly alter the resolution of printed structures. Test patterns were printed on the two substrates PI (Kapton®) and PQA1M, which differ in the surface texture and surface energy (37.5 mN m⁻¹ and 31.5 mN m⁻¹, respectively). Figure 5.2
Fig. 5.1 Stroboscopic image series of an ejected drop of the gold nanoparticle-based (a) and polyimide (PI)-based (b) ink.

shows images of the wetting behaviour and structures printed on Kapton® (left column) and PQA1M (right column). As it can be seen from Figure 5.2a, the contact angle of the gold ink on Kapton® is significantly lower than the contact angle on PQA1M (Figure 5.2b and no exact values could be measured due to complete wetting of the ink on the substrate. Using a 10 pL cartridge, the width of a printed single-droplet line on Kapton® was about 90 µm, which was significantly higher than the width of a line printed on PQA1M ∼ 40 µm, compare Figure 5.2c and d. Using a 1 pL cartridge the width of a single droplet on PQA1M could be further reduced to approximately 27 µm. Whereas the larger feature size of gold structures on Kapton® enable printing MEAs with the low-resolution design (layout 1), the small structures on PQA1M facilitate printing of small-scaled MEAs with a higher electrode density (layout 2). This significant reduction of the pattern’s dimensions on PQA1M in contrast to PI is assigned to the lower surface energy as well as to the more planar surface and a better pinning of the ink on the PQA1M substrate. Since only small amounts of ink were prepared for each printing sessions, batch-to-batch variations may induce slight differences in the printed structures.

After printing conductive tracks, the feedlines of the MEA have to be insulated to prevent leakage currents during cell measurements. Figure 5.2e and f show images of test squares printed on Kapton® (1 layer) and PQA1M (3 layers), respectively. In both cases the squares exhibited a smooth outline. However, a small elevation could be seen at the rim of the squares, which increased for a decreased drop spacing and
for a higher number of printed layers. This is attributed to the so-called coffee-ring effect. This effect originates from a flow driven by surface tension from the centre to the edge upon evaporation of the ink solvent. The nature of the coffee-ring effect has been extensively described elsewhere.[140, 224] The coffee-ring effect is not assumed to significantly affect the MEA performance as long as the central parts of the insulation layer are fully covered with dielectric material.
5.4 Printed gold microelectrode arrays

5.4.2 Printed gold microelectrode array

Subsequently, the conductive gold ink and insulating PI ink was printed to fabricate two types of microelectrode arrays. The lower-resolution layout was printed on a Kapton substrate for the demonstration of electrophysiological recordings. The second layout printed on PQA1M exhibits a higher electrode density with a better resolution of printed structures. This layout was used to demonstrate that devices comparable to conventional clean-room fabricated MEAs can be produced using solely inkjet printing technology. From an optical point of view, the overall success rate of printing electrodes for layout 1 and layout 2 was 83% and 65%, respectively. In Figure 5.3(a) and (b), the first layout with 28 feedlines was printed on the Kapton® foil. The final MEA mounted to a substrate carrier can be seen in Figure 5.3(a). Figure 5.3(b) shows a microscopic image of the central electrode area. Optical measurements revealed an electrode area of $8.4 \pm 2.2 \times 10^{-9}$ m$^2$. Assuming a circular opening, this corresponds to an electrode diameter of $110 \pm 21$ µm. The electrode pitch is approximately 400 µm, which is a factor of 2 to 4 larger than the pitch typically seen in conventional MEAs for in vitro cell recordings.[225] Nevertheless, both, the pitch and the size of the electrodes could be reduced by optimizing the printing parameters as described above. Figure 5.3(c) shows the second layout with a printed array of 64 microelectrodes with a pitch of 200 µm and an electrode diameter of approximately $33 \pm 6$ µm. To fabricate these MEAs, PQA1M was used as a substrate due to its low surface energy and printing was performed with a 1 pL cartridge to obtain smaller drop diameters. However, the electrode openings exhibited deviations in the dimensions and shape, which is attributed to different surface chemistry of the printed gold and the substrate and an
Fig. 5.4 (a) Five traces of action potentials of cardiomyocyte-like HL-1 cells recorded by five printed microelectrodes of layout 1. (b) The action potential was stimulated chemically by noradrenaline. The expected increase in the beating frequency of the HL-1 cells could be recorded by the printed MEA.

associated local uncontrollable flow of ink. In Figure 5.3d an example of an individual microelectrode is shown. After printing, the impedance of individual microelectrodes was investigated.

5.4.3 Extracellular recordings using printed gold MEA

For the extracellular detection of action potentials, a confluent monolayer of cardiomyocyte-like HL-1 cells was cultured on the printed MEAs (layout 1). The cells showed spontaneous beating behavior indicating cell viability. Action potential were recorded after 3 to 4 days in vitro and exemplary traces are shown in Figure 5.4 a. The action potentials occurred periodically at a frequency of 0.5 Hz, which is in the typical physiological range of 0.1 Hz to 2 Hz. The peak-to-peak (P2P) amplitude of the signals recorded on different electrodes varied strongly between 27 µV and 49 µV resulting in an average amplitude of P2P\text{mean} = 36 \pm 7 \text{ µV} (n=11). To evaluate the noise of the recorded signal, the root-mean-square (RMS) value was determined for the recording electrodes. The averaged RMS value was RMS = 2.1 \pm 0.4 \text{ µV}, indicating a low noise level. The average signal-to-noise value is SNR = 17. The signal amplitudes are somewhat lower compared to recordings obtained with clean-room fabricated gold MEAs, which also exhibit significant variation but typically lie in the range of several 100 µV or mV.[226]

The variations in the signals’ amplitudes can have several reasons. Heterogeneity of the cell layer and a possible change in the cell-electrode coupling, depending on the cell-substrate interactions, will have a large influence on the signal amplitude.
Furthermore, the particular cell passage as well as partial coverage of the sensors could lead to a variation in the recorded signal quality. Additionally, the variations of the electrode area and corresponding changes in the impedance will influence the noise level of the recordings. In addition to the recording of spontaneous activity, the samples were stimulated chemically by adding the neurotransmitter noradrenaline. Adding noradrenaline increases the beating frequency of the cells. In this experiment, we can observe this effect as depicted in Figure 5.4 b. The plots show recordings after addition of 0, 20, and 40 µL of noradrenaline (top to bottom). As can be seen, the spike rate of action potentials increases as a response to the added neurotransmitter. In this particular recording, the spontaneous frequency of 0.33 Hz changed to 0.38 Hz and 0.58 Hz after adding 20 and 40 µL of noradrenaline solution, respectively. In total, this demonstrates that the all-printed MEAs can be used for bioelectronic investigations, e.g., as disposable devices for testing drug effects. In the future, I envision that further refinements of the printing process, may enhance the resolution and density of active sensor array devices. Furthermore, it might be possible to also apply printed microelectrodes for stimulation of cells. To this end, investigations concerning the stability of the printed electrode layer during prolonged stimulation should be investigated.

5.5 Printed carbon microelectrode arrays

5.5.1 Characterization of carbon printed patterns

The whole design and fabrication process of our printed flexible carbon MEA chips (called “pFlex-MEAs”) is shown in Figure 5.5a and can be achieved in less than one day (including sample encapsulation). The schematic illustrates the simple image-based (e.g. bitmap) design of a chip and the subsequent printing of three inks on a flexible polyethylene naphthalate (PEN) substrate (Figure 5.5b): conducting nano-silver ink for the feedlines and bond pads (1. step, Figure 5.5b), conductive nanoporous carbon ink as electrode material (2. step, Figure 5.5b) and a dielectric polyimide (PI) ink as insulator (3. step, Figure 5.5b). The aligned multilayer-printing of the first two conductive inks forms 64 individual silver/carbon lines on the flexible and transparent PEN substrate. The underlying silver ink serves as a highly conductive feedline shunt but is not printed in the vicinity of the sensing area in order to avoid any contact with cells or electrolyte. The whole chip layout is then printed on top of the silver with carbon ink.
A macro-image of a printed chip is shown in Figure 5.5c. Highly porous carbon lines are printed in the center of the chip forming a line shaped array with 64 channels. PFlex-MEAs was investigated with several microscopy techniques to assess the printing quality on the micro- and nanoscopic scale. Figure 5.5d shows a microscope (DIC) image of the center of a chip prior to the printing of the insulating polyimide layer. The chip is completed by printing a passivation on top of the feedline (Figure 5.5e). The surface quality and lateral resolution were analyzed with scanning electron microscopy (SEM). The carbon is printed with a spatial accuracy which is within the position accuracy of the printer (< 1 µm) and covers the silver feedline in a nearly seamless transition (Figure 5.5f). The high-quality printing results are further shown in Figure 5.5g revealing a uniform structural form with a feedline width of about < 28.5 ± 1.2 µm, where two
adjacent carbon feedlines are separated by only $< 2 – 3 \mu m$ in distance. A dielectric polymer layer with a thickness of approximately 700 nm is printed on top of the silver and highly porous carbon in such a way that only the bond pads and microelectrodes are exposed. The apertures in the passivation on top of the microelectrodes have a diameter of about $30 \mu m$ (Figure 5.5f) with a highly porous electrode.

The best results in terms of resolution require a precise understanding of different physical, chemical and hydro-dynamical properties of an ink droplet that interacts with the surface and previously printed structures such as neighboring droplets (Figure 5.6a). The discussion of many common printing parameters, their underlying characteristics and optimizations can be found in detail in literature.\[227–229\] I have previously demonstrated how we can control the printing velocity for fabricating micro-gap carbon electrode devices for biosensing application (see chapter 3) Here, I optimized the printing process to resolve the problem of fluid migration to the parent drop. The refined process shown in the following solves fluid migration by adjusting two parameters during printing: the drop distance and the printing velocity. These parameters are essential to achieve a high-resolution printing result for fabricating a line microelectrode array design. In order to elucidate this effect, I first printed the same structures with six different drop distances (Figure 5.6b). The resolution variation ranges from too few drops per area (Figure 5.6b upper row) to excessive overprinting with shortcut electrodes (Figure 5.6c lower row). Although a useful printing resolution with a pitch between 15 to 20 $\mu m$ is easily determined, adjacent feedlines are still short cut via a bulges at the tip of the feedlines (Figure 5.6c “center”). Using a drop distance of 20 $\mu m$, I investigated further the printing velocity in order to eliminate the fluid migration to the printed parent drop (Figure 5.6c “center”). These bulges are caused by dynamic instability of the printed liquid line as the substrate holder moves mechanically from one line to the next with a velocity $v_{\text{stage}}$. This effect can be also observed near 90 degree corners (Figure 5.6c “corner”). The dominant factor to decrease instability in the printed liquid line, is the reduction of the printing speed. For a higher stage velocity, the flow rate is strongly minimizing bulges to be barely visible as seen in Figure 5.6c “corner” at $v = 9.3$. In essence, these two examples show that a systematic investigation and testing for a given substrate-ink combination and printed structure is imperative, but can be conducted within a short time span due to the nature of digital printing. The final pFlex-MEAs were analyzed height profile using 3D laser interferometry (Figure 5.7a), which showed a very homogeneous topography. A corresponding surface profilogram across nine carbon lines is shown in Figure 5.7b. The peak height of an electrode feedline typically reaches 700 – 1000 nm. The profile
of an individual feedline is depicted in Figure 5.7c. Consequently, the porosity of the nanoporous carbon electrodes was investigated with focused ion beam/scanning electron microscopy (FIB/SEM) cross sectioning and imaging method on the nanoscale. The distribution of agglomerated particles and voids is uniform throughout the layer (Figure 5.7d).

### 5.5.2 Electrochemical characterization of carbon MEA

The electrical performance of the carbon and carbon/silver-traces was determined with individual conductivity measurements: the conductivity $G_{\text{Ag}} = 1.3 \pm 0.1 \times 10^{-5} \Omega^{-1} \text{m}^{-1}$ and $G_{\text{Carbon}} = 1.5 \pm 0.2 \times 10^{-2} \Omega^{-1} \text{m}^{-1}$ for silver and carbon, respectively ($n = 8$). The overall feedline resistances are typically (for differing lengths and geometries) far below $1 \text{K}\Omega$. The highly conductive silver layer functions as a shunt resistance thus it is feasible to span even longer feedline traces (e.g. for implants) without significant ohmic voltage drops. In addition to the electrical characteristics I investigated the even more important electrochemical performance which is of crucial importance to understand for a physiological application. Electrochemical impedance spectroscopy measurements were performed in phosphate buffered saline solution (PBS) and fitted the data (Figure 5.7e) with a simplified Randles circuit ($R(RC)$) consisting of a capacitance $C$ and a resistance $R_2$ connected parallel in series with a resistance $R$. Nanoporous carbon exhibited a specific interfacial capacitance of $878 \pm 2 \mu\text{F/cm}^2$, which is in accordance with literature.[230] Non-porous carbon electrodes on the other hand have shown lower specific capacitance values the range of about $2$ to $30 \pm 2 \mu\text{F/cm}^2$,[231–233] being similar to noble metals used for conventional microelectrodes.[49] The high specific capacitance of our sub-micron thick carbon layer is primarily caused by the high porosity of the printed carbon film containing aggregates of nanoparticles in the range of $40 – 200$ nm. Thus, a very high effective surface area forms high interfacial capacitance while keeping a low electrode footprint. High interfacial capacitance imposes low electrode noise for recordings and is therefore beneficial for a high signal noise ratios.[234]

The electrochemical performance of the printed carbon electrodes was further investigated with cyclic voltammetry (CV). Figure 5.7f shows a typical CV curve measured in PBS with $0.5 \text{mM}$ ferrocene dimethanol solution at a potential scan rate of $20 \text{mV/s}$ . The curve shows the standard electrochemical behavior for a microelectrode including capacitive double layer effects apparent in the current offset of the forward and backward sweeps. I calculated the electrochemically active area from the diffusion-limited steady-state current obtained at $450 \text{mV}$ following the Equation 5.1 for current at a micro-disk electrode.[235] The measured peak-to-peak current lies in the range
5.5 Printed carbon microelectrode arrays

Fig. 5.6 (a) Schematic illustrating of piezoelectric inkjet printing and major parameters that are relevant for printing high-resolution structures, (b) series of microscope images (DIC) of the same structure printed with silver at same parameters for varying droplet distances $d$ (scale bars 200 µm), (c) series of microscope images (DIC) printed with varying stage velocity $v$ (200 µm).

The current of 1.9 nA. This value is similar to the expected steady-state current of 1.8 nA for a micro-disk electrode. The discrepancy can be explained by variations of the exact
Fig. 5.7 (a) 3D laser interferometer scan of interdigitated nanoporous carbon feedlines (dashed line indicates height profile in (b), scaled 20x in z-axis for better visibility), (b) height profile of printed feedlines indicated in (a) by dashed line, (c) individual height profile of a carbon feedline, (d) scanning electron microscopy (SEM) image of a focused ion beam (FIB) cross section through a single layer of printed nanoporous carbon and polyethylene naphthalate substrate in the center of a feedline (indicated in (c) by blue marking), (e) electrochemical impedance spectroscopy of nanoporous carbon (electrode with an area of 5.6 mm², 1 Hz - 100 kHz bandwidth), (f) cyclic voltammetry scan of nanoporous carbon in 0.5 mM ferrocene dimethanol solution (20 mV/s scan rate).
5.5 Printed carbon microelectrode arrays

![Image of live/dead staining of HL-1 cells grown on pFlex-MEA](image)

**Fig. 5.8** live/dead staining of HL-1 cells grown on pFlex-MEA (green indicates living cells, red indicates dead cells, scale bar 100 µm.

![Image of time traces of seven nanoporous carbon electrodes recording extracellular HL-1 action potentials](image)

**Fig. 5.9** Time traces of seven nanoporous carbon electrodes recording extracellular HL-1 action potentials. Each channel is indicated by a different color, the different traces are vertically offset for clarity.

geometry of the electrodes. Furthermore, the model itself does not capture the detailed geometrical features of the sensor. The discrepancy can be explained by the geometrical assumption of this model, which neglects diffusion of molecules to the electrode sides with a finite thickness of approximately 700 nm.

\[
I_{ss} = 4 \times nF \times D \times c \times r
\]  

Where Faraday constant \( F = 9.65 \times 10^4 \text{ C/mol} \), the diffusion coefficient \( D = 6.4 \times 10^{-6} \text{ cm}^2/\text{s} \) for FcMeOH, the radius \( r = 0.001 \text{ cm} \), and the concentration \( c = 5 \times 10^{-7} \text{ Mol/cm}^3 \).

Other carbon-based materials such as graphene, carbon nanotubes (CNT) and diamond are known to feature a large electrochemical potential window, where no water splitting occurs. Therefore, they are advantageous for cellular stimulation purposes in neuroscience, where high currents can stimulate larger areas. I performed CV scans in PBS to determine the potential range before the onset of either oxygen or hydrogen
evolution, which potentially damages the electrode and the cells residing in its vicinity (see Figure 5.7).

5.5.3 Extracellular voltage recordings using printed carbon MEA

In order to assess the electrophysiological performance, we chose a very widely used cellular, electrogenic model system for proof of principle experiments: cardiomyocyte-like HL-1 cells. HL-1 cells (Figure 5.8) was cultured on the pFlex-MEAs and recorded propagating action potentials across the cellular network (n= 8). The cells showed a viability above 95% in average as depicted in Figure 5.8. Utilizing a home-built low-noise and high-impedance MEA amplifier system, the spontaneous activity of HL-1 cells was recorded extracellularly (Figure 5.9) as a voltage over time characteristic on six channels in parallel. The cellular action potentials couples primarily capacitively into the electrode and causes a modulation of the electrode potential.[236] The six exemplary traces are shown in Figure 5.10 depicting signal-noise-ratios of up to 19 with a maximum cell signal amplitude of 0.96 ± 0.02 mV$_{pp}$ and a background noise of about 50 µV$_{pp}$. The recording capabilities of the printed nanoporous carbon electrodes feature a low background noise of 15 − 50 µV$_{pp}$ at 3 KHz bandwidth (depending on electrode size variations). The extracellularly recorded amplitudes of planar microelectrode can scale from around noise level to about 1% of the intracellular potential difference in case of well sealing cells [237], which is in accordance with our approximately 1 mV recordings. The recording capabilities can be further compared to other microelectrode approaches for the same cell-type, like planar, metal MEA approaches, nanocavity electrodes,[238] and even CMOS arrays for cellular recordings [239] in terms of signal-to-noise ratio and signal amplitude with similar electrode diameters.

The HL-1 cells were spontaneously mechanically contracting on the device, and subsequently, action potentials were recorded with a firing rate of 0.51 ± 0.08 Hz. Furthermore, the cells were stimulated chemically with noradrenaline, leading to a typical firing rate increase of 20.2 ± 4.8%. The signal propagated across the confluent cell monolayer on the chip and could be detected along with the time shift (Figure 5.11) of the propagating wave using the in-line array of the electrodes. The propagation velocity of the HL-1 cells was determined to be about 25.4 mm/s and is similar to reported values in literature.[240] Although the chips are meant to be disposable they were reused for several HL-1 cultures (including up to 4 cleaning cycles) over the course of several weeks in order to indicate if the chip stability is suitable for further
Fig. 5.10 Exemplary time traces of individual action potentials on seven electrodes.

Fig. 5.11 Plot of relative action potential peak time propagating through the cellular network as a function of the lateral distance.

long-term experiments, which would be required for the investigation of neuronal cell networks. The pFlex-MEAs are stable in electrolyte such as cell culture medium on the time scale of several weeks with no visible degradation in any of the printed layers. Although the chips are meant to be disposable they can and were reused to stress test the stability of the pFlex-MEAs. There is no noticeable degradation in optical and electrical performance visible after four cleaning cycles indicating that these chips could be used for long-term studies.
5.6 Conclusions

The aim of this chapter was to fabricate a high resolution microelectrode array (MEA) for extracellular recordings using solely inkjet printing technology. To this end, gold and carbon MEAs were printed and evaluated for action potential measurements from HL-1 cells.

The gold MEAs were printed in two steps. First, a gold ink was used to print conductive tracks on a flexible polymer-based substrate. In a post-processing step, the printed gold was thermally sintered to achieve conducting feedlines. In a second step, the feedlines were passivated using a polyimide-based ink. In total, two microelectrode array designs were printed. The first layout contained a MEA with 28 electrodes printed on polyimide foil. In further experiments, the resolution of the printed MEAs was improved with a second layout using a low surface energy polyethylene-naphtalate substrate. On the other hand, the carbon MEAs were printed in three steps. First, a silver nanoparticle ink was printed for the feedlines and bond pads. Second, a nanoporous carbon ink was printed as electrode material. Third, a dielectric polymer ink was printed as insulator. The printed carbon MEA was further investigated electrochemically showing a specific interfacial capacitance of $878 \pm 2 \, \mu\text{F/cm}^2$.

Finally, HL-1 cells were cultured on the gold and carbon-MEAs and recorded propagating action potentials across the cellular network. A peak-to-peak amplitudes of about $36 \, \mu\text{V}$ and $960 \, \mu\text{V}$ were recorded using gold and carbon MEA, respectively. The cellular origin of the recorded signals was demonstrated by chemically stimulating the cells with noradrenaline, which lead to an increased frequency in the recorded signals.

Overall, both gold and carbon MEAs reached a very high-resolution printing result on flexible substrates with good electrical and outstanding electrochemical characteristics suitable for cellular recording and stimulation. Although the inkjet technology inherently limits the resolution of the structures, I believe that this approach can be used in the future to provide low-cost disposable alternatives to microfabricated electrode array devices.
Chapter 6

Printed microelectrode array: part II

The following chapter was adapted in part from the following work:

- Nouran Adly, Hossein Hassani, Quang A Tran, Matthias Balski, Alexey Yakushenko, Andreas Offenhäusser, Dirk Mayer and Bernhard Wolfrum, "Observation on chemically protected polydimethylsiloxane: towards crack-free PDMS" DOI: 10.1039/C7SM01457K. The chapter was reproduced with permission from Soft Matter Journal.[1]

- Nouran Adly, Sabrina Weidlich, Silke Seyock, Fabian Brings, Alexey Yakushenko, Andreas Offenhäusser, and Bernhard Wolfrum, "Printing microelectrode arrays on soft materials: from PDMS to gummy bears", manuscript submitted for publication.
6.1 Overview

As we saw in chapter 5, high resolution MEA devices could be fabricated for in-vitro applications using solely inkjet printing technology. As I mentioned in the introduction of chapter 5, implantable silicon-based electrode arrays provide a promising opportunity to monitor neuronal activity, treat neuronal disorders, or restore lost functions in the event of brain and spinal cord injuries. However, the mechanical mismatch between classical solid-state implants and soft nervous tissue may lead to long-term failure of the functional probes. As a result, several biocompatible and soft substrates have been investigated for cellular interfacing, including PDMS (polydimethylsiloxane) and hydrogels. Nevertheless, adapting classical microfabrication methods to new substrates and electrode materials for cellular signal recording and stimulation remains challenging. In the current chapter, I address this challenge by developing high resolution MEA devices on soft substrates such as PDMS and gelatin that can be used for cellular recording.

The results of this chapter are divided into two parts: the first part (section 6.4.1) focuses on preparing PDMS substrate for printing high resolution structures. I demonstrate a new chemical modification method which renders the surface hydrophilic for several days and is effective in preserving the elasticity of the PDMS surface at the nanoscale level. The proposed method overcomes problems associated with oxygen plasma treatment of PDMS such as surface cracks and fast hydrophobic recovery. The second part (section 6.4.2) shows how we can apply the mentioned modification to print MEAs on PDMS substrate. In addition, I demonstrate the versatility of the printing technology by depositing high-resolution microelectrode arrays on top of different soft materials such as PDMS, agarose, and gelatin-based substrates, including gummy bears. Finally, the printed MEAs on PDMS and gummy bear are employed for in-vitro extracellular recording of action potentials from cardiac HL-1 cells.

6.2 Introduction

6.2.1 An alternative approach to render PDMS surface hydrophilic

Over the last few decades, silicon-based elastomer polydimethylsiloxane (PDMS) has been gaining high popularity and widespread usage in biological and biomedical research due to its flexibility, elasticity, low cost, gas permeability, biocompatibility and optical
transparency.[241–249] However, the surface of PDMS is intrinsically hydrophobic, which has a negative impact for applications that require a good wettability with aqueous solutions, including small-scale microfluidic devices, biosensors and cell analysis systems. For instance, a microfluidic device requires good wettability inside the fluidic channels to avoid poor fluid mixing.[250] Biosensors and cell analysis devices also require a hydrophilic surface to avoid biofouling problems from non-specific adsorption and cell or bacterial adhesion, which could alter the performance of the device.[251] Consequently, several surface modification methods have been developed to improve the wettability of PDMS surface. These methods fall broadly into two main categories; gas-phase processing such as air/oxygen/argon plasma oxidation, ultraviolet (UV)/ozone irradiation, and wet chemical treatment methods such as sol–gel coatings, silanization and dynamic modification using surfactants.[252–261] Depending on the application, the surface wettability of PDMS has to be maintained for short-term (up to several hours) or long-term (several days or weeks) duration. However, long-term modification of PDMS surfaces remains an on-going challenge. Fast hydrophobic recovery has been observed after surface modification by plasma oxidation, which may potentially lead to irreproducible results in applications that critically depend on the wetting state of the surface.[262–266] The recovery is primarily caused by loss of polar groups on the PDMS surface, due to their reorientation from the PDMS surface to the bulk, as well as migration of low-molecular-weight oligomers from the bulk to the surface, which is thermodynamically a more favorable state.[252, 267–271] There is a plethora of publications on attempts to stabilize the hydrophilicity degree of PDMS surface.[263, 272–276] For instance, removing the low-molecular-weight or uncured polymeric chain from the bulk by a two-step extraction/oxidation process has been shown to render the PDMS surface hydrophilic for 7 days.[277] Another approach to delay the hydrophobic recovery of plasma oxidized PDMS is thermal aging based on extended curing mechanism as demonstrated by Eddington et al.[278] Although the exposure to oxygen plasma is the most commonly employed method for increasing the wettability of PDMS, this procedure results in a brittle thin layer of a silica-like crust.[267, 279] This layer suffers a mechanical instability due to the elasticity mismatch between the stiff oxidized surface and the soft PDMS bulk underneath.[280] Consequently, the inevitable stress can lead to the formation of cracks on oxidized PDMS surface. Although a limited control on the pattern of the cracks has been demonstrated,[281–284] often the cracks occur as an unwanted side effect. There are many drawbacks associated with cracked PDMS surface. For instance, the deposition of metallic structures and homogeneous coatings on a cracked PDMS surface poses a
challenge for fabricating electronics on a flexible PDMS substrate.[285, 286] Moreover, plasma oxidation of PDMS has been used extensively by many groups to facilitate biological cell attachment on the PDMS surface.[287] However, the oxidation of PDMS leads to an increase in Young’s modulus of the PDMS surface.[288–292] In the present work, we demonstrate how to prevent crack formation on PDMS surfaces, while increasing the surface energy, to render the surface hydrophilic. This is achieved using a chemical modification of the surface. First, 3-mercaptopropyl trimethoxysilane (MPTMS) is covalently bonded to PDMS to obtain a hydrophilic surface.[293–296] A further increase of the surface wettability is achieved by oxygen plasma treatment. MPTMS-coated PDMS shows no sign of cracks, even after oxygen plasma exposure, as represented by the schematic drawing of PDMS surface in Figure 6.1. Moreover, using atomic force microscopy (AFM) as a nano-indentation method, we show for the first time that this surface modification effectively maintains the elasticity of the PDMS surface, even after oxygen plasma exposure. By varying the exposure time, we investigate the crack density on pristine PDMS surface after exposure to different oxygen plasma doses. Additionally, the effect of chemical treatment prior to oxygen plasma exposure on hydrophobic recovery is studied.

![Fig. 6.1 Schematic illustration of the effect of O₂ plasma exposure on PDMS substrates (a) with and (b) without MPTMS modification.](image-url)
6.2 Introduction

6.2.2 Printing microelectrode arrays on soft substrates

As mentioned in the introduction of chapter 5, MEA devices have attracted enormous interest due to their wide use in different types of applications such as cellular recording, biosensors, and drug screening.[297–307] Perhaps one of the most promising applications of MEA devices are biomedical implants in which the MEA serves as a vital tool for monitoring or restoring biological functionality.[308, 309] Very recently, an implanted neural microelectrode array interface has been developed, which is capable of restoring voluntary control of locomotion after traumatic spinal cord injury.[310, 311] In this case, MEAs with platinum-silicone electrodes have been patterned on a PDMS substrate and used for successful long-term in-vivo recordings. Nevertheless, establishing a reliable communication link between a biological cell and an electrode is currently a challenging task due to the mechanical mismatch between the soft biological tissues and the rigid electronic chip.[312, 313] One important factor to consider when evaluating electronic interfaces for bioimplants is the Young’s modulus of the biological tissue, which lies in the range of 100 kPa to 10 kPa for tissue of the central nervous system (CNS).[314–316] In contrast, electronic implants exhibit very high elastic moduli in the range of giga Pascal for rigid silicon based chips.[317] Therefore, numerous studies have been conducted to investigate the structural biocompatibility (including shape and flexibility) of different substrate materials for future implants.[318–323] However, adding electronic functionality to these soft substrate material still remains challenging due to technical limitations arising from standard fabrication methods. Recently, bioactive coating of microelectrodes using hydrogels has been introduced in an effort to enhance the biocompatibility of the rigid metal biological interface.[324–327] Likewise, several methods have been investigated to fabricate bioelectronic interfaces on flexible substrates such as polyimide [302, 328] and parylene [321] or to transfer a metallic pattern onto PDMS substrates.[294, 329] Unfortunately, these methods require expensive instrumentation and long fabrication processes. As an alternative, inkjet printing has recently proved to be a versatile alternative fabrication tool for patterning high-resolution microstructures with complex electrode geometries on the micrometer scale.[134, 142, 175, 212, 330–332] A major advantage of the fabrication using inkjet printing is the possibility of changing the structure design in flight. Inkjet printing eliminates the need for pre-patterned lithographic masks and thus allows for the adaptation of different geometries in a cost and time efficient manner. Another feature of this method is the ease of incorporation of new emerging ink materials such as PEDOT: PSS (Poly(3,4-ethylenedioxythiophene) doped with poly(4-styrenesulfonate)),[333] which could serve as a better electrode material compared to
gold for cellular interfaces.[334] Similar to the MEA presented in chapter 5, I used carbon nanoparticles as an electrode material due to carbon’s good electrical properties, long electrochemical stability, wide electrochemical safe potential, and high charge injection capabilities for electrical stimulation of nervous tissues.[335–342].

Here, I shed light on the process required for ink-jet printing high-resolution microelectrodes on different soft substrates. I print MEAs on PDMS, agarose, and even gelatin-based substrates including candies (gummy-bears). For the first time, I introduce hydrogel-MEAs, which are challenging to pattern using classical photolithographic methods. Following the successful printing of MEA designs on various substrates, proof-of-principle measurements were conducted with HL-1 cells for determining the feasibility of printed MEAs in cellular recording.

Following the successful printing of MEA designs on various substrates, we cultured HL-1 cells on the printed MEAs. The cells showed high viability and exhibited spontaneous mechanical contraction. Due to the increase of the surface to volume ratio provided by the nanoporosity of the printed carbon electrodes printed on PDMS substrates, we could record cellular action potentials with amplitudes of up to 906 µV$_{pp}$ and a background noise of about 62 µV$_{pp}$, yielding a signal-to-noise-ratio of 14.6. We demonstrate advanced printing methods for printing on PDMS substrates with high-resolution printing features. In this communication we address two main challenges: printing metals or conductive electrode lines and covering it with a dielectric polymer on top of PDMS. We show for the first time fully printed MEA devices on soft PDMS and gelatin substrates. The printed MEA has been used for stimulation and electrical recording from electrogenic cells.

6.3 Materials and methods

PDMS substrate preparation

A 5-inch silicon wafer was silanized in order to generate a repellent surface. The silanization of the wafer was performed by initially activating the surface using oxygen plasma (0.8 mbar, 3 min, 80 W) and afterwards an immediate vapor deposition of perfluorooctyltrichlorosilane (FOTCS) 97% (from Alfa Aesar) to achieve covalent linking. Vapor deposition was performed at 45 mbar for 90 minutes in an argon atmosphere. The silicone elastomer polydimethylsiloxane (PDMS) samples (Sylgard 184 from Dow Corning) were prepared by manually mixing the curing agent and base material at a ratio of 1:10 by mass. This ratio results in a substrate with Young’s modulus of 2.5 MPa as reported previously.[343] In order to cast the elastomer substrate, 10 mL
of the mixture was poured on the FOTCS-coated wafer and subsequently degassed in a vacuum chamber at room temperature. The curing of PDMS was done at 60 °C overnight. Afterwards, the cured PDMS substrate was easily peeled from the wafer.

**Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (FT-ATR-IR)**

The spectra were recorded using a Vertex 70 IR spectrometer (Bruker) with Diamond Platinum ATR accessory and an RT-DLaTGS detector. With a scanner velocity of 10 KHz and 6 mm aperture, 32 spectra were recorded for both PDMS samples. A background spectrum recording was performed before loading each sample.

**Energy-dispersive X-ray spectroscopy**

An EDX detector from a Magellan XHR scanning electron microscope (FEI, Hillsboro, OR) was used for elemental analysis of PDMS substrate before and after chemical modification. All the samples were coated with a carbon layer of 10 – 15 nm prior to spectra recording. They were measured with an accelerating voltage of 10 kV and a 30 s collection period. The data were analyzed with the software provided with the instruments.

**Oxygen plasma**

The PDMS substrate was oxidized using oxygen plasma (100-E plasma system, Technics Plasma GmbH) at 40 W and 0.2 mbar pressure for 5 min, unless stated otherwise.

**Laser interferometry**

Laser interferometric measurements were conducted with a Keyence VK-X130K laser scanning microscope at a wavelength of 658 nm. Surface scanning was performed with Nikon CF Plan 100x/0.85 and CF Plan 50x/0.80 (Nikon Co., Japan) air objectives. The resolution in z-direction was 5 nm. Dust particle spikes were removed from the data for a better visibility.

**Crack density analysis**

In order to quantify the effect of oxygen plasma on the crack density of PDMS surfaces with and without MPTMS coating, oxygen plasma was applied on both samples, for up to 10 minutes. After the exposure, 8 regions with the area of 5.7 mm² were
randomly selected for each sample. Using an in-house developed code based on the Image Processing Toolbox of MATLAB® (R2016b, The MathWorks, Inc., USA), all the images were processed and the total length of the cracks in each image was calculated as described in the supporting information. Finally, dividing the lengths by the area provided the crack densities.

**Chemical modification**

Surface modification of PDMS was performed using (3-Mercaptopropyl) trimethoxysilane 95% (MPTMS) from Sigma-Aldrich. (Warning: MPTMS may be harmful upon inhalation or skin contact) The treatment was carried out in the fume hood by immersing the PDMS substrate in 1:200 MPTMS solution prepared in ethanol for 1 hour. Next, the PDMS substrate was washed with deionized water and dried with nitrogen gas. Hydrolysis was initiated by immersing PDMS in 1 mM HCl solution for 1 hour. Finally, the substrate was washed with deionized water and dried using a stream of nitrogen.

**Contact angle measurement**

The wetting behavior of the PDMS substrate was investigated by contact angle measurement. The sessile drop technique using an OCA H200 instrument (DataPhysics Instruments GmbH) in room temperature was performed. A 2 µL drop of deionized water was dispensed on top of PDMS surface. The acquired image of the water on the sample was taken using an integrated camera. The drop profile of a liquid-vapor interface was extracted and fitted by the Young–Laplace function provided by the OCA H200 software. The contact angle at the liquid–solid interface was assigned according to the fitted profile. Measurements were repeated five times for each case. A small contact angle below 90 °C indicates a high wettability and therefore a hydrophilic surface, while a large contact angle above 90 °C corresponds to a hydrophobic surface.[344]

**Bond strength test**

In order to evaluate the effect of the surface modification on the bonding capabilities of PDMS to a glass substrate, a PDMS device with a dead-end channel was used. The microfluidic channel dimensions were $1 \times 1 \times 20 \, \text{mm}^3$ (width, depth, length) with an outlet diameter of 3.5 mm. The contact area of the PDMS with the glass substrate was $(250 \pm 60 \, \text{mm}^2)$. The pressure was gradually increased and monitored using an inline pressure gauge until the PDMS was separated from the glass, at which point
the pressure was recorded. The measurement was performed 3 times for each type of sample.

**Atomic force microscopy**

AFM measurements in contact mode were performed using a Nanoscope Multimode 8 (Bruker) microscope with an E type piezoelectric scanner and an aluminium-coated antimony silicon cantilever from Bruker, with a nominal spring constant of 5 N/m, frequency of 148 kHz and the tip radius of 8 nm. For each sample, deflection curves were acquired at 3 random positions on the surface. Measurements were conducted up to a deflection threshold of $\sim 60$ nm.

**Inkjet printing**

Microelectrode arrays were fabricated using an OmniJet 300 inkjet printer. Silver nanoparticle ink was used on PDMS samples only. The gummy bear and the agarose based MEAs were printed using carbon ink prepared as described in section 3.3.2 in chapter 3. Prior to printing, all inks were sonicated in a sonication bath for 5 minutes and added in 1 pL cartridges for printing silver and carbon inks. However, 10 pL cartridges were used for printing the passivation layer. The sintering and curing steps were conducted on a precision hot plate (CT10, Harry Gestigkeit GmbH, Germany) for PDMS substrate. However, in case of gummy bears and agarose gel, samples were cured using a photonic curing system with six 500 µs long pulses of 100 V.

**Carbon ink preparation**

The carbon ink in this work was prepared as mentioned in section 3.3.2, chapter 3.

**MEA design layout**

As shown in Figure 6.2, the micro-electrode chip consists of 64 individually addressable carbon electrodes with an inner diameter of $35 \pm 4 \mu m$ and center to center spacing of $50 \pm 5 \mu m$. The size of the final printed chip is $12 \text{ mm} \times 12 \text{ mm}$. Figure 6.2 shows a schematic illustration of the design of the chip and printing process on PDMS substrates. In a first step, silver nanoparticle ink is printed to form the feedlines and bond pads of the chip as shown in Figure 6.2a. The contact pads are used later for connecting the MEA to the electronic amplifier headstage. Next, the carbon nanoparticle ink is printed in a way that covers the silver feedline and forms the electrode area for cellular measurements (Figure 6.2b). This will ensure that only carbon will be exposed to the
electrolyte solution. Finally, a passivation ink is used to cover all the chip except for the contact pads and small electrode opening in the center of the chip. For printing on other substrates, we skipped the first step of printing silver ink in order to have a MEA chip for future in-vivo applications. Therefore, I printed directly a layer of carbon ink followed by the passivation layer.

Fig. 6.2 Sketch of the device principle and printing procedure. In print step 1, a 12 mm × 12 mm outer feedlines layer is printed with silver nanoparticle ink. In print step 2, a 4 mm × 4 mm inner feedlines layer is printed with carbon nanoparticle ink. In print step 3, a 9 mm × 9 mm passivation layer is printed with polyimide ink.

**Oxygen plasma**

Prior to printing, PDMS substrates were exposed to O\(_2\) plasma for 15 seconds at 40 W and 0.2 mbar pressure, unless stated otherwise.

**Gummy bear substrate preparation**

using the same silicon mold used in step-1, commercial jelly bears from Haribo\(^\circledR\) (Haribo GmbH Co. KG, Bonn, Germany) were melted and casted on the silicon mold. Subsequently, substrate was cleaned by ethanol and washed with deionized water. Finally, the substrate was immersed in deionized water for 8 hours before printing.
6.3 Materials and methods

Agarose substrate preparation

Gels were prepared by dissolving 3 g of agarose (Sigma-Aldrich) in 100 mL of buffer. The gel was poured in a petri dish and kept in the refrigerator. The thickness for agarose gel was 3.5 – 5 mm.

Electrical characterization

The resistance of the printed test structures was measured using a multimeter (Voltcraft Plus VC 960, Conrad, Germany). The sheet resistance was measured using a four point probe (Jandel CYL-HM21, Bridge Technology, USA).

Electrochemical characterization

Prior to electrochemical measurements, all the chips were cleaned by ethanol for 5 minutes followed by 5 minutes in water. A glass ring with a height of 10 mm and a diameter of 7 mm was glued to the MEAs using polydimethylsiloxane (PDMS) in order to create a reservoir. Electrochemical experiments were performed using a Biological potentiostat (VSP-300 potentiostat from BioLogic Science Instruments). All experiments were carried out in a supporting electrolyte of phosphate buffered saline (1x) using 0.5 mM solution of 1,1-ferrocene dimethanol (Sigma-Aldrich) in PBS (pH 7.4) as a redox tracer unless stated otherwise. The signals were recorded vs a Ag/AgCl reference electrode (Super Dri-ref SDR 2, World Precision Instruments, USA).

Cellular recording

Prior to cell culture, the chips were sterilized by incubation with 70% ethanol for 10 min, followed by rinsing with sterile bidistilled water thrice. They were coated with 2.5 µg/cm² fibronectin from bovine plasma in calcium and magnesium free PBS at 37 °C for 1 h. They were rinsed once with supplemented Claycomb medium just before cell seeding. Cardiomyocyte-like HL-1 cells were maintained in Claycomb medium supplemented with 10 v% fetal bovine serum, 100 µg/ml Penicillin-Streptomycin, 0.1 mM (±) Norepinephrine (+)-bitartrate salt and 2 mM L-Glutamine in a humidified incubator at 37 °C and 5 % CO₂. The medium was changed daily. Once confluency was reached, the contracting cell layer was first washed and then detached by incubation with 0.05 % trypsin-EDTA at 37 °C. Trypsin digestion was then inhibited by addition of supplemented Claycomb medium and the cells were sedimented by centrifugation at 200 rcf for 5 min. They were resuspended in pre-warmed, supplemented Claycomb medium and a small volume was added to the center of each chip. The cells were left
to adhere in a humidified incubator at 37 °C and 5% CO₂ for 30 minutes. Afterwards, 500 µm of medium were added to each chip. The medium was exchanged daily until confluency was reached. Once the confluent cell layer was beating, action potentials were recorded employing a 64 channel MEA amplifier system developed in house. The system consists of a headstage connected to a main amplifier which is connected to the controlling PC via a high-resolution A/D converter. The headstage holds the MEA chip and provides a voltage amplification of 10. The main amplifier was operated at a gain of 100 for all measurements, yielding a total amplification of 100. Data acquisition are controlled through a in-house developed LabView software (National Instruments, Austin, USA), which allows the definition of the recording parameters such as gain and filter settings. We limited the effective bandwidth with a high pass filter (AC coupling) and a low pass filter (high frequency cutoff) from 1 Hz to 1 KHz for all measurements reported here.

6.4 Results and discussions

6.4.1 Substrate preparation for inkjet printing

Surface modification

Using FT-IR and FT-ATR-IR, the surface chemistry of pristine PDMS along with different modifications of PDMS, namely: oxidized PDMS (PDMS–O₂ plasma), MPTMS-coated PDMS (PDMS–MPTMS) and oxygen plasma treated MPTMS-coated PDMS (PDMS–MPTMS–O₂ plasma), are characterized (Figure 6.3). The spectra of all the samples show the main characteristic peaks of the PDMS surface.

The sulfhydryl (SH) peak at 2550 cm⁻¹ in the FT-IR spectrum of PDMS–MPTMS confirms that MPTMS was successfully coated on the PDMS surface (Figure 6.3b). The presence of sulfur on PDMS–MPTMS was also confirmed by EDX (see Figure 6.3. 2S in the supporting information). In FT-ATR-IR spectra, the doublet peaks at 1000 – 1100 cm⁻¹ (Figure 6.3a) correspond to the asymmetric (vₐ) and symmetric (vₛ) stretching vibrations with a high absorption coefficient of siloxane bonds.[345] The two peaks appearing at 1259 cm⁻¹ and 800 cm⁻¹ have been reported to originate from the in-plane bending or scissoring and out-of-plane oscillations of the Si–CH₃ bond, respectively.[346, 347] The peak around 3000 cm⁻¹ represents the asymmetric and symmetric stretching of the methyl groups on the PDMS surface.[348] To reveal the details of the spectra between 3000 and 4000 cm⁻¹ that are dominated by the large absorption coefficients at low wavenumbers, we zoom into that region in Figure
As seen in this figure, PDMS–O$_2$ plasma shows a broad peak in the range of 3000 – 3700 cm$^{-1}$, which reveals the partial replacement of methyl groups (CH$_3$) with hydroxyl groups (OH), resulting in the formation of polar silanol groups (Si–OH) on the PDMS surface.[255, 349, 350] Therefore the formation of a brittle silica-like layer on the surface is expected, which has been previously reported.[351] Interestingly, unlike PDMS–O$_2$ plasma, PDMS–MPTMS–O$_2$ plasma shows a very similar spectrum to pristine PDMS and PDMS–MPTMS in the mentioned range. This observation indicates the protective role of MPTMS to the PDMS surface against oxygen plasma. We assume that the added mercapto-containing compound (in this case MPTMS) reacts with the carbon–carbon double bonds (C=C) that are available in the low molecular weight oligomers of PDMS. The reaction is known as thiol-ene reaction, which is based on click chemistry.[352–354] This results in the formation of thioether groups (C–S–C), causing fixation of PDMS oligomers and preventing their migration to the surface.[266] On the other hand, the oxidative cleavage or ozonolysis reaction
is responsible for the cleavage of the carbon double bond in the molecule of siloxane oligomers of PDMS into R-OH. However, the oxidative cleavage reaction in this case is hindered by the thioether group and therefore adding mercaptosilane may inhibit fast oxidation.

![Fig. 6.4](image)

**Fig. 6.4** Elemental Analysis of the (a) pristine PDMS and (b) PDMS–MPTMS. The analysis verify the presence of surface coating of PDMS with MPTMS due to the presence of the sulfur peaks at 2.3 and 2.46 KeV.

**Hydrophilicity of chemically modified PDMS surface**

The hydrophobic recovery is an ongoing problem in the realization of reliable microfluidic systems, particularly for bioassays that require prolonged storage before use. In order to evaluate the changes in the surface energy of PDMS, we performed contact angle measurements using the sessile drop method. The surface contact angles of water on pristine PDMS compared to different PDMS modifications as a function of aging time in air (humidity 45 − 60 %, room temperature) are shown in Figure 6.5.

As expected, PDMS surface without any physical or chemical modification is inherently hydrophobic with a static angle of about 109° ± 3°, also consistent with literature values.[355, 356] It can be seen that oxygen plasma treatment (60 seconds) reduces the contact angle down to 70° ± 7°. Here, we applied a mild oxygen plasma treatment to obtain surfaces with higher contact angles, which are suitable for material deposition with water-based inks using e.g. inkjet printing. A longer exposure time to oxygen plasma (600 s) showed a stronger effect of decreasing the contact angle to 20° ± 5°. As seen in Figure 6.5, PDMS–O₂ plasma undergoes fast hydrophobic recovery during the first day of the treatment, as reported earlier.[265, 357] In the case of PDMS–MPTMS the contact angle is reduced down to 80° ± 4°. Moreover, the contact angle remained much more stable over the duration of 9 days in comparison with
6.4 Results and discussions

Fig. 6.5 Water contact angles as a function of the aging time during air exposure, measured on surfaces of pristine PDMS, PDMS–MPTMS, PDMS–MPTMS–O$_2$ plasma and PDMS–O$_2$ plasma.

PDMS–O$_2$ plasma. The contact angle measurements of PDMS–MPTMS–O$_2$ plasma revealed a stronger hydrophilic character of the surface compared to PDMS–MPTMS and even PDMS–O$_2$ plasma. Initially, the contact angle decreased to 64° ± 7°. After 3 days, it approached the state of PDMS–MPTMS sample. Additionally, to assess the usefulness of the method for microfluidic devices where a hydrophilic PDMS surface is bonded to a glass substrate, we found that the PDMS–MPTMS sample on a glass substrate can withstand a bursting pressure of up to 150 ± 8 kPa.

MPTMS modification and plasma-induced crack spacing

It was observed that with the increase of the plasma exposure time, the crack density on PDMS–O$_2$ plasma samples increases steadily in a decelerative manner. However, no microscopic cracks could be observed in case of PDMS–MPTMS–O$_2$ plasma (see Figure 6.6). This indicates that treating PDMS surface with MPTMS can form a barrier that prevents the crack formation due to oxygen plasma exposure.
**Fig. 6.6** (a-d) Optical microscope images of PDMS–O$_2$ plasma samples with different oxygen plasma exposure times. Scale bars represent 400 µm. (e) Crack density on PDMS–O$_2$ plasma and PDMS–MPTMS–O$_2$ plasma versus oxygen plasma exposure time. Without MPTMS treatment, cracks start to appear on the surface of PDMS immediately, whereas no crack was observed on PDMS–MPTMS–O$_2$ plasma samples.

**Atomic force microscopy**

In order to confirm that MPTMS treatment prevents the formation of a silica-like crust, we investigated the flexibility of the samples by acquiring deflection curves at three distinct positions for each sample and a glass substrate as reference. Figure 6.7 shows the deflection curves, averaged over the three measurements. According to the curves,
O₂ plasma-treated PDMS has a hard surface. In addition, extended plasma treatment leads to an even stiffer surface, suggesting an increase in the thickness of the silica crust. On the other hand, MPTMS treatment itself does not strongly influence the elasticity of PDMS surface. Moreover, it notably preserves the elasticity of the surface after oxygen plasma exposure, confirming the absence of a crust on the surface. The results of AFM measurements confirm the assumption that the chemical modification minimizes alterations in hardness and elasticity of the PDMS surface due to oxygen plasma exposure. Therefore, it can be concluded that MPTMS coating may form a protective barrier against physical alterations caused by oxygen plasma.

Fig. 6.7 Averaged deflection curves vs. the z-displacement measured on 6 different substrates (N = 3 for each sample). Five different surface modifications of PDMS are compared with glass as a rigid substrate. The average and the maximum of the standard deviations in deflection over the whole data points are 1.1 nm and 5.2 nm, respectively.

6.4.2 Printing microelectrode arrays on soft substrates

Printing lines on PDMS

Developing functional electronics on soft materials requires the printing of electrically continuous lines. Therefore, it is important that adjacent ink droplets, which are deposited on the substrate are connected to a functional entity. However, this is typically difficult to achieve with a water-based ink containing the functional material
and a hydrophobic substrate such as PDMS. Our pristine PDMS substrates exhibited a static contact angle of PDMS was $109^\circ \pm 3^\circ$ as discussed in section 6.4.1. The high contact angle consequently leads to a breakup of the deposited liquid structure due to dewetting, effectively causing discrete islands of printed liquid similar to condensed water droplets on a cold surface. One way to avoid dewetting of ink on PDMS surfaces is to implement a multilevel matrix deposition (MMD) method, in which few drops separated by a defined distance in the X- and Y-directions are printed each time until a whole film is completed. This method has been adapted to print on a wide variety of substrates as reported previously.[296, 358]

Another way to overcome the dewetting problem is to increase the substrate surface energy. For instance, chemical modification of PDMS surfaces has been applied to tune the PDMS wettability. As discussed in the previous section (section 6.4.1) Chemically modifying PDMS surfaces using MPTMS can be utilized for improving the surface wettability and enhancing the adhesion of the deposited ink.[343]

In order to fine-tune the PDMS surface energy to comply with our printing resolution, we control the degree of surface modification using MPTMS by changing the incubation time. Figure 6.8 shows the printing results of carbon ink on the PDMS substrate with different MPTMS incubation times using the same line pattern. As seen in Figure 6.8c, the water contact angle decreases with longer incubation time. Optimal conditions for continuously formed lines are observed after approximately 60 minutes. As our ultimate goal is the fabrication of MEAs with small electrode sizes, we contained printing lines structures by limiting the incubation time to 60 minutes. Larger features size can be patterned on PDMS by increasing the wettability of the surface. Thus, a wide range of features ranging from high-resolution individual lines of 30 $\mu$m up to a few millimetre-wide structures can be printed by tuning the MPTMS incubation time.

**Printing passivation polymer film on PDMS**

Fundamentally, the microfabrication of electronic devices for applications in wet environments, requires the patterning of a dielectric material, which serves as a passivation layer. In the case of microelectrode arrays for electrophysiological or electrochemical recordings, the passivation layer must be biocompatible and provide insulation of the feedlines versus conducting electrolytes. Moreover, it needs to be chemically resistant against the medium supporting the cell culture. For this reason, we chose a polyimide (PI) insulation material, which has already demonstrated good passivation properties in clean-room fabricated microelectrode arrays.
6.4 Results and discussions

Fig. 6.8 Effect of MPTMS incubation on printed line formation. (a) Microscopic images of printed carbon lines with a fixed drop spacing of 20 µm versus the incubation time of MPTMS, scale bars represent 200 µm. (b) Schematic drawing of the ink spreading on a PDMS substrate. (c) Measured contact angles of a water drop as a function of the incubation time of MPTMS. (d) Optical microscope images of printed PI ink on PDMS as a function of oxygen plasma exposure time. The printer software is provided by a pattern, which creates a rectangular shape as shown in the third image of this sequence. All structures were printed with a fixed DPI (dots per inch) of 846. Scale bars represent 200 µm.
Fig. 6.9 Multilayer printing process of carbon MEA on PDMS using (a) silver ink as a first layer (b) carbon ink as an electrode material deposited in the second layer and (c) PI ink as a passivation ink. The electrode opening is indicated by the red arrow in (c). Scale bars represent 200 µm.

As I am aiming for a fully printed device, it is crucial to choose a suitable ink and to understand possible factors that could induce passivation failure. One common problem in printing continuous passivation films is dewetting, which is especially important for covering areas that are larger than two printed drops. Considerable work has been reported and applied in order to avoid dewetting when printing films either by using algorithm-generated spacings or by radically changing the surface energy of the used substrate.[228, 359] As we discussed earlier, one can change the PDMS wetting degree to a desired state by changing the incubation time with MPTMS. However, it is not possible to combine two different wetting states: one for printing fine structures while the other enables the printing of large films. Obviously, there is no universal wetting state that can meet all the requirements for printing small and large structure for different inks. In this case, printing multilayer electronic devices bearing carbon lines and passivation films on PDMS, requires changing the surface energy of the PDMS surface again to a degree that matches the requirements for good wetting in order to form a passivation film. In other words, if one initially modifies the PDMS surface to be completely wetting in order to homogeneously cover the surface with a passivation layer, the microelectrodes will be shorted as seen in Figure 6.8a. As I presented in section 6.4.1 that the MPTMS treatment acts as a protective layer and prevents spontaneous crack formation upon plasma treatment. We took advantage of the chemical modification applied to print the microelectrodes and examined the effect of different plasma doses on the wetting of the passivation ink on PDMS surfaces. Figure 6.8d shows the effect of the applied plasma time on the quality of the printed passivation layer at fixed DPI (dots per inches).
For the fabrication of microelectrodes, I aim at covering a precise area of the substrate while keeping the active electrode uncovered. This is crucial for contacting the chips with the electronic headstage and for preserving a small electrode opening during microelectrode fabrication. Figure 6.8d shows a series of images taken immediately after printing of a passivation layer. We can see that after 150 seconds of plasma activation time, the passivation film could be printed in controlled way. In a similar manner, we printed a passivation film on top of the carbon electrode, which has been previously printed on the PDMS substrate as discussed earlier. The final devices are shown in Figure 6.9. The passivation was printed in a way that only a small carbon area is exposed to the liquid as seen in Figure 6.9c.

Characterization of printed MEAs

The effect of thermal treatment on the conductivity of printed carbon nanoparticles, we measured the sheet resistance for test pattern. Figure 6.10a shows the sheet resistance obtained at various temperatures on the PDMS substrate. The resistance of printed carbon decreased with increasing the thermal treatment which is likely due to removal of hydrocarbon molecules and other functional groups. As seen in Figure 6.10a, the resistance showed a large increase at a temperature of 150 °C n = 10 most probably due to thermal expansion of the PDMS. Therefore, a temperature of 120 °C was chosen to be used for thermal annealing of carbon.

To evaluate the stability of the printed microelectrode device on PDMS upon bending, we investigated the resistance of printed lines after applying repetitive cycles of bending experiments. For this experiment, the substrate was bent to a radius of 5 and 10 mm and relaxed several times followed by electrical characterization. Figure 6.10b shows the relative change in the resistance, $\Delta R/R_0$, where $\Delta R = R - R_0$, $R$ and $R_0$ are the resistance for the bent and relaxed state, respectively. As indicated in Figure 6.10b, after 20 cycles for a bending radius of 10 mm, the resistance increased only by 10%. This observation demonstrates the good mechanical stability of the carbon electrode printed on PDMS substrate.

The electrochemical performance of the printed MEAs on PDMS was also evaluated prior to extracellular recording. First, to gain some insight into the intrinsic potential windows of printed carbon on PDMS, cyclic voltammogram (CV) measurements were recorded in PBS solution (Figure 6.10c). The CV scan shows a relatively featureless region within the water window in the range of -2 to 2 V (all potentials vs. Ag/AgCl). The absence of any other peaks within the working potential of the carbon electrode confirms also the absence of redox active contaminants. Consequently, we demonstrated
Fig. 6.10 Characterization of the printed PDMS carbon MEA. (a) Sheet resistance vs temperature curves of carbon film on PDMS, error bars represent standard deviation ($n = 10$). (b) The bending stability of carbon electrodes printed on PDMS, relative resistance as a function of bending cycles to 5 (black line) and 10 mm (red line) radius of curvature. (c) Inert potential range for printed carbon electrode compared to evaporated gold electrode. (d) Typical Nyquist plot obtained for the bare electrode (red circles). The obtained spectra was fitted using a Randles equivalent circuit $R_{ct}$ values (blue circles). The measurements were performed in the presence of $\text{Fe(CN}_6^{3-/4-}$ as the redox probe frequency range between 1 Hz and 100 KHz and 200 mV as a biased potential.
that selecting carbon as an inert and conductive electrode material is suitable for electrochemical applications.

Second, to get a better understanding of the printed electrode impedance, we performed electrical impedance spectroscopy (EIS). Figure 6.10d shows typical Nyquist plot for the printed PDMS MEA. In order to characterize the interfacial electron transfer on the printed MEA, Faradaic impedance measurement were performed using a ferrocyanide-ferricyanide couple as a redox molecule Figure 1d presented by Nyquist diagram. The measured data was fitted with Randles circuit as discussed in chapter 2. The electrochemical charge transfer resistance \( R_{ct} \), which is observed from the semicircle region of the Nyquist plot, was determined to be 21.5 KΩ.

Figure 6.11 shows typical Bode spectra measured using the printed PDMS MEA in PBS, the data was fitted with a \( R_e (R_{ct} C) \) equivalent circuit, where \( R_e \) is the resistance of the electrolyte solution, \( R_{ct} \) is the charge transfer resistance and \( C \) is the electrical double layer capacitance. The electrical double layer capacitance was in the range of 800 – 900 µF/cm². This high double layer capacitance value results from the porous electrode nature is in good agreement with previously reported values.[7, 232]. The electrochemical non-faradic impedance recording from all 47 electrodes printed on PDMS are also shown in Figure 6.12. The results show clearly the ratio of the non-functioning to working electrodes after fabrication. Notably, 41 carbon electrodes were working with low impedance values in the range of \( 7 \times 10^{-5} \) Ω measured at 1 KHz, with good consistency over 40 electrodes, while 6 electrodes showed a high impedance
Fig. 6.12 Impedance measurement of 47 electrodes printed on PDMS.

due to failure to connect to the electronics. The failure can probably be attributed to a poor bonding to the chip carrier.

**Extracellular voltage recordings on soft MEAs**

In order to evaluate the capability of the printed MEA for biomedical application, extracellular recordings of cardiomyocyte-like HL-1 cells were performed on the PDMS MEAs. The excellent cell viability as shown from the live/dead staining in Figure 6.13, coupled with the fact that the HL-1 cells were spontaneously contracting, suggests that these devices are biocompatible. On the other hand, finding electrode materials with low elastic modulus leads to minimizing the mechanical mismatch between the tissues and the electronic device. The printed carbon electrodes have an elastic modulus of 1 MPa.[360] This low elastic modulus makes carbon a much softer electrode material for cellular recording than the stiffer metal electrodes such as gold (elastic modulus ca. 78 GPa), iridium (elastic modulus ca. 525 GPa) and platinum (elastic modulus ca. 250 GPa).[317, 361] To determine whether the nanoporous carbon structures enable the electrical coupling between the cultured cells and the electrode surface, electrical signals from HL-1 cells were recorded using three printed chips. Figure 6.14 shows an example of the electrical signal recorded on 13 different electrodes on the same MEA. The HL-1 cells exhibited spontaneous action potentials with a firing rate of 2.3 Hz.
Fig. 6.13 Final device and demonstration of the printed MEA on PDMS (a) Photograph of printed carbon MEAs on PDMS-substrate, (b) final chip bonded to printed circuit board and encapsulated for use in cell culture, (c) live/dead staining of HL-1 cells grown on PDMS MEA (scale bar 100 µm). (d) beating of HL-1 cells grown on nanoporous carbon electrodes over a time span of 2 s, (e) action potential recording at a higher time, (f) overlay of two individual extracellular recordings channels the temporal shift indicating signal propagation.
Fig. 6.14 Printed MEA on soft hydrogel substrates for extracellular recording. (a) Photograph of printed carbon MEAs on a gummy bear substrate. (b) Final chip bonded to printed circuit board with HL-1 cells culture. (c) Exemplary photograph of a printed MEA on a gelatin substrate. (d) Action potential recording from HL-1 cells using printed carbon microelectrodes on a gummy bear substrate, traces are offset in y-direction for clarity of representation. (e) HL-1 cells stimulation with noradrenaline (NA). (f) Photograph of a printed MEA on an agarose substrate. (g) Microscopic image of printed MEA on an agarose substrate.
The maximum cell signal amplitude recorded was 906 \( \mu V_{pp} \) and a background noise of about 62 \( \mu V_{pp} \), which is comparable to our reported values using printed gold and carbon MEAs on PEN substrates (refer to chapter 5). The results are also comparable with previously reported values of HL-1 recordings using nanocavity electrodes [362] and pillar electrodes.[363]

We further tested the printed MEAs on gelatin-based gummy bear substrates for cellular recording. Gelatin was chosen as substrate for several reasons. First, it is a soft material with a Young’s modulus in the range of 100 – 102 kPa.[364] Second, it has been used as a scaffold for tissue engineering and proved to be an excellent material for repairing traumatic injuries to the brain as it improves the brain-tissue reconstruction.[365] Third, it exhibits antibacterial and haemostatic effects,[366, 367] which has been shown to enhance tissue healing after mechanically inserting the electrodes to the desired tissue location.[368, 369] Therefore, it does not only prevent foreign body reactions, but also supports the surrounding tissue to heal after implantation of electronic devices.

Experiment to measure HL-1 action potential was performed using printed MEA on gelatin substrate (see Figure 6.14). Action potentials were recorded from 8 channels with a maximum amplitude of 442 \( \mu V_{pp} \) and a background noise of approximately 80 \( \mu V_{pp} \). Spontaneous mechanical contraction could be observed microscopically, resulting in a firing rate of 1.3 Hz. To further examine the signal specificity, the cells were chemically stimulated with noradrenaline (NA), a catecholamine that triggers a sympathetic response. As expected, the firing rate of the spontaneous action potential was increased up to 1.8 Hz upon addition of 4 \( \mu L \) of a 10 mM noradrenaline (NA).

Overall, the cells showed high viability and exhibited spontaneous mechanical contraction as observed by the live/dead staining. Due to the increase of the surface to volume ratio provided by the nanoporosity of the printed carbon electrodes printed on PDMS substrates, we could record cellular action potentials with amplitudes of up to 442 mV_{pp} and a background noise of about 80 mV_{pp}, yielding a signal-to-noise-ratio of 14.6.

6.5 Conclusions

In summary, the aim of the present chapter was to study new substrate material for cellular recording. In order to achieve this, I first investigated the realization of crack-free PDMS based on chemical protection, using MPTMS. This technique allows tuning of the PDMS hydrophilicity using a chemical modification combined
with oxygen plasma. Moreover, modifying the surface with MPTMS can stabilize the hydrophilicity of PDMS at least up to 9 days. The use of MPTMS prior to oxygen plasma treatment prevents the formation of the silica crust and, therefore, helps in preserving the mechanical properties of the pristine PDMS surface. This simple and fast surface treatment can also be applied for \textit{in-vitro} studies of cellular physiology on soft interfaces and microfabrication of flexible electronics on PDMS substrates.

Secondly, I designed and fabricated soft MEAs on PDMS, gelatin and agarose substrates. A straightforward process was investigated which exploits the poor wetting properties of carbon and PI inks on hydrophobic substrates such as PDMS. In this section, I addressed two main challenges: printing conductive electrodes and feedlines and insulating the feedlines with a dielectric polymer on top of PDMS. I showed for the first time fully printed MEA devices on soft PDMS and gelatin substrates. I presented solutions to circumvent major printing problems in printing small lines and large films printed on the same substrate. Moreover, I have introduced gelatin and agarose as a substrate for MEA fabrication. The printed carbon MEAs showed good mechanical stability and electrochemical proprieties. Our results demonstrated a high double layer capacitance will enhance the signal to noise recorded during cellular measurements. This will simultaneously allow to use the printed electrodes in electrical stimulation of electrogenic cells due to the higher safe-charge-injection limit. Soft MEAs were used for \textit{in-vitro} measurements of action potentials and successfully recorded activity from HL-1 cells, validating the suitability of the printed chips for electrophysiological measurements. The printing method presented in this chapter can be exploited in future work, for the printing of dense multilayer microelectrode arrays, in order to meet the needs in neuroscience applications \textit{in-vitro} and \textit{in-vivo}. 
Chapter 7

Conclusions and Outlook

7.1 General conclusions

This thesis focuses on the utilization of inkjet printing technology for fabrication of electrochemical devices for biosensing and bioelectronic applications. The results were loosely divided into two main ideas, namely molecular and cellular biosensing.

In the topic of molecular biosensing, two types of biorecognition elements were investigated: antibodies for protein detection and PNA for nucleic acid sensing. For protein detection, I developed an immunosensor to detect CRP protein in human serum using printed technology. In this approach the antibodies bind covalently to the printed gold surface by exposing the antibodies to UV radiation prior to the gold surface. The UV photons are absorbed by tryptophan residues and then transferred to the Cys-Cys. As a result, a disulfide bridge is opened, forming free thiol groups, which can covalently bind to the gold electrode in controlled orientation. As demonstrated in chapter 2, the proper orientation of the immobilized antibody by the photonic approach was characterized and compared to random immobilization strategies by electrochemical methods using printed micro electrodes. The selectivity of the developed immunosensor was investigated via detection of CRP, showing lower cross-reactivity with a non-target solution compared to the target antigen. The biosensor was further tested for the detection of CRP in human serum, showing a high discrimination ability. Overall, I have demonstrated a new approach for the fabrication of biosensors based on photonically activated antibodies and printed technology for the selective and sensitive detection of CRP protein.

In the case of nucleic acid sensing, a sensitive electrochemical biosensor for the detection of an HIV-related gene, based on the interactions between DNA and PNA was investigated. The ssDNA detection was performed using electrochemical redox
cycling as an amplification strategy. Redox cycling is an electrochemical technique that amplifies faradaic current through repetitive oxidation and reduction events between two closely-placed electrodes. As discussed in chapter 3, a novel approach was investigated for fabricating micro-gap electrodes using inkjet printing without any surface pretreatment. The electrochemical behavior of the printed micro-gap device and the sensitivity during redox cycling mode were studied. Sequentially, ssDNA marker sequences encoding the HIV-1 nef gene were chosen, as they are clinically overexpressed in the early stage of the disease among HIV-positive patients. Fundamental aspects such as the electrochemical characterization of the genosensor and evaluation of the immobilization strategy using PNA were also discussed. Finally, the sensitivity and the selectivity of the ssDNA detection scheme using printed micro-gap sensors were evaluated. Thus, a novel approach for direct, sensitive, fast and reproducible DNA sensing was presented which opens a promising path towards direct ssDNA detection at the point of care.

At the end of the micro-gap work, a limitation in the lateral resolution in inkjet printing was pointed out. Afterwards, the vertical resolution of inkjet printing by printing vertically stacked microelectrodes was studied. In this part, the fabrication of the electrochemical redox cycling device was based on the vertical stacking of two conductive electrodes made of carbon and gold nanoparticle inks. In such configuration, the two electrodes are parallel to each other while being electrically separated by a layer of polystyrene nanospheres. This design allowed for the printing of two electrodes with a very small distance between them, down to 100 nm. This design led to a considerably improved sensitivity, as explained in chapter 4. The results obtained with the vertically stacked design suggests a novel fabrication approach for printing multilayer nanoporous redox cycling devices with a tunable nanometer gap that provides electrochemical sensing.

The second part of the results, as mentioned above, was focused on cellular biosensing. To this end, several electrode and substrate materials were investigated for extracellular voltage recordings using printed technology. First, the successful fabrication of MEA devices in a rapid prototyping approach based on inkjet printing was presented. As discussed in chapter 5, two types of electrode materials were investigated for fabricating MEA on flexible polymer-based substrates: gold and carbon. The results obtained from HL-1 cells grown on the printed gold-MEA demonstrate the feasibility of using printed MEAs for recording action potentials. The printed gold MEA showed a signal-to-noise ratio of $\approx 17$. The cellular origin of the recorded signals was demonstrated by chemically stimulating the cells with noradrenaline, which led
to an increased frequency in the recorded signals. On the other hand, the printed carbon MEA showed an improved signal-to-noise ratio. It is worth noting that printing of a chip for cellular measurements takes less than 60 minutes and costs less than three cents per chip. Therefore, I overcome plenty of the current limitations of the conventional fabrication approaches namely: high costs, inflexible substrates, and lack of rapid prototyping capabilities.

Moreover, various substrates were investigated as alternatives to the traditional options of silicon and glass, such as PDMS, gelatin and agarose. For the PDMS substrate, I first optimized the surface energy of PDMS for printing MEA. Here, two surface modifications were used to tune the surface energy of the PDMS; the first modification was performed using MPTMS to print small, fine structures of carbon ink. This modification approach was investigated by FT-ATR-IR and EDX in order to confirm the surface modification. The modification was evaluated further by contact angle measurements to investigate the wetting behavior of inks on the PDMS substrate. It was observed that MPTMS modification renders the PDMS surface hydrophilic, persistent for multiple days. The second surface modification was oxygen plasma exposure. Interestingly, exposure of MPTMS-treated PDMS to oxygen plasma revealed a stronger hydrophilic effect of the surface compared to MPTMS-treated PDMS without plasma treatment. Furthermore, since the applicability of MPTMS modification to microfluidic devices requires a remarkable adhesion between the structure and substrate, the withdrawal pressure of PDMS-MPTMS samples on a glass substrate was measured.

![Fig. 7.1](image)

**Fig. 7.1** (a) Unshrunken Shrinky Dink substrate with inkjet printed MEA pattern using carbon ink. (b) The same sample after being baked at 180 °C. Note the shrinkage in the structure upon baking.

Crack appearance on a PDMS surface is a common problem with exposing PDMS to oxygen plasma, which has a negative impact on PDMS applications such as the deposition of metallic structures and homogeneous coatings. One important advan-
Conclusions and Outlook

Fig. 7.2 SEM images of discrete dot array printed using carbon nanoparticle ink. (a) Inkjet printed dots array after backing. (b) and (c) Wrinkles and crumples introduced to the printed structures upon baking the Shrinky Dink substrate.

One advantage of MPTMS modification, apart from being simple and fast, is that it prevents spontaneous crack formation on the PDMS surface upon oxygen plasma exposure. Therefore MPTMS modification and oxygen plasma treatment make an effectively combination. After covering the PDMS surface with MPTMS and successfully printing the electrodes, the surface was exposed to oxygen plasma for printing passivation films. Finally, to explore the potential of the printed PDMS and gelatin–based MEAs, *in-vitro* extracellular signal recordings of HL-1 cells were successfully demonstrated. The results presented in chapter 6 suggest that manufacturing soft implants is possible using inkjet printing.

To conclude, the feasibility of applying inkjet printing for fabricating sensitive biosensors and cell-chip platforms has been investigated. This thesis represents a step toward the design of soft bioelectronic devices for next-generation biosensor and bioelectronics devices.

### 7.2 Outlook

This thesis shows how printing technology can be used in extracellular recordings. One challenge facing printed technology is the size of the printed drop, which is limited by the volume of the jetted drop. A novel approach that would meet this challenge is the use
of a thermoplastic substrate that can easily shrink down the printed feature size. Upon heating above their glass transition temperature, these substrates experience shrinking. Figure 7.1 demonstrates the concept of a shrunk MEA. The advantages of thermal shrinkage include (a) significant reduction in size, (b) the feasibility of using a higher sintering temperature than that used in a PEN substrate and (c) shrinkage-induced wrinkles in the electrode surface that are expected to decrease electrode impedance. Figure 7.2 shows size reduction upon heating of the printed MEA on a Shrinky-Dink substrate. As seen in Figure 7.2, the printed carbon dots exhibit “wrinkled flowers” which may find its potential application in many Fields such as tissue engineering.

Another promising approach that can be employed for interfacing cells is the use of a biocompatible conducting polymer such as poly (3,4-ethylenedioxythiophene)
doped with polystyrene sulfonate (PEDOT:PSS). Several groups have investigated this material and demonstrated an improved cell-device coupling compared to typical metal electrodes. In this context, developing flexible PEDOT:PSS based microelectrode arrays was tested in preliminary experiments. Figure 7.3 shows initial results for the fabrication of PEDOT:PSS MEA using printed technology, featuring high-resolution lines of conducting PEDOT:PSS. In order to investigate the biocompatibility and suitability of the printed PEDOT:PSS MEA for biological systems, as shown in the initial tests in Figure 7.4, cell-surface testing was conducted using cardiomyocyte-like HL-1 cells. The printed samples were initially coated with gelatin/fibronectin prior to cell culture. Cells were seeded on the printed test lines and exhibited a healthy growth. After seeding, the cells formed a confluent layer within 2-3 days, mostly on the printed area. In this stage, the cells showed contraction.

In my opinion, the main objective of future work should be devoted to developing inkjet-printed biocompatible MEAs on biodegradable substrates such as agarose or gelatin. Overall, this method can offer biocompatible and highly flexible devices for next-generation neural implants.
References


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