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Princip delovanja tranzistorja s krmilno elektrodo v elektrolitu kot imunosenzor

Magistrsko delo

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**Table 1:** Quantities and symbols
List of Abbreviations

- Ab - antibody
- AC - alternating current
- AFP - Alpha fetoprotein
- AgNP - silver nanoparticle
- AI - Avian influenza
- ALP - alkaline phosphatase
- AMS - accelerator mass spectrometry
- ATR - attenuated total reflection
- AuNP - gold nanoparticle
- AV - avidin
- BSA - Bovine serum albumin
- CCD - Charge-coupled device
- CDR - complementarity determining region
- CEA - Carcinoembryonic antigen
- CL - Chemiluminescence
- CLIA - Chemiluminescence immunoassay
• CNT - carbon nanotube
• CPE - constant phase element
• CRP - C-reactive protein
• cTnI - cardiac troponin I
• CVD - chemical vapour deposition
• DAC - digital-to-analog converter
• DAPE - diaminodiphenyl ether
• DC - direct current
• DNA - Deoxyribonucleic acid
• DI-H$_2$O - de-ionized water
• EDC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
• EDL - electric double layer
• EDLC - electric double layer capacitor
• EGFET - electrolyte-gated field-effect transistor
• EGOFET - electrolyte-gated organic field-effect transistor
• EIS - electrochemical impedance spectroscopy
• ELISA - enzyme-linked immunosorbent assay
• ESI - electrospray ionization
• FET - field-effect transistor
• HAT - Human alpha thrombin
• HAU - hemagglutinin units
• HBsAg - Australia antigen
• HEMT - high electron mobility transistor
• HIV - Humman immunodeficiency virus
• HRP - horseradish peroxidase
• HTS - high throughput screening
• ICAT - Isotope-coded affinity tags
• IDE - inter-digitated electrodes
•IDES - inter-digitated electrode structure
• ISFET - ion-sensitive field-effect transistor
• KT - Ketamine
• LC - liquid chromatography
• MALDI - matrix assisted laser desorption/ionization
• MHA - 6-Mercaptohexanoic acid
• MIP - molecular imprinted polymer
• MOSFET - metal-oxide-semiconductor field-effect transistor
• MPA - 3-Mercaptopropionic acid
• MUA - 11-Mercaptoundecanoic acid
• MWNT - multi-walled carbon nanotubes
• NHS - N-hydroxysuccinimide
• PBS - Phosphate-buffered saline
• PCT - Procalcitonin
• PI - polyimide
• PL - phospholipid bilayer
• PMDA - pyromellitic dianhydride
• PML - promyelocytic leukemia
• PSA - prostate-specific antigen
• PVD - physical vapour deposition
• QCM - Quartz crystal microbalance
• RAC - Ractopamine
• RARα - retinoic acid receptor alpha
• RCA - rolling circle amplification
• RIA - Radioimmunoassay
• RT - Reverse transcriptase
• RU - response units
• SA - streptavidin
• SAM - Self-assembly monolayer
• SERS - surface-enhanced Raman scattering
• SiNW - sillicon nanowire
• SP - surface plasmon
• SPR - Surface plasmon resonance
• SPRI - surface plasmon resonance imaging
• SWNT - single-walled carbon nanotubes
List of Abbreviations

- TIR - total internal reflection
- UV - ultraviolet
List of Abbreviations
Razširjen povzetek

V magistrski nalogi se osredotočimo na izdelavo in razumevanje delovanja biosenzorja (angl. biosensor) za zaznavanje različnih bolezn, t.i. imunosenzor (angl. immunosensor) na osnovi detekcije biomarkerjev (angl. biomarkers), kot so protitelesa, antigeni in drugi specifični proteini.


1
Zaradi potrebe po zanesljivih, hitrih, cenovno ugodnih, kvantitativnih in kvalitativnih diagnostičnih metodah, se na področju analitične kemije poleg že obstoječih metod hitro razvijajo številne nove. Med najbolj uveljavljenimi in prepoznavnimi metodami sta encimskoimunski test ELISA (angl. enzyme-linked immunosorbent assay) in kemiluminiscenca (angl. chemiluminescence), ki temeljita na označevanju proteinov z konjugiranimi encimi. Masna spektrometrija spada med zahtevnejše in dražje analitične metode, a ponuja celosten vpogled v sestavo vzorca vse do posameznih peptidov. Biosenzorji, katere odlikuje izjemna občutljivost, selektivnost, prenosnost, hitrost in ugodnost, predstavljajo korak proti enostavni, ugodni diagnostični metodi za uporabo na mestu oskrbe (angl. point-of-care diagnosis).

Biosenzor je analitična naprava, ki vsebuje sistem za biokemično zaznavanje, njegova uporaba pa sega na številna področja, kot so analitična kemija, klinično diagnosticiranje, nadzor okolja in zivil ter nenazadnje tudi obramba in varnost. PrviKoncept biosenzorja, kot naprave za merjenje glukoze v bioloških vzorcih, je bil predstavljen leta 1962. Vse od takrat so biosenzorji priča hitremu in nenehnemu razvoju, zaradi napredka na področjih nanotehnologije, biomedicinske tehnike, elektronike in biokemije. V zadnjem času je veliko pozornosti namenjene elektrokemičnim senzorjem na osnovi poljskega tranzistorja (angl. field effect transistor, FET). Tovrstne naprave ponujajo zaznavanje v realnem času, brez potrebnih oznak (angl. label-free) biomarkerjev, z zmožnostjo zmanjševanja velikosti in integracije z obstoječo elektroniko. Glavna prednost biosenzorjev na osnovi poljskega tranzistorja je neločljivo povezana zmožnost ojačitve signala. Uporaba najnovejših nanotehnoloških materialov, kot so ogljikove nanocevke (angl. carbon nanotubes, CNTs), silicijeve nanožice (angl. silicon nanowires, SiNW) ali samostojna plast grafena (angl. graphene sheet), kot osnovni gradnik kanala tranzistorja, dodatno pripomorejo k izboljšanju lastnosti biosenzorjev. V aplikacijah za biokemično zaznavanje se pogosto uporablja posebna izvedenka poljskega tranzistorja, t.i. tranzistor s krmilno elektrodo v elektrolitu (angl. electrolyte-gated field effect transistor, EGFET). Pri tovrstnih tranzistorjih vrata tranzis-
Razširjen povzetek

torja in kanal namesto oksida ločuje elektrolit. Ob priključitvi napetosti na elek-
trodo se na stiku elektrolit-kovina in elektrolit-polprevodnik pojavi t.i. dvoplasticni
kondenzator (angl. *electric double layer capacitor, EDLC*), katerega električno
polje uravnava tok v kanalu tranzistorja.

Da senzor lahko uspešno zaznava prisotnost proteinov, je potrebna ustrezna
funkcionalizacija površine senzorja. Za uspešno pritrditev bioloških receptorjev
na površino elektrode iz zlata se najpogosteje uporablja t.i. plast iz samostojnih
molekul (angl. *self-assembly monolayer, SAM*). Le-ta poskrbi za dobro pasivacijo
površine elektrode in hkrati nudi mesto za uspešno pritrditev protiteles, ki se ne
vežejo neposredno na površino iz zlata. Nanos nove dielektrične plasti na stik
elektrolit-kovina, se odraža v spremembah dvoplastnega kondenzatorja. Zaradi
neprepustnosti plasti na stiku, se protinaboj (angl. *screening charge*) v elektrolitu
nahaja dlje od elektrode, kar povzroči povečanje debelini kondenzatorja. Ob
novi, nižji dielektrični konstanti materiala v kondenzatorju in povečani debelini,
se kapacitivnost dvoplastnega kondenzatorja zmanjša. Spremenljiv dvoplastni
kondenzator na stiku elektrolit-kovina skupaj z dvoplastnim kondenzatorjem na
stiku elektrolit-polprevodnik sestavlja napetostni delilnik. Potencial elektrolita,
ki je definiran z razmerjem obeh kondenzatorjev vpliva na tok tranzistorja. V
primeru imunosenzorja, aktivno površino na elektrodi vrat tranzistorja sestavljajo
protitelesa. Ob stiku s testnim vzorcem, protitelesa ujamejo in vežejo nase ciljne
antigene. Tako se zaradi dodatne plasti na elektrodi in novega dielektričnega ma-
teriala kapacitivnost dvoplastnega kondenzatorja zmanjša. Ker je padec napetosti
na manjšem kondenzatorju večji, se potencial elektrolita ustrezno zmanjša, kar
vodi k manjšemu izstopnemu toku tranzistorja. Na tem mestu nas zanimi ali
omenjen princip delovanja senzorja deluje in v kolikšni meri sprememba debelini
dvoplastnega kondenzatorja vpliva na izstopni tok. Ustrezne meritve pridobimo s
pomočjo merjenja elektrokemične impedance elektrode, padca napetosti na kon-
denzatorju, ki se formira na planarni elektrodi senzorja, ter merjenja tranzis-
torskega toka ob različnih debelinah dielektričnega materiala na planarni elek-
trodi.

Meritve padca napetosti na spremenljivem dvoplastnem kondenzatorju smo opravili z voltmetrom ter osciloskopom. Pri tem smo funkcionalizirano planarno
raziskovalni povzetek

elektrodo imunosenzorja potopili v PBS in preko referenčne elektrode povezali z diskretnim 1\(\mu\)F kondenzatorjem, ki je služil kot stabilna referenca. Opisano serijsko vezavo dveh kondenzatorjev smo vzbujali s kvadratnim signalom v primeru meritev voltmetra, ter kvazi-sinusnim signalom v primeru meritev z osciloskopom. Padec napetosti na elektrodah z različnimi debelinami pasivacijskih plasti je sovpadal z rezultati merenja impedance elektrokemičnega spektra. Največji padec napetosti je bil prisoten na planarni elektrodi, ki je imela najmanjši dv plastni kondenzator t.j. najdebeljšo dielektrično plast. Padec napetosti na planarni elektrodi s tanjšimi plastmi pa se je ustrezno zmanjšal. Meritve z osciloskopom so dodatno poskrbele za vpogled v potek signala, kjer je moč opaziti tudi fazno razliko med vzbujanim in izstopnim signalom. Tudi ta je odvisna od debeline dielektrične plasti na planarni elektrodi.

Da debelina dielektrične plasti igra pomembno vlogo pri odzivu imunosenzorja na osnovi tranzistorja, smo preverili še s tranzistorskimi meritvami. Senzor na osnovi tranzistorja smo pred tem okarakterizirali s tipičnimi tranzistorskimi meritvami izhodne karakteristike (angl. output characteristics) in prehodne karakteristike (angl. transfer characteristics). S pomočjo le-teh lahko določimo linearno območje delovanja (angl. linear region) in območje nasičenja (angl. saturation region) tranzistorja, transkonduktanco (angl. transconductance), pragovno napetost (angl. threshold voltage), t.i. on-off razmerje (angl. on-off ratio) morebitno histerezo (angl. hysteresis) in druge karakteristike. To nam je v pomoč pri izbiri merilnega postopka, izbiri delovne točke tranzistorja in kasneje optimizacije delovanja. Ker smo želeli doseči čim boljši odziv senzorja, smo potisnili točko delovanja senzorja v območje nasičenja, kjer je izstopni tok kvadratno odvisen od krmilnega potenciala. V primeru meritve odziva na različne debeline dielektrične plasti na elektrodi, smo senzor vzbujali z enosmerno napetostjo na priključkih vrat in ponora tranzistorja. Odziv senzorja med elektrodo s plastjo merkaptoopionične kisline in elektrodo s plastjo merkaptoheksanoične kisline je znašal 150 nA. Glede na to, da se debelina omenjenih plasti razlikuje v zgolj nekaj ogljikovih vezeh, lahko sklepamo, da je predstavljen imunosenzor izjemno
občutljiv na dielektrični material nanešen na planarno elektrodo vrat senzorja. Razlika med plastjo merkaptopheksanoične kisline in merkaptopoundekanoične kisline je znašala 440 nA.

Meritve v tej magistrski nalogi prikazujejo princip delovanja in hkrati nakazujejo možnost realizacije predlaganega imunosenzorja za merjenje različnih bolezni, na podlagi prisotnosti različnih biomarkerjev. Z meritvami smo dokazali, da je odziv senzorja tesno povezan z debelino dielektričnega materiala nanešenega na planarno elektrodo senzorja.

**Ključne besede:** tranzistor s krmilno elektrodo v elektrolitu, EGFET, biosenzor, imunosenzor, dvoplastni kondenzator
Abstract

The field of biosensors, utilizing nanotechnology, bioelectronics and electrochemistry is rapidly developing due to a growing demand for affordable and simple diagnostic devices. These devices are applicable in a variety of fields such as environment and food diagnosis, analytical chemistry, clinical diagnosis, safety and defense. Electrolyte-gated field-effect transistor (EGFET) based biosensor exploiting its inherent signal amplification is offering a viable solution towards extremely sensitive, selective, simple, reliable and affordable diagnostic device. In the field of clinical diagnosis, the sensitive monitoring and quantifying of the biomarkers is required for successful early discovery and treatment of disease. Immunosensor is a special type of biosensor, using antibodies as a biorecognition layer on the surface of the device. These devices based on a EGFET structure are designed to detect the binding of antibody-antigen immunocomplex and offer a high sensitivity and selectivity.

By applying potential on the gate electrode of EGFET, an electric double layer at the metal-electrolyte interface is introduced, due to the screening charge in the electrolyte. Thus, a double layer capacitor is formed on metal-electrolyte and semiconductor-electrolyte interfaces, constructing a capacitor voltage divider. By functionalization of the gate electrode and upon binding event of the analyte, the double layer capacitor changes due to introduction of new dielectric material. The change of the capacitor is reflected in the change of the electrolyte potential, which is modulating the output current of the transistor.

This thesis focuses on the sensor development, more specific, on proper functionalization of base layer for further immobilization of biorecognition layer. A
A dense and defect-free insulating layer is important for the proposed sensing mechanism. Electrochemical impedance measurements, voltage divider measurements and transistor measurements are conducted to see the effect of insulating layer thickness on the change of double layer capacitor.

The results of impedance measurements show that the electropolymerization of electrode with polymers like Phenol and Tyramine results in the unstable insulating layer. Due to instability of mentioned polymer layers on the gold surface, the immobilization of alkanethiol SAM layer is utilized. According to the measurements, electrode with SAM based insulating layer has a higher impedance than bare gold, indicating a good and stable layer formation. Stability was examined in 1X PBS and DI-H$_2$O. Different increase of the impedance, using MPA, MHA and MUA SAM layers indicated the change of capacitor due to functionalization of the electrode. The voltage drop across functionalized electrode, as well as signal waveform was investigated. As it turns out, the capacitor with thicker SAM layer has the highest voltage drop, while the capacitor with thinner SAM layer has the lowest. With that, the potential of the electrolyte is changing, upon the introduction of dielectric material on electrode surface. To check the response of transistor output current on the thickness of the insulating layer, transistor measurements were done. Results show, that the 150 nA change in the current is present, when the thickness of the SAM layer changes by only a few carbon chains. These measurements are indicating a very high sensitivity of the proposed device. Further, the response of the sensor due to a new dielectric material immobilized on the electrode surface is detected and therefore the principle proven.

**Keywords:** electrolyte-gated field effect transistor, EGFET, biosensor, immunosensor, electric double layer capacitor
Chapter 1

Introduction

Cancer, as one of the leading causes of death in modern society, is a major public health problem worldwide. It is an enormous global health burden, present in every region and socioeconomic group. According to the data of American Cancer Society, cancer is responsible for about 1 in every 7 deaths worldwide. The estimated new cancer cases and deaths in United States for previous year reached above 1.5 million and 0.5 million, respectively [1]. By 2030, about 21.6 million new cancer cases and 13 million cancer deaths are expected worldwide [2], based just on the growing and ageing of the population. These numbers might further increase, due to the adoption of the western lifestyle, such as smoking, physical inactivity and poor diets, associated with income growth. Even though, the cancer death rate dropped for 25% from 1991 to 2014 [1], the survival rate should be further increased. Many studies have concluded, that the early stage diagnosis of disease and immediate treatment significantly increase the patient survival rate [3, 4, 5]. Therefore, diagnosis of the cancer disease in the early stage is very important. One of the major challenges in early diagnosis is the low level concentration of specific protein, called biomarker, as there might be only a slight difference between healthy individual and cancer patient. Further, apart from concentration, the important indicators of some cancer types are also the ratios between multiple related biomarkers [6]. Hence, highly selective and
sensitive methods are needed, to measure cancer diagnosis markers at ultra-low concentrations. There are many existing diagnostic tools, such as enzyme-linked immunosorbent assay (ELISA), which are not sensitive enough, time consuming and rather expensive. Despite large efforts, simple and affordable tests for the cancer diagnosis are still missing.

The possible solution to overcome these issues are biosensors, offering high specificity and sensitivity, speed, portability and low-cost. The basic concept of the biosensor was introduced by Leyland C. Clark in 1962, as a glucose measurement tool in biological samples. Ever since, the field of biosensors has experienced incredible progress in technology and applications, utilizing innovations in electrochemistry, bioelectronics and nanotechnology [7]. Biosensor is an analytical device incorporating a biological sensing element, used in the field of analytical chemistry, clinical diagnosis, environment diagnosis, food monitoring, as well as defense and security.

In general biosensor consist of a biological/molecular recognition system and a physio-chemical transducer connected in series. The detection techniques in biosensors can be classified into label-free and label-based [8]. While label-based technique exploits specific properties of labels attached to the biomarkers, the label-free detection does not require labeling of biomarkers. Recently more attention has been given to label-free techniques, as they can provide information on molecular interactions, cellular responses, selectivity, affinity as well as binding kinetics [8]. Based on the transducer, label-free biosensors are divided into optical and non-optical. Non-optical transducers are further divided into platforms exploiting acoustic waves, electrochemistry and micro-calorimetry. Among them, electrochemical biosensor has drawn much attention over the last few years. Electrochemical biosensors are molecule sensing devices that turn biological recognition event into a useful electrical signal [9]. These sensors are typically prepared by modifying the surface of the electrodes using biomaterials such as enzymes, DNA or antibodies. Together with the development of nanotechnology, these electrical devices play an important role towards point-of-care diagnosis.
Among variety of electrochemical sensors, field effect transistor (FET) based biosensors are very suitable candidates for biosensing application, due to their ability of real-time label-free sensing, use of small sample volumes, and potential miniaturization and integration with electronics [8]. Main advantages of the FET biosensor are direct translation of analytes interaction on the surface into a readable signal and inherent signal amplification. The introduction of nanomaterials like carbon nanotubes (CNTs), silicon nanowires (SiNW) or graphene sheets as transducers further improved the properties of FET biosensors [10]. A concept of ion-sensitive FET (ISFET), appropriate for biosensing applications have been developed in the early 1970s [11]. Further modifications of FET architecture has led to electrolyte gated FET (EGFET), where gate electrode is separated from channel with electrolyte. Here the information transfer takes place by modification of electric field across double layer capacitor formed on the electrolyte-semiconductor interface. Biochemical interaction on the functionalized surface of nanomaterial based semiconductor channel or gate electrode induces a readable signal response. Biosensor with biomarkers, like antibodies, as a biorecognition layer on the surface of FET architecture is known as immunosensor (ImmunoFET) [8, 10]. These analytical platforms are designed to detect the binding of antibody-antigen immunocomplex on the transducer surface, and have been developed as an alternative to conventional immunoassays. Hence, immunosensors play an important role in monitoring and quantifying the concentration of biomarkers in a body. Detecting the presence and concentration of antibodies, antigens or microorganisms in the body fluids is serving as an indicator for a certain disease such as cancer, bacterial or viral infections. Immunosensors with nanomaterial based transducers exhibit excellent selectivity and sensitivity, real-time response, cost effective fabrication and are therefore excellent interfaces for point-of-care devices.
The biosensing technology is a broad and multidisciplinary field, rapidly evolving since the introduction of first analytical techniques. As the need for rapid, quantitative, qualitative and reliable diagnostic technique is growing, new methods are being developed in addition to well established ones. Analytical tools are widely applied in clinical chemistry, bioanalysis, pharmaceutical, toxicological, environmental and food analysis. Enzyme-linked immunosorbent assay (ELISA) and Chemiluminescence are widely used assays employing labels like enzymes to produce a measurable signal. More complex analytical tool is mass spectrometry, measuring the mass-to-charge ($m/z$) ratio of peptides, providing a comprehensive insight of analyte composition. Even though mentioned techniques are well established and widely used, biosensors like Quartz crystal microbalance (QCM) and Surface plasmon resonance (SPR) as a label-free, cost efficient and rapid technique are emerging. Especially electrochemical biosensors based on field effect transistor (FET) architecture employing nanomaterials gained much interest as promising sensitive and quantitative point-of-care devices. A brief review of mentioned diagnostic tools, its capabilities and drawbacks together with recent developments is given in this chapter.
2.1 Enzyme-linked Immunosorbent Assay

Radioimmunoassay (RIA) and Immunofluorescence were immunodiagnostic tests performed in the 1960s and 70s. Due to development and perfection of methods to label antigens with isotope or fluorescent labels, these tests resulted in good sensitivity and selectivity. However, because of expensive equipment, strict regulatory controls, short life of reagents of RIA method and Immunofluorescence with tedious and time consuming procedure which could not be easily automated, a search for alternative methods and labels was needed [12]. Enzyme labels linked to antibodies or antigens enabled immunological and enzymatic activity of complexes. A simple and rapid but one of the most sensitive immunoassay technique to detect and quantify protein biomarkers immobilized on a solid surface is an Enzyme-linked immunosorbent assay, also known as ELISA [13]. It was published by Engvall and Perlmann in 1971 [14], as a quantitative assay for Immunoglobulin G from rabbit, using more stable enzyme-antigen conjugates. This assay is based on antibody antigen complex, and can identify and quantify target analyte by producing a visible signal usually in colour change due to enzyme labels. Apart from medical laboratories, ELISA test may be used also by manufacturers of in-vitro diagnostic products, regulatory bodies, external quality assessments and proficiency-testing organizations [15].

This high throughput assay relies on washing of the unbound biomarkers in between the steps allowing the detection of bound markers only. Even though many variations like in-cell or multiplex microarray ELISA tests have been developed, the traditional procedure can be performed in four different formats named direct, indirect, competitive and sandwich ELISA. Basic difference of these four formats in terms of capture biomarkers and enzyme-linked biomarkers is presented in Figure 2.1

In indirect ELISA method, the antigen of interest is immobilized to the solid phase passively. Upon the addition of the test sample, primary antibodies specific for the antigen are forming a complex. After a washing procedure to remove
2.1. Enzyme-linked Immunosorbent Assay

Figure 2.1: The core difference of capturing and sensing mechanism using enzyme-linked biomarkers in direct, indirect, sandwich and competitive ELISA.

any unbound antibody, an antiglobulin enzyme conjugate or secondary enzyme conjugated antibody is subsequently added to bind to the primary antibody. To get a signal, an enzyme substrate or chemiluminescent substrate is added, resulting in a measurable signal proportional to the amount of antigen present in the well [13, 12].

The procedure where primary antibody, specific to an antigen immobilized to the plate, is enzyme linked, is called direct ELISA method. The advantage of direct method is shorter time of procedure, since there is no secondary antibody involved to bind to a primary antibody [13].
A very popular format is sandwich ELISA. In this method, instead of antigens, primary capture antibodies specific to a target antigens are immobilized to the solid phase. Unlike other methods, where the amount of antibody present in the sample is measured, this method measures the amount of antigen in the sample. Antigens in the test sample form complex with capture antibodies, while any unbound antigen is washed during the washing procedure. Subsequently an enzyme linked antibody specific to the antigen is added, trapping the antigen between detection and capture antibody. Upon the addition of enzyme substrate a measurable signal is produced. Even though sandwich ELISA does not need the purification of the sample before the analysis, and has a higher sensitivity than indirect or direct method [13], finding an antibody pair with matching properties like binding affinities, stability, etc. is often a difficult task. However these properties are directly related with the selectivity and specificity of a sandwich ELISA [13, 12].

Fourth format is competitive ELISA, where enzyme linked antigen is mixed with antigen in the test sample. They are competing for the antibody immobilized to the solid phase. In parallel, only enzyme linked antigens are added to the well, forming a complex with immobilized antibody. Upon the addition of enzyme substrate, the difference in the signal between two wells corresponds to the amount of unknown antigen from the test sample [12].

In 2016 Tao Liao et al. suggested an ultrasensitive ELISA method based magnetic beads and enzyme labeled gold nanoparticles to detect procalcitonin (PCT) [16]. PCT is a specific marker for severe sepsis caused by bacterial infection [16]. In their work authors suggested an improved ELISA method utilizing magnetic separation for catalization of enzymatic substrate, sensitive enough to detect PCT as low as 20 pg/mL. Sandwich ELISA was modified, using carboxyl modified magnetic beads on which capture antibody was chemically conjugated through classic EDC/NHS protocol. detection antibody specific to PCT was immobilized on gold nanopraticle (AuNP) via electronic and hydrophobic interaction. Additionaly, horseradish peroxide (HRP) was immobilized to the surface
2.1. Enzyme-linked Immunosorbent Assay

of AuNP to catalyze the substrate oxidation, forming a colorimetric signal. Upon
the addition of PCT and modified AuNP to the magnetic beads with capture
antibody, a complex of antibody/antigen/antibody is formed and isolated with
magnetic separation. Since surface of AuNP can hold multiple antibodies and
multiple HRPs for catalyzation, a higher sensitivity and higher colorimetric sig-
nal can be reached. As the level of PCT in human body is 0.5 ng/mL, reported
detection limit of 20 pg/mL, which is 5-fold lower than that of conventional ELISA
confirms the improved sensitivity of suggested ultrasensitive ELISA method with
magnetic beads and gold nanoparticles.

Another ultrasensitive method was reported by Jiajie Liang et al. [17]. This
method is using Raman signal as the signal generating system, combining surface-
enhanced Raman scattering (SERS) ELISA with aggregated silver nanoparticles
(AgNPs) for ultra sensitive analyte detection. Enzyme labeled ELISA controls
the dissolution of Raman reporter-labeled AgNPs through hydrogen peroxide, as
an effective oxidizing agent, generating a strong Raman signal when analyte is
present. Using this assay, prostate-specific antigen (PSA) and adrenal stimulant
ractopamine (Rac) were detected in whole serum and urine at the concentrations
of $10^{-9}$ ng/mL for PSA and $10^{-6}$ ng/mL for Rac.

R. de la Rica and M. M. Stevens reported a plasmonic sandwich ELISA detect-
ing disease biomarkers with naked eye using colouring nanoparticle solution [18].
In this work, the enzyme is linked to the complex through interactions of en-
zyme decorated streptavidin and biotinylated secondary antibodies, attaching to
the detection antibody of sandwich ELISA. The biocatalytic cycle of the enzyme
generates coloured nanoparticle solution. In the presence of hydrogen peroxide in
large concentrations favours the formation of non-aggregated spherical nanoparti-
cles, and the solution is expected to acquire red colour. If the concentration of the
hydrogen peroxide decreases due to the presence of biocatalyic action of enzyme
catalase slowing down the kinetics of crystal growth, aggregated nanoparticles are
formed, producing a blue colour of solution. Since the difference between red and
blue is clear, the detection with the naked eye is possible. With this modified
method of ELISA, PSA and HIV-1 capsid antigen p24 were detected in whole serum at the ultra low concentration of 1 ag/mL.

Recently Yudong Wu et al. reported an ultrasensitive method of enhanced Fluorescence ELISA based on human alpha thrombin (HAT) triggering fluorescence with enzyme-antibody labeled gold nanoparticles (AuNP) [19]. It is based on a classic sandwich ELISA method, using capture and detection antibody to form a sandwich complex. Nanoparticle probes are labeled with detection antibodies and HAT enzyme. A bisamide derivative of Rhodamine with quenched fluorescence is serving as a substrate for HAT. Upon enzymatic hydrolisis fluorescence quenched bisamide Rhodamine substrates are cut into weak fluorescence monoamide Rhodamine (background signal), and then cut further on to a strong fluorescent Rhodamine, greatly contributing to the lower background signal. The concentration of alpha fetoprotein (AFP), commonly used biomarker specific for people suffering from primary hepatic carcinoma [20], is crucial to monitor to improve the cure rate, especially in the early stage of the disease. With this method an excellent detection of AFP with used dual labelled probe was reached, yeilding a distinguishable signal for concentration of 10 ag/mL. This excellent detection performance was explained by high loading efficiency of HAT and detection antibodies on AuNP probes enhancing concentration, enzyme based signal amplification, and low background signals. Further, the detection of HBsAg biomarker for Hepatitis B disease was checked, where detection limit reached $5 \times 10^{-4}$ UI/mL.

### 2.2 Chemiluminescence

Another very much exploited method of Immunoassay derived from radioimmunoassay is chemiluminescence or Chemiluminescence immunoassay (CLIA). As a versatile, ultrasensitive analytical tool, it is widely applied in bioanalysis, pharmaceutical, clinical and environmental analysis, due to its wide dynamic range and high sensitivity [21]. Luminescence is a term to describe the emission of a visible or near-visible radiation, generated when an electron transits from excited
2.2. Chemiluminescence

state back to ground state [22, 23]. Different types of luminescence differ from the source of energy to obtain an excited state. In the case of chemiluminescence, the source of energy is the chemical reaction [23]. This can create enough energy to push the electron to the excited state. The return to the ground state can be accompanied with the emission of a photon, detectable as a light pulse signal [21]. However, excited electron can also lose energy due to chemical reaction, deactivation upon collision and others [23], which are undesirable in chemiluminescence. The intensity of emission depends on the rate of chemical reaction and overall efficiency of chemiluminescence reaction [24]. Thus, the intensity is directly proportional to the concentration of analyte or biomarker [25]. CLIA is very similar to the ELISA procedure. Its methods can be indirect, using enzyme markers, or direct using luminophore markers [22]. Further, both methods can be competitive or non-competitive. Most commonly used solid-phase are 96-well plates [21], precoated with capturing protein, for the purposes of immunoassay. Recently, magnetic microspheres replaced solid-phase microplates, providing larger surface area for utilization of higher concentrations and increasing sensitivity [21].

**Figure 2.2:** Chemiluminescence analysis for antigen (left), or antibody (right) detection in the test sample. This is a labeled method, using enzyme-linked antibodies, to chemically react with luminescent substrate and produce a light signal.
Activation of substrates requires chemical or enzymatical reactions, associated with immunological reaction [22]. In general, labels are separated into two groups. Luminol, isoluminol and its derivatives are the most commonly used as substrates that are consumed in the reaction process. While luminol is more efficient in free state and is therefore mostly used in enzyme labeled immunoassay, Isoluminol derivatives show excellent efficiency in substrate labeled immunoassay [23]. Lately, nanoparticles gained on popularity, because of their unique properties and good biocompatibility [21]. Other group consist of labels that catalyze the production of light, such as enzymes, alkaline phosphatase (ALP) or horseradish peroxidase (HRP). These labels require suitable substrates. Nevertheless, constant search and development of new chemiluminescence labels is present, needed for a very low limit of detection.

Key advantage of Chemiluminescence is a wide dynamic range, which implies a higher analytical sensitivity and the ability to accurately detect concentrations of biomarkers in non-diluted samples [22]. CL additionally offers also high signal intensity, specificity, stability, absence of toxicity and low consumption of reagents [23, 22]. Due to mentioned properties, CLIA is able to detect the presence of biomarkers at extremely low concentrations of zeptomole ($10^{-21}$ mol) [22]. Limited test panels, closed analytical system and high cost of assay are limitations of CLIA.

Microassay technology is an emerging field in analytical chemistry. To reduce analysis time, cost of reagents and use of laboratory space, Chemiluminescence microassay was developed as a powerful tool, for a rapid multiplex analysis of complex samples [25]. It consist of reactive spots with diameter in a micro range functionalized with biochemical selective receptors like DNA, aptamers or antibodies, placed on a supporting material. Analytical microassay is enabling multianalysis, due to possibility of generating several sets of quantitative data for different classes of analyte in a short time [25]. Photons from different spots are detected with an appropriate optoelectric imaging sensors, usually CCD cameras. Labels on each spot produces a signal that quantitatively correlates with
the amount of labeled analyte. After the calibration with multiplex standards, analyte concentrations can be quantified. Sensitivity of chemiluminescence microassay depends on reaction time, sensor chip in camera used, surface chemistry and readout system, to prevent photons from outside entering the CCD chip [25].

J. Chen et al. proposed a chemiluminescence detection of a p53 DNA, based on hairpin assembly triggered cyclic activation of a DNA machine [26]. Here, two hairpin DNA structures are used (H1, H2), responsible for binding with target DNA, hybridization, formation of H1-H2 complexes and displacement of targeted DNA. This displaced p53 DNA can then start a new hybridization cycle leading to formation of multiple H1-H2 complexes. Formation of HRP-mimicking DNAzymes can catalyze the oxidation of luminol, by hydrogen peroxide (H$_2$O$_2$). Cyclic process is the key to an amplified CL signal, resulting in a ultrasensitive method, with a limit of detection estimated to 0.85 fM. Reported amplification is label free, does not need washing or separation steps, making it very simple and cost-effective [26].

Chemiluminescence assay on microchip, based on DNAzymes functionalized gold nanoparticles for ultrasensitive detection of thrombin, was reported by J. Wang et al. [27]. DNA aptamers were immobilized on the surface of a microchip, while gold nanoparticles were conjugated with aptamers and rolling circle amplification (RCA) template. After the recognition and capture of thrombin by aptamers on a microchip and nanoparticles, second amplification with RCA reaction process was performed. Finally, chemiluminescence was achieved by catalytic effect of DNAzymes in a luminol-hydrogen peroxide (H$_2$O$_2$) system. Different concentrations of thrombin dissolved in a PBS solution were tested with this method. CL assay showed a good linear response in the range from 1 pM to 25 pM. The result is a sensitive and quantitative analysis capable of detecting the concentration of thrombin as low as 0.55 pM. Method showed good results in circumstance of real sample, however a 10 fold dilution of serum was necessary.

Another dual amplification scheme based on gold nanoparticles was employed by X. Hun, B. Liu and Y. Menga [28], for the lung cancer biomarker cytoker-
atin 21-1. Here catalytic circuit in which the addition of one single stranded DNA leads to the release of more than one output strand was presented. Whole network is enabled by trigger DNA resulting in a release of two complementary DNA-labeled gold nanoparticles. Further, nanoparticles are dissolved to gold ions, which catalyze the luminol-hydrogen peroxide system. by taking advantage of dual amplification introduced, this gene assay has a 6 fM detection limit [28].

In future, further improvements are expected with the developments of new platforms, such as flow-injection CLIA, ensuring a more efficient regent mixtures, and reduction of incubation time, 2D resolution for multiplex immunoassay and the magnetic nanoparticles assisted CLIA, which will result in additional increase of efficiency.

2.3 Quartz Crystal Microbalance

Apart from ELISA an chemiluminescence, as a highly selective and sensitive tools for biomarker detection, other methods, utilizing different analyte properties are used. The type of the transducer highly depends on the nature and physiochemical properties of sensitive material used. Since not all surfaces necessarily posses optical, electrochemical or magnetic properties, a labeling is usually needed for appropriate detection. However, this limitation or requirement can be overcome with sensing a mass, as a fundamental property. Quartz crystal microbalance (QCM) is based on a gravimetric or mass sensitive transducer [29]. QCM biosensors are highly sensitive to the changes of mass loading, where very little change in mass due to the analyte detection (binding) can be easily detected. AT-cut quartz crystal wafer between two electrodes on the opposite side is connected to the oscillating circuit, and covered with a thin receptor material, usually gold. Transverse wave generated by oscillating circuit propagates perpendicular to the quartz surface driving QCM to resonate at characteristic frequency [29, 30]. In the event of analyte-receptor interaction, a signal is produced in a form of a frequency change. QCM combined with appropriate detection material or receptor
layer is highly sensitive small device, capable of detecting tiny alterations in the surface mass, usually in the range of ng/cm² [29]. However, this type of label-free sensors need a reference system, to distinguish between specific and non-specific bindings on the sensor surface [31]. The principle of a QCM based gravimetric biosensor is presented in the Figure 2.3.

![Figure 2.3: Basic principle of QCM based gravimetric biosensor. Upon the detection of analyte, the mass on the transducer increases, and the frequency drops. Source: [29]](image)

The foundation for chemical and bio sensing using QCM was laid with the work of Sauerbrey [32], calculating the frequency changes as a function of mass deposited or adsorbed on the surface of a gravimetric device. The relation between resonant frequency shift $\Delta f$, and mass change $\Delta m$ is given by Sauerbrey equation [32]:

$$\Delta f = -f_o^2 \frac{\Delta m}{A \sqrt{\rho_q \mu_q}};$$  \hspace{1cm} (2.1)

where $\Delta f_o$ is the fundamental frequency, $\Delta m$ the change of mass, $A$ active area of the sensor surface, while $\rho_q$ is the density and $\mu_q$ the shear modulus of the quartz crystal. This equation is slightly modified if the measurements are done in the liquid medium. It is important to take into the account also acoustic wave properties of the liquid medium, when the surface of QCM sensor is in
contact with liquid. Modified Sauerbrey equation describing liquid loading effect on frequency change is

$$\Delta f = -f_0^{3/2} \sqrt{\frac{\rho_l \mu_l}{\pi \rho_q \mu_q}},$$

(2.2)

where $\rho_l$ is the density of the liquid and $\mu_l$ viscosity of the liquid.

Furthermore, both equations show, that the response of the sensor can be improved with higher fundamental frequency $f_0$. Unfortunately, to achieve higher fundamental frequency, quartz crystal wafer have to become thinner, which will result in a fragile device. Therefore, fundamental frequency is actually a fabrication and device performance limiting parameter. Typically, frequency range of QCM sensors in liquid medium is between 5 MHz and 20 MHz [29], however some reports showed frequency of operation as high as 110 MHz [33]. Another quite important parameter determining the sensing performance is device area. Bigger electrode area means bigger receptive layer, which results in higher sensitivity of the device. Usually there is only one active electrode area per device, but there are innovative ideas of multichannel QCM, detecting multiple different analytes at the same time.

Synthetic antibodies based on molecular imprinted polymers, natural antibodies, DNA and aptamers are potential receptor layers for sensitive detection of different diseases. These layers can be printed on the electrode using soft lithography procedure. This is a surface imprinting technique, for producing a biomimetic surfaces, allowing the transfer of precise structural details of analytes onto synthetic polymer surface. Molecular imprinted polymers (MIPs) are artificially designed receptors, also known as artificial antibodies, which can mimic the molecular recognition of a natural antibodies. This low cost and ease of fabrication method offers better stability and selectivity [29] and comparable sensitivity method for a receptor layer, with the detection limit below 1 ng/mL [34]. C. Lu, Y. Zhang, S. Tang en al. reported a bimimetic sensor for the detection of human immunodeficiency virus type 1 (HIV-1) related protein glycoprotein 41 (gp41), based on epitope imprinting technique [35]. Gp 41 is an important indicator for determining the extent of HIV-1 disease progression. Dopamine is a
2.3. Quartz Crystal Microbalance

great material for MIP due to its high hydrophilicity and biocompatibility [35], and was polymerized as a functional monomer and crosslinking agent on QCM surface. Alongside, synthetic peptide of gp41 fragment was used as a template to functionalize QCM sensor. Both gp41 and dopamine were combined during polymerization, leaving imprinted recognition sites complimentary in shape, size and distribution of functional groups upon the removal of the template. Reported detection limit for gp41 is 2 ng/mL. Good results show some advantages of epitope imprinting technique such as high selectivity and sensitivity for the template molecule and its mother protein. Additionally, sensor was tested in human urine sample as well, to prove the feasibility of this approach also in real samples.

Natural antibody based QCM biosensors are suitable for rapid diagnosis with high selectivity and high specificity. As a product of immune system to protect the body, natural antibodies are special proteins responsible for detection and neutralization of pathogens [36]. Like synthetic antibodies they function as a selective receptors offering site specific affinity with viral shell proteins or with proteins released in the body by the viruses [29]. D. Li, J. Wang, R. Wang et al. reported a rapid detection of pathogenic avian influenza (AI) H5N1 virus, using a nanobeads amplified QCM immunosensor [37]. Polyclonal antibodies against AI H5N1 were immobilized on gold surface of QCM coated with SAM layer. Additionally, antibodies were immobilized onto nanobeads, used for further amplification of the binding reaction as a mass enhancer. After the immobilization of gold surface with capture antibody, a target analyte is added, and the mass on the surface increase due to complex formation. To enhance the change in the mass further more, nanobeads functionalized with antibodies are added, to bind with target analyte, forming sandwich-like structure. According to reported results, using nanobeads amplifies the response signal much better at lower concentrations than higher, making this method especially useful for a very sensitive detection. Reported limit of detection for this method is 0.128 hemagglutinin units (HAU), where the linear quantitative relationship for enumerating target viruses was found [37].
Recently, Y. Yang, Y. Tu, X. Wang et al. reported a label-free immunosensor with the variation in resonant frequency of a QCM for the ultrasensitive, portable or on-spot detection of Ketamine [38]. Such detection is important for clinical and forensic field, since Ketamine is used as a recreational drug with hallucinogenic and dissociative effect [39]. Immobilization of KT antibodies on the QCM gold covered surface was done using well known protocol of self assembly monolayer (3-mercaptopropionic acid, MPA) and activation with EDC/NHS. Immobilized capture antibodies are responsible for capture of Ketamine, resulting in increased mass of QCM and shift in resonance frequency. Sensor was tested in urine samples, which were diluted 1500-fold. Detection limit of 0.86 pg/mL was reported, with good stability and great anti-interference ability.

2.4 Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a label-free detection method, emerged as a suitable and reliable platform in clinical analysis for biomolecular interactions [40]. It is widely used in biomedical, proteomics, genomics and bioengineering fields due to the possibility to measure interactions in real-time with high sensitivity, high-throughput and quantitative monitoring [40, 41]. Since 1990s when it was first introduced, SPR has been proven to be one of the most powerful technologies to determine affinity, specificity and kinetic parameters during the different binding of many biomolecules such as protein-protein, DNA-protein, receptor-drug, virus-protein and others [40]. Even nowadays SPR biosensors are an established method for measuring molecular interactions such as antibody-antigen, ligand-receptor or protein-nucleic acid [42]. Therefore SPR is an acceptable method for disease diagnosis, drug discovery, food-borne pathogen detection and others.

Surface plasmon (SP) was observed in 1902 by Wood [43], with the photon-excited electrical resonance oscillation in small metallic particles, and gained a wide research interest ever since. Explanation of SPR was achieved in 1968 by
Otto [44], as the portion of the incident photon energy couples through metal at the certain incident angle, resulting in the movement of the conducting electrons in the metal surface layer due to excitation. This electron movements are called plasmon, and propagates parallel to the metal surface, generating an evanescent field. This phenomenon occurs when incident light under conditions of total internal reflection (TIR) hits the interface between medium with higher refractive index (glass) and medium with lower refractive index (Au) [45]. Even though fully reflected light beam does not lose any energy at the interface, it leaks an electric field (evanescent field) into the low refractive index medium. The amplitude of this evanescent field decreases exponentially with the distance from the interface [42]. SPR angle at which resonance occurs, under conditions of constant light source wavelength and thin metal surface, is dependent on the refractive index of the material near the metal surface. Upon the small change of the refractive index of the sensing medium, due to biomolecule attachment, SPR angle has to be adjusted or plasmon can not be formed [40].

In 1971 Kretschmann [46] proposed Kretschmann configuration of attenuated total reflection (ATR) coupling, which have become the commercial configuration of SPR biosensor. In this configuration, the incident light is employed using a glass prism with high refractive index. It is placed on the opposite side of the thin metal layer as presented in the Figure 2.4.

Most of commercial SPR biosensors use an optical method, to measure the variations of the refraction index in close vicinity of the sensor surface (200 - 300 nm) [45, 47]. Detection is accomplished by measuring the changes in the reflected light obtained on a detector. Furthermore, by monitoring reflected light intensity, the amount of surface concentration can be quantified [40]. As mentioned, the angle is dependent on the refractive index of adsorbed layer. In case of protein detection, the difference between refractive index of the buffer layer and the adsorbed layer can be easily converted to thickness and mass of the adsorbed layer [42]. To capture biomarkers, capture molecules are immobilized on the surface of the SPR sensor. When the solution containing target biomarkers
is applied, binding via affinity interaction occurs, consequently inducing the increase of refractive index. Here resonance, or response units (RU) are used, to describe the change in the signal, where 1 RU is equivalent to critical angle shift of $10^{-4}$ degrees. Detection limit of SPR biosensors depends on multiple factors such as molecular weight, optical property, binding affinity and surface coverage of the capture molecules. Nevertheless typical detection limit is on the order of 10 pg/mL [40].

One drawback of a standard SPR biosensor using flow cells as presented in Figure 2.4, is that it is not offering a high throughput screening (HTS). A modified version of SPR, called SPR imaging (SPRI) was developed to overcome this obstacle. Using rapid optical arrays, this method can monitor hundreds of samples with biomolecular interaction simultaneously and have potential applications in the high-throughput screening of drugs and biomarkers [40, 47]. Not eliminating the advantage of label free detection, SPRI’s value lies in its capability to analyze whole biochip via a charge-coupled device (CCD) camera as presented in Figure 2.5. Thus, biochips are prepared in an array format, where individual arrays

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**Figure 2.4:** Basic measurement principle of SPR biosensor. Capture molecule is immobilized on the sensor surface, while the target biomarker flows across it. Light source, prism and detector are placed on the opposite side of thin metal layer, producing and capturing a signal response. Source: [45]
offer SPR information simultaneously. In contrast to scanning angle and scanning wavelength SPR methods, SPRI is performed at a constant light wavelength and a constant angle [47]. Therefore any change in the reflected light intensity captured by CCD camera is proportional to the variation of the refractive index near the thin metal surface.

Figure 2.5: General measurement setup and principle of detection using SPRI method. [47]

Prostate specific antigen detection using microcontact imprinting based SPR sensor was proposed by G. Ertürk, H. Özen, M. Tümer et al. [48]. Due to advantages of microcontact imprinting and SPR methods, developed sensor exhibits high sensitivity and selectivity, satisfactory re-usability and stability and good accuracy in detection of PSA in clinical samples. PSA-MIP SPR chip was produced by polymerization of glass stamp with immobilized proteins placed on the monomer on the surface of the sensor. Chip was tested with the PSA concentrations in the range from 0.1 ng/mL to 10 ng/mL in 1/4 diluted human serum samples. Difference in the reflectivity of the light was measured as a signal. The limit of detection was determined to be approximately 90 pg/mL ($18 \times 10^{-14}$ M), which is comparable to other existing methods for PSA detection.
B. Guo, B. Wen, W. Cheng et al. most recently presented an enzyme- and label-free SPR biosensor detecting fusion gene based on DNA self assembly hydrogel with streptavidin encapsulation [49]. With a very interesting design principle of the sensor, using two types of X shaped polymers with DNA strands to form the aptamer-based network hydrogel nanostructure, they achieved a large enhancement of the SPR signal. Developed strategy utilizes ultra sensitive detection of promyelocytic leukemia, retinoic acid receptor alpha (PML/RARα) with limit of detection of 45 fM.

### 2.5 Mass Spectrometry

Mass spectrometry is a central analytical technique in the field of proteomics, to analyze the protein composition and their quantity in a complex biological mixture [50]. It is a method providing quantitative and qualitative information based on mass-to-charge (m/z) ratio of the ions in the sample [51]. At first, mass spectrometry was limited to small molecules, until 1981 when ionization of larger molecules such as proteins was introduced with fast atom bombardment ionization method [52]. The ability to ionize larger molecules was further improved by electrospray ionization (ESI) [53], which was easily connected to in-line liquid chromatography (LC) allowing the possibility to analyze complex mixtures. Together with ESI, a matrix assisted laser desorption/ionization technique (MALDI) for ionization and possible vaporization of larger molecules, presented a big advancement in mass spectrometry [50].

Each mass spectrometer consists of at least three major components: ion source, mass analyzer and ion detector, as presented in Figure 2.6. Ion source is responsible for ionization or vaporization of the sample. Some of the most common used ion sources are previously mentioned ESI and MALDI, as well as electron impact ionization and chemical or thermal ionization [51]. After the ionization of the sample, ion beam is focused into a mass analyzer. In this stage, ions are separated by their m/z ratio. Different types of mass analyzers such as
2.5. Mass Spectrometry

Figure 2.6: Schematic representation of mass spectrometer. Three basic components are Ion Source, Mass Analyzer and Ion Detector. Additionally vacuum system is used to control an maintain the pressure within the mass spectrometer, while computer system is used to collect and analyze the data. [51]

time-of-flight, magnetic sectors, quadrupole and ion trap, are characterized with upper mass limit, transmission and resolution [51]. Electron multipliers or microchannel plates are most commonly used ion detection systems. Here, the strike of the ion against the plate triggers a cascade of electron emission, resulting in a measurable current [51]. Apart from those three major components, computer system is needed to record and process the data, while vacuum pump is used to control the pressure within the mass spectrometer. Very low pressure ($10^{-6}$ to $10^{-8}$ Torr) [51], far below atmospheric pressure, maintained in the system, is necessary to prevent unwanted ion collisions. Those could alter the path of ions, and produce reaction products or loss of the charge [54]. Different combinations of mass analyzers and ion sources have unique properties in terms of resolution and accuracy, and are selected according to desired analysis requirements [50, 51]. Therefore the comparison of instrument performance can be a controversial topic, as properties very much depend on the experimental setup, type of application and nevertheless sample analyzed [50]. Even though a very low limit of detection is often reported for individual peptides, this limits can be off for several orders of magnitude for a very complex biological samples. Thus, a sample preparation strategy is of great importance in complex samples such as serum or plasma. Most commonly used is the depletion of highly abundant proteins in order to enhance
the detection sensitivity and analytical dynamic range of targeted analysis [55]. This means that in general, accuracy and reproducibility of the measurements are reduced for the low abundant proteins.

Mass spectrometry can give information on the identity of the protein, the amount of it present in the sample, and possible modifications the protein contains [50]. This is the biggest advantage of mass spectrometry over other protein analysis methods. Traditionally, ELISA is the most common method used for quantification of the proteins in the sample, providing a good sensitivity and high throughput[55]. Validating a biomarker candidate can be very straightforward in the cases where ELISA assay or high quality antibody already exist. However, ELISA method is limited in most novel protein discoveries, where there is a lack of high specificity antibodies[55]. Another advantage of the mass spectrometry is the possibility to analyze hundreds of proteins from a single injection where the analysis can be non-targeted, enabling to compare the abundance of a yet unknown protein. However, the analysis with a mass spectrometer is time consuming and costly, especially when the cost for a mass spectrometer is considered.

In general, every proteomics workflow has three distinctive stages. First stage consists of isolation of protein samples from biological source, which can be optionally fractionated. Further, proteins are digested by enzymes at specific amino-acids sites into smaller peptides. In the second stage the content of the sample is measured with mass spectrometer resulting in a large data set with recorded $m/z$ ratios and their intensities. In the last stage, large data sets are analyzed by appropriate software tools, to deduce the amino acid sequence from the measured $m/z$ and to quantify the proteins in the sample based on the intensities measured [50].

The identification of proteins is software assisted by entering the list of peptide masses from the resulting spectra, along with the information of which enzyme was used for digestion and possible protein modifications. Software then uses amino acid sequence information stored in the database for the analyzed
organism, to predict all possible peptide masses that could occur in the sample. Comparison between peptide masses that were measured with tandem mass spectrometry process and those that were predicted, gives the list of possible peptides and therefore proteins identified in the sample together with the probability that the identification is correct. For the clinical diagnosis especially, knowing not only which proteins are being expressed by the organism, but also their abundancy, is crucial. Commonly, mass spectrometry is used to measure the relative difference in protein abundance between two different samples. This can be done directly, without using any chemical labeling, by calculating the ratio between the intensities measured for specific peptide in two samples. More accurate quantification of individual proteins in a complex samples can be done by labeling the peptides with chemical labels. One of the most used quantification techniques is Isotope-coded affinity tags (ICAT), developed in 1999 [51]. Labeling not only assures more accurate relative quantification but also enables absolute quantification. In ICAT approach, protein in one sample is tagged with heavy isotope, while protein from other is tagged with light isotope [55, 51]. Both samples are then combined and enzymatically digested. Resulting peptides are then separated by liquid chromatography (LC) and analyzed by mass spectrometry [56]. The result is shown in the ratio of the intensities between heavy- and light- labeled peptides, which are separated on the \( m/z \) scale exactly by known mass difference of used isotopes [55, 51]. When instead of labeled sample labeled standard peptides of known concentration are used the ratio between the intensities of peptides can directly inform us of the absolute concentration of the protein of interest.

Recently, P.Geyer, N.Kulak, G.Pichler et al. focused on developing a robust, rapid and highly reproducible plasma proteomics workflow for potential use in a clinical context [57]. In order to achieve a rapid workflow, optimal for high throughput, all depletion steps of highly abundant plasma proteins were omitted. 1\( \mu \)L of plasma was harvested from a single fingerprick blood sample of 5 \( \mu \)L. Using ordinary amount of digestion enzyme resulted in an adequate protein digestion after 1 h. To determine maximum information content per unit time, single-run
gradient time for peptide separation was reduced from 100 min to 20 min, as only 12 additional proteins are identified with longer gradient time [57]. Combination of liquid chromatography with lowered gradient time and optimized sample preparation, allowed for high throughput. The boost of protein identification by 39% was reached with the use of matching library, consisting of undepleted plasma of 10 individuals and plasma depleted of 20 highest abundant proteins. A total of 347 protein groups were identified, of which 285 were detected in all 10 individuals. The duration of the entire workflow was taken down to less than 3 h.

N. Forsgard, M. Salehpour and G. Possnert reported a method for detection and quantification of ultra low concentrations of $^{14}$C-labeled biologically active compounds in biological fluids by accelerator mass spectrometry (AMS) [58]. The problem of limited sensitivity range below picomolar range due to natural level of $^{14}$C in biological samples taken from living organisms was circumvented with separating of labeled markers from biological matrix. Labeled drug was separated from the sample and collected in fractions using liquid chromatography separation method. $^{14}$C-deficient compound was added to the fractions and analyzed by AMS. The reported detection limit of 0.45 fM shows the remarkable sensitivity of used method, able to detect biomarkers in human plasma in the attomolar range.

### 2.6 FET-based electrochemical sensors

Due to a strong demand for fast, inexpensive, reliable and sensitive point-of-care diagnosis test, Field Effect Transistor (FET) based biosensors are believed to be the technology of choice. The miniaturization and integration into a portable device is already well developed, while their main advantage is the inherent signal amplification. FETs, as most widespread family of transistors, are very sensitive to changes in the surface potential, which is widely exploited in the analytical diagnosis field [59]. Metal-oxide-semiconductor FET (MOSFET) is the most
common architecture, where two electrodes, source and drain, are separated by a semiconducting material. Gate, as a third electrode, is insulated from the rest of the structure with an oxide. By applying a potential across the insulator, the current between source and drain electrode can be modulated by means of an electrical field. More appropriate variant of the FET has been developed, and can be found in the literature as ion-sensitive FET (ISFET) or electrolyte-gated (organic) FET (EGFET, EGOFET). In this architecture, gate electrode is separated from the semiconductor with an electrolyte as presented in Figure 2.7.

![Figure 2.7](image.png)

**Figure 2.7:** Schematic representation of EGFET architecture. Gate is separated from the semiconductor, source and drain by electrolyte. With potential applied to the gate electrode, the electrical double layer forms on the both interfaces (metal-electrolyte and semiconductor-electrolyte).

In the case of a p-type transistor and impermeable semiconductor, when negative voltage is applied on gate electrode, anions in electrolyte drift away towards semiconducting-electrolyte interface, while cations are attracted to the gate-electrolyte interface. The ions at the interfaces screen the charges in the gate electrode, resulting in the formation of electrical double layer (EDL) and accumulation of carriers in semiconducting channel [59, 60, 10].

More specifically, EDL consists of compact single layer of ions and solvent
molecules, known as Helmholtz layer, and diffuse layer of free ions [59]. Due to layers of opposite charge acting as a dipole, electrical field across the interface is established. In the quasi-static operation, nearly all voltage applied drops across the EDL and little drop is present in the bulk solution [60]. Accordingly, EDL at the interface can be considered as nanometer thick capacitor. Capacitance of electrical double layer capacitor (EDLC) is a complex function, dependent on concentration of solution and potential applied, but a good estimation can be given with the following, simplified equation

\[ C_{\text{edl}} = \frac{\varepsilon_0 \varepsilon_r A}{\lambda_D}. \]  

(2.3)

Here, \( \varepsilon_0 \), \( \varepsilon_r \), \( A \) and \( \lambda_D \) stand for electric permeability in vacuum, dielectric constant, area of capacitor, and Debye screening length, respectively. Since Debye length is in the order of 1 nm, attainable capacitances are high, usually a few tens of \( \mu \text{F/cm}^2 \) [59, 60]. This feature enables the operation of such transistors at low potentials. Debye length is defined as a measure of net charge carrier’s electrostatic effect in the electrolyte solution, decreasing in magnitude by \( 1/e \) every \( \lambda_D \). It is given by the equation

\[ \lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon_r k_B T}{2N_A q^2 I_s}}, \]  

(2.4)

where \( \varepsilon_0 \) is the electric permeability in vacuum, \( \varepsilon_r \) is the dielectric constant of a medium, \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( N_A \) is the Avogadro number, \( q \) is the charge and \( I_s \) is the ionic strength of the solution.

It is generally accepted that the detection of charge or potential changes due to adsorption of analyte is possible only within the Debye length [61, 62, 63]. According to the theory, Debye length decreases with increasing ionic strength of the solution because of the charge screening effect [64], which affects the sensitivity of such devices in real samples. Even though, lowering of ionic strength might be a solution, dilution require complex procedures, and may affect the stability and activity of biological species.

However, recent studies reported the detection beyond Debye length is possible, with a capacitive mechanism of detection [65, 66]. G. Palazzo et al. reported
2.6. **FET-based electrochemical sensors**

A device for C-reactive protein (CRP) detection in high ionic strength (0.15 M) phosphate-buffered saline solution, mimicking body fluids [65]. The biological sensing layer was composed of phospholipid bilayer (PL), followed by streptavidin (SA) or avidin (AV) proteins and CRP antibodies. They reported the change in the current of an EGOFET, with a formation of PL/SA(AV)/Ab/CRP layer, independent of the charge of protein layer underneath. Explanation for this peculiar feature was found with the study of capacitance changes upon binding event. Gating system capacitance was modeled by series configuration of fixed capacitance accounting for Helmholtz double layer, Gouy-Chapman diffuse layer capacitance and Donnan’s capacitance due to the presence of protein layer. Study therefore implies the possibility to measure a response associated with the binding at the distance far beyond Debye length, as well as the dominant effect of the outermost protein layer on electric transconductance [65]. Different beyond Debye length detection mechanism was reported by C.Chu et al. [66]. In their study, a high electron mobility transistor (HEMT) functionalized with antibodies to directly detect proteins including Human Immunodeficiency Virus-1 Reverse Transcriptase (HIV-1 RT), Carcinoembryonic Antigen (CEA) and C-reactive Protein (CRP) was used, to overcome the problem of detection in high ionic strength solutions. Result showed that mentioned proteins can be effectively detected in high ionic strength solutions and real samples without any dilution. Voltage drop and current gain depend on the gap between the gate electrode and the channel. Solution in between acts as a capacitor. As voltage drop across the capacitor linearly decrease with the thickness of capacitor, there is a presence of potential gradient through the solution. Thus, electric field across bulk solution is not zero. Reported high sensitivity obtained with smaller gap is attributed to the extended potential gradient through the solution, leading to protein detection [66].

The role of electrochemical biosensing, especially with FET based sensors, has gained interest in the last decades. Sensitive detection and quantification of bio-analytes and biomarkers, is a driving force for innovations in many vital fields. For example, EGFET based nanobiosensors for detection of glucose
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[67, 68], pH value [69, 70, 71] or ions [72, 73] are used in a broad field of analytical chemistry. A sensitive monitoring of pollutants and contaminants, such as different chemical compounds, heavy metals, toxins or pathogens is needed in food [74, 75] and environment diagnosis [76, 75, 77]. The biggest and probably most important field of application for biosensors is in proteomics and clinical diagnosis, where FET based sensors, also known as ImmunoFETs, are used for detection and quantification of biomarkers [66, 78, 79, 80, 81, 82, 83]. The concept of FET-based immunoensors was introduced by Shenck in 1978 [10]. Since this kind of sensors usually operate in contact with a solution, EGFET architecture is most appropriate. Recognition system of immunoensors can be introduced to either gate-electrolyte or semiconductor-electrolyte interface and consist of biomarkers, like enzymes, cellular receptors, antibodies, nucleic acids or artificial bio-mimetic materials [10]. Gate functionalization is usually realized through self assembly monolayers (SAMs), exploiting a well known thiol-gold chemistry [84]. On the other hand, functionalization of semiconductor can be achieved with different methods including modification of semiconductor surface with proper functional groups. Immunoensors are capable of providing analytical information by forming specific biological recognition complexes such as enzyme-substrate, antigen-antibody and others. Therefore, they are useful tool for monitoring and quantifying presence of specific biomarkers in body fluids. Further biofunctionalization of sensor’s surface is done with immobilization of antibodies or antigens (biomarkers). It is important, that immobilization results in controlled coverage and optimal orientation of biomarker receptors, to maintain the optimal biological activity and minimal non-specific adsorption. Since antigens and antibodies are usually charged, binding event effects the electric field and electric double layer, resulting in the modulation of carrier flow. Changes in current can be correlated to the analyte concentration [76].

FET based immunoensors have excellent sensitivity, due to their inherent signal amplification, where even single biological event is capable of inducing a change in the response. Besides high sensitivity, FET based immunoensor fea-
2.6. FET-based electrochemical sensors

tures simplicity, low cost, portability, flexibility, it needs small sample volumes and enables real-time detection and quantification [85]. Under the ideal conditions, it can detect clinically meaningful range, varying in the order of µM to sub fM, while sensing under real conditions has proven to be challenging. For instance, in undiluted phosphate-buffer saline solution (0.15 M ionic concentration) mimicking physiological fluids like blood, urine or serum, Debye length is far smaller (0.8 nm) than actual size of antibody (10 - 12 nm).

Recently, nanomaterials used in FET-based biosensing have attracted a great deal of attention. Although the prominent characteristics of different nanomaterials such as silicon nanowires (SiNWs), carbon nanotubes (CNTs), graphene or nanoparticles vary, they have become extremely attractive for the construction of semiconducting and sensing layers. Owing to their nanoscale dimensions, these materials exhibit unique physiochemical properties, including high surface area and surface-to-volume ratio, increased chemical and thermal stability, biocompatibility, ease of functionalization and remarkable electrical properties [10, 86]. For instance, graphene as a semiconducting channel was used for pH detection and protein adsorption [69] and prostate cancer biomarker detection [87]. Silicon nanowires were used for detection of tumor necrosis alpha and interleukin 8, as typical biomarkers of oral squamous cell carcinoma [80], prostate specific antigen in human serum [88], cardiac troponin I (cTnI) biomarker for acute myocardial infarction [89] and pH and ion sensing [62]. Carbon nanotube based FET biosensors were used to detect C-reactive protein as inflammatory biomarker associated with cardiovascular diseases [90], Interleukin-6 as a major mediator of the inflammatory response, involved in variety of diseases such as rheumatoid arthritis, myeloma and even cancers [91] or detection of bacteria and viruses such as Escherichia coli O157:H7 and the bacteriophage T7 respectively [92].
Chapter 3

Device Fabrication

Electrolyte gated FET-based protein sensor with carbon nanotubes forming semiconducting channel, was designed and developed with available micro-fabrication technology, following well-established procedures. Device was fabricated with photolithography steps in a controlled environment, using spin-coating technique for photoresist application, UV-light exposure and development of photoresist for patterning. Thermal evaporation of Chromium (Cr) and Gold (Au) and liftoff were used for electrode deposition. Finally, spray deposition of carbon nanotubes (CNTs) solution was used for the semiconductor channel. All steps are described in details in the following sections.

3.1 Substrate preparation

Flexible, 75 µm thick, 300HN Kapton polyimide (PI) substrate, developed by DuPont, was used as the substrate of our sensors. Polyimides are a representatives of thermally stable heteroaromatic polymers [93], constructed of imide monomers. They can be classified as thermoplastic, condensation or addition types [93].

Low temperature condensation reaction of aromatic pyromellitic dianhydride (PMDA) with 4,4’-diaminodiphenyl ether (DAPE), to a precursor polyamic acid followed by cyclodehydration, produces polyimide as seen in Figure 3.1, commer-
cially known as Kapton [94, 93].

Figure 3.1: Chemical structure of pyromellitic dianhydride and 4,4’-diaminodiphenyl ether (PMDA/DAPE), also known as Kapton. Source: [94]

Owing to its excellent physical, thermal, electrical and chemical properties, Kapton has been used in many application areas. It has a great chemical resistance, as there are no known organic solvents for the film. Additionally, properties are maintained over a wide temperature range, from −269 °C to 400 °C [95]. Kapton is most widely used as a high temperature insulating material in electrical and electronic applications, as well as in aerospace industry. Other fields of application include flexible printed circuits, magnetic tapes and solar sails [93].

Kapton sheets were cut into smaller 6 cm × 6 cm squares, to fit the layout of the protein sensor and opening of UV exposure light. In order to clean and prepare them for photoresist application, samples were immersed in Acetone and Isopropanol bath for 2 min and dried with N$_2$ gas gun.

3.2 Photolithography

Photolithography, also known as optical lithography or UV lithography, is a technique of transferring the pattern to a thin film or bulk substrate. UV light is used to transfer the desired pattern from an optical mask, to the light sensitive photoresist on a substrate. Exposure to the intense light causes chemical changes, allowing some parts of photoresist to be removed by a special solution known
as developer. There are two types of photoresist, positive and negative. Using negative photoresist, the exposed part is polymerized, making it difficult to dissolve in developing solution. On the other hand, exposure of positive photoresist to the light makes such chemical changes that resist becomes more soluble in the developer. Accordingly, positive or negative masks for pattern transfer can be used. In general photolithography consists of photoresist application, light exposure and development. This cycle can then be repeated multiple times with different masks to achieve complex design with multiple layers.

![Figure 3.2: The layout of the optical mask of developed EGFET-based immunosensor.](image)

Four gate electrodes are equally distant from the IDES channel in the middle. Squared electrodes pads at the bottom are used for electrical connection with the measurement setup.

The 2.5 cm × 2.5 cm square shaped layout of our developed EGFET-based protein sensor is presented in Figure 3.2. The channel of the transistor is constructed of 12 inter-digitated electrodes (IDEs), 2.4 mm wide and 0.1 mm high, with the spacing of 75 µm. Four circular gate electrodes with the diameter of 3.75 mm are equally distant from the channel, excluding any differences in properties due to channel-to-gate distance. Squared electrode pads at the bottom are used for connection with measurement setup pins. This optical mask was used in photolithographic steps, to transfer the sensor layout to the Kapton substrate.
3.2.1 Spin coating

Spin coating is a technique to deposit uniform thin layer of photoresist on the substrate surface. Spin coating process consist of dispense step, high-speed spin, and drying step. In the dispense step a small amount of liquid is delivered to the middle of the substrate at rest or slow spinning. After dispense step, there is usually a ramp of accelerating to a relatively high-speed, to spread the liquid across the substrate, and thin it to close to final thickness. With the rotation of the sample at high-speed, centrifugal and viscous forces act on a liquid of constant viscosity, resulting in a uniform film, growing ever thinner [96]. With the film getting thinner, the flow of the remaining liquid slows, as resistance to flow grows with thinner films. Therefore, the thickness of the film depends on the angular velocity, viscosity of the liquid and duration of spinning [97]. Drying step is needed to eliminate any excess solvents from the film, thus hardening the film of photoresist.

For spin coating, we used microprocessor-controlled spin coater SCC-200. It offers rotational speed up to 12 000 rpm, two rotational speed ramps and targets, active vacuum suction of the sample and exchangeable rotational platforms for substrates [97]. Pre-cleaned Kapton samples were fixed in the middle of spinning disk by applying the vacuum through the small opening in the center of the disk seen in the Figure 3.3. While in the rest state, approximately 0.2 mL of ma-N 1420 negative photoresist was spread across the entire surface using pipette. ma-N 1420 has a high wet and dry etching resistance, spectral sensitivity in the range from 300 to 410 nm, good thermal stability of resist pattern up to 160 °C, it is easy to remove and is based on safe solvents [98]. The spinning of 30 s at 3000 rpm results in 2µm film thickness [98]. In our experiment, after the photoresist was applied, the spinning started with 5 s ramp up to 5 rps for 5 s. Afterwards, high-speed spin at 50 rps was applied for 30 s. To dry the resist, evaporate excess solvent, samples with resist were soft-baked on a hot plate at 100 °C for 2 min.
3.2. Photolithography

Figure 3.3: Microprocessor-controlled spin coater SCC-200 used for spin coating of ma-N 1420 negative photoresist on Kapton samples. Exchangeable rotational platform has a small opening in the middle to fix the sample with vacuum.

3.2.2 UV light exposure

30 minutes prior to the use, a UV lamp has to be powered in order to heat up. We used UV lamp exposure apparatus, presented in Figure 3.4. Optical mask with sensor layout (Figure 3.2 on page 43) attached on a piece of glass was fixed on a mask holder, using a vacuum pump. Mask holder has an opening in the middle, where the optical mask should be placed, for the light to transfer the pattern on the substrate. Once the mask holder is placed, Kapton sample was inserted under the mask holder and centered, so that the entire desired pattern is aligned with the substrate. Finally, substrate was pressed against the mask, to establish a direct contact. It is important that the printed side of optical mask is facing the Kapton samples. If the mask is not in direct contact with the photoresist, the light can polymerize also the photoresist under the covered parts, leading to the defects in a sensor layout. Exposure of photoresist to the UV light was done
In our case, the exposure was done for 15 seconds for 15 sec, where exposed parts of photoresist polymerize. As the photoresist is light sensitive, it is of great importance that prior to photolithography steps all unwanted light sources, having a spectra in the range where photoresist is sensitive, are eliminated.

### 3.2.3 Development

After the exposure to intense light, exposed parts of photoresist chemically differs from non-exposed ones. In this process a special solvent called developer is used, whose purpose is to dissolve either exposed parts (in the case of positive photoresist), or non-exposed parts (negative photoresist). The result is a final resist structure with the pattern of the optical mask.

Since we used negative photoresist, chemical structure of all sample parts that were covered by the optical mask, as seen in Figure 3.2, remained unchanged.
3.3 Thermal evaporation

Hence, after development, photoresist was dissolved from those parts, leaving sensor pattern in the resist layer. To achieve this, samples were dipped into the ma-D 533S developer for exactly 1 min, and subsequently into the de-ionized water (DI-H$_2$O) to stop the developer action. To dry the samples, blow drying was done with N$_2$ gas gun. Leaving samples exposed for too long in the developer may result in dissolving also the parts of photoresist that did polymerize. This may consequently lead to the short circuit connections upon metallization of the pattern. On the other hand, sample not being exposed to the developer long enough may lead to insufficient resist removal, and upon metallization result in open circuit connections. While 54 seconds exposure yielded short circuit connections, 1 min exposure resulted in defect-free sensor pattern.

3.3 Thermal evaporation

Thermal evaporation is one of the physical vapor deposition (PVD) techniques. Because of the simplicity it is a common method for a thin-film deposition. The process is performed in a closed chamber under a high vacuum. Material is heated, so the surface atoms have sufficient energy to detach from surface, and coat the substrate attached at the top of the chamber. Basic schematic representation of Thermal evaporation configuration is presented in Figure 3.5. The pressure in the chamber must be low enough, so the mean free path is longer than the distance between evaporation source and substrate. In other words, average distance an atom or molecule travels before it is deposited on a substrate, without colliding with any unwanted particle and disturbing the path in between, must be ensured with a high vacuum. Usually the required pressure is below 10$^{-6}$ mbar [99, 100]. If the coating process is done in a poor vacuum, close to atmospheric pressure, the deposited layer may be non-uniform and rough.
**Figure 3.5:** Schematic representation of thermal evaporation chamber. Vacuum pumps ensure the low pressure in the chamber, usually around $10^{-6}$ mbar. Evaporation source is placed on the resistive thermal boat, and heated by applying high current. Sample is placed on the top of the chamber on a slowly rotating plate, to ensure evenly distributed, uniform coating. Source: [100]

The metal to be deposited on the substrate is heated employing resistive boat-like shaped holders, known as "boats". A very high current (around 300 A) is applied across thermal boats, made from tungsten, molybdenum or ceramic materials [100], to heat and eventually melt the metal piece placed on it. During the ramping up or cooling down the metal sources, special shutters placed in the chamber protect the samples from uncontrolled deposition. With further temperature increase, pool of melted metal in the boat cavity evaporates, creating a vapor cloud reaching the substrate placed above the source. Instead of boats, resistive coils from similar material can be utilized, on which horseshoe shaped source material is placed. Maintaining a high current flow is crucial for evaporation process. With increasing or decreasing the current, the rate of evaporation can be controlled.
3.3. Thermal evaporation

Figure 3.6: Sample after the development of chemically changed photoresist. The pattern of the sensor layout is visible, as there is no more resist on it. After the development, samples are ready for evaporation.

For deposition of thin film metal layers on the sensor structure, we used Univex 250 experimental system by Leybold. Samples with the developed resist, as seen in Figure 3.6 were fixed on the rotating plate and placed in the chamber. The rotation of 5 rpm ensures uniform thin coating. A fresh piece of chromium (Cr) and gold (Au) were placed in the separated thermal boats. Our metallization layer was formed by depositing 10 nm of Cr and 100 nm of Au. Gold is commonly used in flexible chemical and biochemical sensors as conducting layer since it has high conductivity, low Young’s modulus and is not susceptible to corrosion products in severe environments [101, 102]. However, main drawback of gold thin films is their weak adhesion on the substrate and therefore adhesion promoting processes are often needed [101, 102]. Hence, two-layer metallization has been used. Chromium is oxidative metal, used as intermediate layer enhancing the adhesion, but also affecting electrical properties [102, 103, 104]. Recently, Putz et al. compared the adhesion and electrical properties of gold and chromium/gold metallization on a
flexible polyimide films, such as Kapton. While adhesion might be better, results show, that the use of brittle metals such as chromium, introduce fractures at low applied strains, leading to decrease of electrical performance [104].

When evaporation sources and samples were in place, the vacuum pumps were activated to establish high vacuum. After 1.5 h, the pressure was low enough (around $10^{-6}$ mbar) to start the evaporation process. In the first stage, the high current passes through thermal boat with chromium, to coat the samples with 10 nm thick adhesion layer. Subsequently 100 nm of gold was coated over the chromium adhesion layer, as a conducting layer. The thickness of each layer is monitored through the mass change of the rotating plate with fixed samples. The final result of thermal evaporation is presented in Figure 3.7.

![Figure 3.7](image)

**Figure 3.7:** Sample after evaporation, coated with 10 nm thick layer of chromium as adhesion layer, and 100 nm of gold as a conduction layer.

### 3.4 Lift-off

The lift-off process, as one of the micro-structuring methods, is applied in the case where coating of metal layers is done on the existing photoresist structures. These are the parts where there is a polymerized photoresist which was not dissolved in
the development solution. In the region where photoresist was dissolved, metallization layers coated the substrate. The subsequent lift-off actually removes the photoresist with the metallization coating thereon, leaving behind only the metal pattern directly on the substrate. A schematic representation of the process is presented in Figure 3.8. In the case of a lift-off, evaporation technique is more suitable than sputtering, as the deposition of metal on the photoresist sidewalls is prevented. For that, the so called "undercut" of the photoresist, where photoresist walls and substrate are not coated, is desirable (Figure 3.8B). Therefore lift-off solvent can easily access the uncovered photoresist parts, making the liftoff process easier. The depth of undercut can be adjusted with the time photoresist is exposed to the developing solution. However, if exposed for too long, chemically unchanged photoresist may dissolve as well, as mentioned in section 3.2.3.
Figure 3.8: Schematic representation of the evaporation and lift-off process. After the development of chemically unchanged photoresist, the undercut marked with red circles (A), is present on the sidewalls of the remaining photoresist. Metal coating is done with thermal evaporation (B). Sidewalls of photoresist and the exposed substrate in the "undercut" area are not coated with metal. Therefore, lift-off solvent can reach the photoresist, and preform clean removal of remaining resist coated with metal layer (C).

In general all organic solvents are suitable for lift-off [105]. However, low-boiling solvents like acetone are not preferred, as one can not speed the process by heating the solution. Further, there is a risk of re-deposition of lifted metal coating, when using quickly-evaporating solvents.

For the lift-off we dipped our coated samples in the beaker full of acetone, and leave the process for over night. To reduce the evaporation of acetone, beaker was covered with aluminum foil. After samples being in the acetone for 12 or more hours, the beaker was placed in the sonication bath, to promote the lift-off of the coated photoresist. Subsequently, each sample was taken from sonication
bath and rinsed with fresh Acetone and Isopropanol [106]. The result of described fabrication is a flexible EGFET-based biosensor layout on a Kapton substrate as seen in Figure 3.9, ready for functionalization and use.

Figure 3.9: The result of fabrication process is flexible sensor with the golden electrodes, lines, connection pads and IDE channel structure. On one 6 cm × 6 cm kapton foil, four sensors are placed.
Chapter 4

Underlying mechanism of capacitive biosensor

Electrochemical biosensors, based on FETs, exploit their inherent property of amplifying the signal. FET device is a voltage regulated current source, where voltage applied on the gate electrode induces an electric field across gate oxide, acting as a capacitor. Oxide capacitor ($C_{ox}$) separates the gate terminal from semiconducting channel placed between source and drain terminals. Due to electric field across oxide capacitor pulling the charge carriers closer, channel in the semiconductor is established. To induce a conducting channel in the semiconductor, the voltage applied on the gate must surpass a certain threshold, also known as threshold voltage ($V_{th}$). As long as gate voltage is lower than threshold voltage ($V_G < V_{th}$), transistor is not conducting any current. When the gate voltage is higher than threshold voltage ($V_G > V_{th}$), and voltage across channel $V_{DS}$ is lower than voltage applied on the gate ($\vert V_{DS} \vert < (V_G - V_{th})$), transistor is in linear regime, conducting a current according to equation

$$I_D = \mu C_{ox} \frac{W}{L} (V_{GS} - V_T) V_{DS}. \quad (4.1)$$

Here, $\mu$ is the mobility of the charge carriers, $W$ is the width of the channel, $L$ is the length of the channel, $C_{ox}$ is gate oxide capacitance, $V_{GS}$ is voltage between gate and source terminals, $V[th]$ is threshold voltage and $V_{DS}$ is voltage between
source and drain terminals. Usually source terminal is connected to ground, which means $V_{GS}$ and $V_{DS}$ are potential applied on the gate and drain terminals, respectively. If the voltage applied to the drain terminal is greater than voltage applied to the gate ($|V_{DS}| > (V_G - V_{th})$), transistor operates in saturation mode and the equation describing a current is

$$I_{DS} = \frac{1}{2} \mu C_{ox} \frac{W}{L} (V_{GS} - V_T)^2.$$  (4.2)

As can be seen from the Equation 4.2, current of the transistor operating in the saturation mode is no longer dependent on voltage applied across the channel ($V_{DS}$). Since $W$, $L$, $\mu$ and $C_{ox}$ are determined and fixed in the manufacturing stage, current can be only modulated by gate voltage $V_G$.

As mentioned in chapter 2.6, the most popular version of transistor for biosensing application is electrolyte-gated FET (EGFET). Upon the voltage applied to the gate electrode a double layer capacitance is induced, due to the screening of the charge in electrolyte solution separating gate and channel (Figure 2.7). According to Equation 2.3, double layer capacitance is in $\mu F/cm^2$ range, orders of magnitude larger than $C_{ox}$. This allows the same transistor current $I_D$ to be obtained at smaller $V_G$ and $V_{DS}$. Further, at fixed voltages, larger $C$ favours larger $I_D$ and vice versa. Introduction of a new biochemical layer modifies the $C$ and induces a change in the sensor current $I_D$. Hence, the easiest and probably most efficient way to sense the biochemical reaction between biomolecular recognition element and biomarker is to immobilize the former on the surface of electrochemical transducer. In general the working principle of such sensors is explained by sensing of the charged biomolecules or ions introduced within the Debye length [61, 62, 63]. However, a small Debye length of high ionic solutions presents a big drawback for biosensing applications, as biorecognition of analyte may happen at the distance far beyond Debye length [10]. In this case, electric field of the screening charge is so small, that the change of the charge due to biochemical reaction is insignificant. Recent research reported the possibility of sensing beyond Debye length [65, 66], where authors proposed different capacitance systems describing the electrolyte-semiconductor interface. Further, some authors
proposed capacitive biosensors [65, 79, 107, 108, 109], with the series capacitor configurations modeling the biolayers functionalized on gold electrode. Majorly, the explanation of the capacitance change is still attributed only to the presence of charged molecules. Therefore the underlying mechanism of capacitance based biosensing needs to be analyzed.

Here, we propose a sensing mechanism based on a capacitance change due to functionalization of the electrode-electrolyte interface. Keeping in mind, that electrical double layer forms at the gate-electrolyte and semiconductor-electrolyte interfaces, a modified schematic representation of transistor symbol with two capacitors separating gate electrode and channel is presented in Figure 4.1, where $C_g$ represents the double layer capacitor formed at the gate-electrolyte interface, while $C_{edl}$ represents the double layer capacitor formed at the semiconductor-electrolyte interface.

![Figure 4.1: Schematic representation of EGFET with capacitors at gate-electrolyte ($C_g$) and semiconductor-electrolyte ($C_{edl}$) interfaces dividing the gate electrode and channel with capacitor divider.](image)

Semiconducting channel is based on a random network of carbon nanotubes (CNTs), applied over IDE structure seen on Figure 3.2. Carbon nanotubes have gained much interest as a conducting and sensing material especially in biosensing applications, due to their remarkable chemical, electrical and mechanical properties [110, 111, 112, 113, 114]. First one to observe this new carbon based structures was S. Iijima in 1991 [115]. They are unique tubular structures formed from graphene sheets of nanometer diameter and large aspect ratio. Quasi-one-dimensional (1D) structure and graphite like shell arrangement of CNTs is the reason for amazing electrical and mechanical properties. Nanotubes can consist
of single graphitic sheet (single-walled carbon nanotubes (SWNTs)), or multiple graphitic sheets (multi-walled carbon nanotubes (MWNTs)) with approximately 0.43 nm of adjacent shell separation [114, 76]. Only two years after the discovery of carbon nanotubes, Iijima and Ichihashi were able to synthesize single-walled carbon nanotubes with arc-discharge method [116]. Nowadays, SWNTs and MWNTs are synthesized either by arc-discharge method, laser-ablation or chemical vapor deposition (CVD) [114, 110, 76]. Rolling a graphene sheet at different angles results in nanotubes with different chirality (Figure 4.2). Chirality is described with chiral vector

\[ C_h = n\mathbf{a}_1 + m\mathbf{a}_2. \]  

along which the graphene sheet is rolled. The nanotube is uniquely specified with indices \( m \) and \( n \) as armchair type \( (n = m) \), zigzag type \( (m = 0) \) or chiral type \( (n \neq m) \). The properties of CNTs highly depend on their diameter, length, chirality and defects [110]. Since chiral vector affect the electronic structure of

\[ \text{Figure 4.2: Representation of chiral vector on a sheet of graphene. Depending on indices } (n,m), \text{ different types of nanotubes can be formed. Source: [76]} \]

graphite sheet, SWNTs can be either metallic or semiconducting. The bandgap
of semiconducting nanotube is inversely related to its diameter. MWNTs on the other hand, are considered to be metallic, as there is a high possibility that at least one shell has metallic behavior [110]. When fabricated under ambient conditions, SWNTs mainly exhibit p-type semiconducting behavior. However, doping of nanotubes to achieve different behavior (n-type) is possible with different methods such as doping, annealing or coating [113]. In terms of biosensing, CNTs offers substantial advantage of size compatibility and biocompatibility for the detection of biochemical analytes. Most biological processes involve electrostatic interactions and charge transfer [112]. As each atom is on the surface of SWNT and therefore exposed to the environment, even small changes in the electrostatic environment can cause change in electrical properties. Further, all-carbon composition provides a natural match to organic molecules. Due to this, CNTs are widely exploited in biosensing application as a semiconducting and sensing layer.

In spite of biocompatibility of CNTs and their extraordinary electrical properties, their stability in different and especially aggressive chemical environments is under question. Hence, gold surface of gate electrode was used for functionalization with biochemical receptors, while CNT based network was used as a channel of transistor based biosensor. The introduction of additional layers immobilized on gate-electrolyte interface affects the double layer capacitance. Based on Equation 2.3, on page 36, the increase of Debye length $\lambda_D$ decreases the capacitance. As the new layer is immobilized on the gold surface, the screening charge moves further from the interface, thus capacitance decreases. It is extremely important, that the immobilized layer is dense and not porous, otherwise ions can penetrate closer to the interface, and the thickness of the capacitor is therefore reduced. Functionalization of the gold electrode is important for the sensing of biochemical analytes. In case of immunosensors, antibodies are most widely used as a biorecognition layer, for sensing changed protein concentrations related to different kinds of diseases. Since the direct immobilization of biorecognition layer on bare gold electrode is not possible, pre-treatment of gold surface is needed.
Passivation of bare gold surface is usually done exploiting self-assembled mono-layers (SAMs). These are highly ordered thin surface films forming spontaneously on the substrate surface by adsorption. In general SAM layers consist of "head" group, which binds to the substrate, and a tail group, possessing a terminal functional group for further functionalization [117]. Surface properties can be precisely tailored by selecting the chemical structures of the SAM molecules. Alkanethiols adsorbed on gold, silver, palladium, platinum or cooper are among most extensively studied class of SAMs [118], as they offer a versatile approach for surface modification. This molecules can introduce a wide variety of organic functionalities like electroactivity, polarity or non-polarity and biological activity. Alkanethiols (HS(CH₂)nX) are a group of molecules with alkyl (C-C)n back bone chain group, thiol (S-H) head group and a functional tail group (X), as seen on the Figure 4.3. The long chain alkanethiols form a densely packed, crystalline like assembly, tilted from the surface generally by 20 to 30 degree [119]. The formation of alkanethiol SAM layer on gold exploits an Au-S bindings with strong affinity. Gold is the most studied substrate for SAM formation, as it is easy to obtain as a thin film, easy to pattern and it does not oxidize below its melting point [118]. Further, it is commonly used in analytical techniques such as QCM, SPR and in electrochemical sensors, for biochemical studies. Terminal end of the SAM can be functionalized with different functional groups including thiol (-HS), amino (-NH₂), hydroxyl (-OH) or carboxyl (-COOH) group [118]. In order to functionalize SAM layer with biorecognition layer, such as capture antibodies, carboxyl functional group is used, to form a peptide (CO-NH) bond with amino group of antibody. Thus, common SAM layer for biochemical or immune sensing applications are formed using 3-Mercaptoglycolic acid (MPA; C₃H₅O₂S) [78, 120, 121], 6-Mercaptopyruvic acid (MHA; C₆H₁₂O₂S) [122, 123], 11-Mercaptoundecanoic acid (MUA; C₁₁H₂₂O₂S) [78, 122] and others [37, 124, 125, 126]. Deposition of good SAM layer may result in a well ordered monolayer with good electrochemical stability and high flexibility. The Faradic current, present at bare gold interface is dramatically reduced at the modified interface, where most of the current is
non-Faradic i.e. capacitive [119]. However, there are still a few issues with SAM layers, like instability at higher temperatures and presence of defects, that needs to be addressed [127]. Especially nanometer hole defects in the SAM layer, known as pinholes, are critical to resolve in order to form a densely packed thin insulator layer on gold electrode. A promising method for SAM pinholes passivation was reported, using polymers like Tyramine and Phenol [127, 128, 129]. Pinholes can be selectively and permanently passivated using electrochemical polymerization, as the oxidation of Phenol or Tyramine occurs exactly on the uncovered gold surface. Further, a replacement of SAM by polytyramine films as metal or silicon passivation layer has been reported [130, 131, 132].

Cross-linking of amino groups of peptides and proteins to the carboxylic functional group of SAM layer is done through a well known and established EDC/NHS protocol. Among few chemical groups that can provide good conjugation of carboxylic group, carbodiimide compounds are most popular and versatile. Water soluble 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is the most widely used carbodiimide for water based cross-linking. In this conjugation process, carboxylic group of SAM layer is activated, to react with free protein amino group and form an amide bond. Reaction of EDC with carboxylic

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**Figure 4.3:** Schematic representation of alkanethiol SAM layer adsorbed on the metal substrate. Alkanethiols consist of back bone alkyl (C-C)\(^n\) chain group (blue), a thiol (S-H) group (red), that forms strong S-Au bonds with the gold substrate and functional tail group (yellow), ready for further functionalization.
group forms an active intermediate O-acylisourea, which is easily replaced by nucleophilic attack from amino groups. However, O-acylisourea intermediate is not stable in water solutions, thus can hydrolyse and the carboxylic group regenerates. Hence, water soluble sulfo-N-hydroxysuccinimide (sulfo-NHS) is added to the EDC process, to improve the cross-linking efficiency. Amine reactive sulfo-NHS ester is formed upon coupling of sulfo-NHS to carboxylic group. Sulfo-NHS ester is considered more stable than O-acylisourea, resulting in more efficient conjugation to primary amines. The process with its intermediate steps and products is presented in Figure 4.4.

Figure 4.4: EDC/NHS protocol scheme. Reaction of EDC with carboxylic acid forms an unstable O-acylisourea intermediate, which can hydrolise. Adding sulfo-NHS produces a more stable sulfo-NHS ester, which is amine reactive. This results in more efficient conjugation to primary amines.

Once the carboxylic acid is active, with an amine-reactive Sulfo-NHS ester attached to it, the primary amines with free amino group (-NH₂) can be conjugated. Thus, recognition layer consisted of proteins or other biomarkers can conjugate to carboxylic group of SAM layer. In biosensing and immnosensing applications antibodies are commonly used as a recognition/receptor layer. An Antibody is an “Y” shaped glycoprotein produced by immune system. Its function is to recognize and subsequently neutralize foreign harmful substances to the body, known
as antigens [10, 133]. Therefore the presence of antigens in the body triggers the production of antibodies by immune system. They bind to the specific antigen region, known as epitope, with a high specificity. Even a minor change in the chemical structure of the antigen can affect the binding affinity. Hence, antibodies must assure strong and stable binding to the antigen, which is specific and able to sense the concentration of target antigens in the required range. Antibody is composed of highly ordered sequences of numerous amino acids formed into two heavy and two light polypeptide chains linked with disulfide bonds [10], as seen in Figure 4.5. Both light and heavy polypeptide chains consist of constant and variable region, where the latter is responsible for binding of the antigen [133]. Variable part of heavy and light chain together forms the hyper-variable region, known also as the complementarity determining region (CDR). While the antibody constant regions are generally conserved among different antibodies with possible minor differences, the variable parts exhibit a high level of sequence diversity [133]. There are two types of antibodies, that mainly differ in specificity and the production. Polyclonal antibodies are a product of multiple immune cells and can therefore bind to the target antigen through numerous epitopes. They lack specificity but are cheap to produce. On the other hand, monoclonal antibodies are generated by immune cells identical to the parent cells. As they recognize and bind to the specific epitopes of antigen, the specificity is higher.

Every biological protein can have net electric charge. The overall net charge of the protein is dependent on the amino acid structure, where individual amino acid can be positively, negatively or neutrally charged. Further, net charge of the molecule is affected also by the pH of the environment. The Isoelectric point (pI) is the pH at which the molecule has no net charge i.e. it is electrically neutral. However, it is difficult to obtain an isoelectric point of a certain protein by adjusting pH. Even slight offset in the pH results in the charged molecule. Also, isoelectric point varies between different proteins and molecules.

Nevertheless, net charge of protein combined with a change of double layer capacitor thickness, due to immobilization of layers, is beneficial for capacitive
underlying mechanism of capacitive biosensor

**Figure 4.5:** Antibody structure with marked heavy and light polypeptide chains, and antigen binding sites (CDR). 3D model of real antibody structure is presented from front and back side. Green part of the antibody represent the heavy polypeptide chain, while the orange presents the light polypeptide chain. The ”Y” shape is distinguishable from the 3D model.

Based biosensing. Without any layer immobilized at the gate-electrolyte interface, the electric double layer capacitance is formed with the thickness approximated by Debye length $\lambda_D$ (see equation 2.3 on page 36). This is the distance from the electrode, where the charge on the gate metal is screened, and electric field decreases in amplitude for $1/e$. Under the assumption that the SAM layer immobilized at the interface is uniform and dense, without any pinholes, screening charge is separated from the interface. Hence we can say that the electrolyte close to the gate electrode is replaced by dielectric layer.

In the case of a parallel plate capacitor without any dielectric as seen on Figure 4.6 A, the electric field $\mathbf{E}_0$ in the capacitor can be found with Gauss law equation

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$$\oint_S \mathbf{E}_0 \cdot d\mathbf{A} = \frac{Q}{\epsilon_0}$$

(4.4)

where $Q$ is the charge enclosed by Gaussian surface. This equation yields

$$E_0 = \frac{Q}{A\epsilon_0} = \frac{\sigma}{\epsilon_0}. \quad (4.5)$$

If the dielectric slab is inserted between capacitor plates, as seen in Figure 4.6 B, dipole moments inside the material will polarize under the influence of the
external electric field. As polarization charge $Q_{pol}$ with opposite sign is induced on the surface of dielectric, the net charge enclosed by Gaussian surface is $Q - Q_{pol}$. By replacing the net charge in the equation 4.4, we get the electric field of capacitor with dielectric material

$$E = \frac{Q - Q_{pol}}{A\varepsilon_0} = \frac{\sigma - \sigma_{pol}}{\varepsilon_0}.$$  \hspace{1cm} (4.6)

The effective electric field $E$ in the capacitor with dielectric is reduced due to the orientation of the dipole moments, which are inducing an opposite electric field. The factor by which the effective electric field across capacitor is changed is known as dielectric constant or relative permittivity $\varepsilon_r$. The effective electric field can be given by introducing $\varepsilon_r$ in equation 4.5 resulting in

$$E = \frac{E_0}{\varepsilon_r} = \frac{Q}{A\varepsilon_0\varepsilon_r} = \frac{\sigma}{\varepsilon_0\varepsilon_r}.$$  \hspace{1cm} (4.7)

The capacitance of the system can be obtained by integrating the electric field across the capacitor. The integration of electric field gives us voltage drop as

$$\Delta V = - \int_{+}^{\infty} E \, dl = -El = - \frac{Q}{A\varepsilon_0\varepsilon_r}l$$  \hspace{1cm} (4.8)

where $l$ is the thickness of capacitor. By using the charge - voltage relation on the capacitor ($C = Q/|V|$), the capacitance is

$$C = \frac{Q}{|\Delta V|} = \frac{\varepsilon_0\varepsilon_r A}{l}.$$  \hspace{1cm} (4.9)
**Figure 4.6:** Schematic representation of A: parallel plate capacitor with no dielectric material inside. Due to applied voltage, the positive charge (+Q) and negative charge (−Q) accumulate on the capacitor plates. Gaussian surface is presented with red square box. B: capacitor with dielectric material inside. The electric field of polarized dipole moments $E_d$ reduce the effective electric field $E$ across capacitor. C: A dielectric slab of thickness $l_d$ inserted in capacitor of thickness $l$ results in partially reduced electric field $E$ across capacitor.

Dielectric material with high permittivity, indicating a high polarizability of dipole moments, allows a greater stored charge at given voltage. If material with high dielectric constant is replaced by the insulator with low dielectric constant, like alkanethiol SAM on gold ($\epsilon_r = 2.7 \pm 0.3$) [119, 135], the capacitance of the system decreases. This is true under the assumption that the SAM layer is dense and uniform, without any defects. Further, inserting an insulating slab of thickness $l_d$ into a capacitor with thickness $l$, as seen in Figure 4.6 C reduces the capacitance of the system. In this case two materials with different dielectric constants are present in the system. The voltage across such system can be calculated as

$$\Delta V = -\int_+^- E \, dl = -E_0(l - l_d) - E_d l_d = -\frac{Q}{A\epsilon_0}(l - l_d) - \frac{Q}{A\epsilon_0\epsilon_r}(l_d), \quad (4.10)$$

and the capacitance is

$$C = \frac{Q}{\Delta V} = \frac{\epsilon_0 A}{l - l_d(1 - \frac{1}{\epsilon_r})}. \quad (4.11)$$
Nevertheless, with impermeable SAM layer immobilized at the interface, ions are physically blocked and the screening of the charge happens at the surface of SAM layer. Thus, while introducing the insulator layer inside the double layer capacitance, the overall thickness of capacitor increases by the thickness of SAM layer $l_{SAM}$. Approximation of the capacitance can be given by

$$C = \frac{\varepsilon_0 A}{\frac{l_{SAM}}{\varepsilon_{SAM}} + \frac{\lambda_D}{\varepsilon_D}}.$$  \hspace{1cm} (4.12)

In the case of MHA, the thickness is approximated to be 0.6 $nm$, while thickness of longer chain MUA is around 1.1 $nm$. Since the Debye length is in the order of $nm$, the increase in the capacitor thickness is significant. Since thickness is inversely proportional to capacitance, increase due to impermeable SAM also contributes to lower capacitance.

With further functionalization biomarker receptor layer is subsequently immobilized on the SAM layer by forming an amide bond. Under the similar assumption, that the bioreceptor layer is dense, the further screening charge separation by the size of biomarker receptors (antibodies) $l_A$ would increase the capacitor thickness and consequently further reduce the double layer capacitance. The approximation of this system can be given by

$$C = \frac{\varepsilon_0 A}{\frac{l_{SAM}}{\varepsilon_{SAM}} + \frac{l_A}{\varepsilon_A} + \frac{\lambda_D}{\varepsilon_D}}.$$  \hspace{1cm} (4.13)

However, recognition layer consisting of biomarkers, such as antibodies, is not as uniform and dense as SAM layer and does not necessarily separate screening charges further from the gate-electrolyte interface. Hence, ions present in the electrolyte can penetrate in the biorecognition layer and are eventually blocked by SAM layer. Nevertheless, the immobilized biomarker receptors displace the electrolyte liquid, and introduce a new dielectric constant. A study by L. Li et al. [136] done on a large set of proteins showed that the average dielectric constant inside the protein is very low ($6 - 7$), while on the surface of the protein it can reach up to 30. The dielectric constant of immobilized bioreceptors being similar or higher than the dielectric constant of replaced medium (electrolyte), would
reduce the effective electric field and consequently increase the capacitance. As presented in the case of SAM layer, new, smaller dielectric constant, regardless of position, reduces the electric field and hence reduces the system’s capacitance. A model, with proteins immobilized on SAM layer introducing a new dielectric material in the capacitor is presented in Figure 4.7.

![Figure 4.7: 3D model representing the immobilized antibodies on the electrode functionalized with SAM layer. Antibodies replace the electrolyte inside the capacitor. Since dielectric constant reaches only up to 30, the electric field across such capacitor is reduced.](image)

Additionally, proteins are known to have net charge, which depends on the amino acid structure and pH. Charge of the biorecognition layer can be presented as fixed charge immobilized on the plane of double layer capacitor. Screening charges stop at the SAM layer, as proteins are considered porous. Charge of the immobilized protein layer may affect the distribution of the screening charge. Thus, Debye length may extend for a small fraction, resulting in the slight increase of capacitor thickness. To really understand and explain the effects of immobilized charge on the capacitance value and charge distribution, an extensive study of Gouy-Chapman theory of double layer structure and Stern’s modification (Gouy-Chapman-Stern) [137] should be carried out.
As explained, the immobilized layers at the gate electrode-electrolyte decrease the capacitance. In Figure 4.1, a capacitive voltage divider is introduced since at both interfaces a double layer capacitor is present. Using a theory of capacitor voltage divider, the potential of the electrolyte ($V_{el}$), driving the current in the channel can be described with

$$V_{el} = \frac{C_g}{C_g + C_{edl}} V_g.$$  \hspace{1cm} (4.14)

Since $C_g$ is smaller due to immobilized SAM and bioreceptor layer, the voltage drop across it is larger. Further, with biomarkers captured by biorecognition layer, the capacitance $C_g$ increase accordingly, and potential of the electrolyte drops further. Channel is driven by electrolyte potential $V_{el}$, which drops upon the sensing of the biomarker analyte. Thus, the current of the transistor drops, providing a signal response.

Having a layout, presented in Figure 3.2, is beneficial for point-of-care sensing application. Four gate electrodes properly functionalized and ready for detection of bioanalyte in a sample have a function of calibrating tool. Test samples of target analyte can be diluted to three different concentrations covering the range in which sensor should be sensitive. Hence, the calibration curve of the sensor response can be obtained on spot. Real sample of human serum or blood applied on the remaining gate electrode produce quantifiable response, due to calibration procedure of first three gate electrodes. Therefore, this sensor should present easy to use, cheap, sensitive and reliable point-of-care diagnostic application.
Chapter 5

Results and Discussion

To investigate the working mechanism of proposed immunosensor, we used different analysis methods. Electrochemical impedance spectroscopy (EIS) was performed to inspect the impedance of gate-electrolyte interface. Voltage measurements and signal waveform monitoring was done, to see the voltage divider based on introduced electric double layer capacitors. At the end, transistor measurements were performed to see the response of the output current on dielectric material, introduced on the electric double layer interface.

5.1 Impedance measurements

In order to have a good, functional immunosensor, an insulating layer immobilization on the gold electrode is of great importance. A dense and uniform insulating layer is desirable to increase the thickness on the interface where double layer capacitor forms, by blocking ions from gold electrode surface. This insulating layer can be presented as a parallel configuration of multiple capacitors. In the case of a defect in the layer, ions could penetrate to the uncovered area of the gold electrode, represented by a capacitor with lower thickness. Thus the capacitance of pinhole is much higher. A defect could therefore represent a dominant contribution to the parallel configuration of capacitors. In other words, even small pinholes in insulating layer are enough to increase the overall capacitance and
may result in non-functional immunosensor. Electrochemical impedance spectroscopy technique should confirm, that insulating layer formed is thick and defect free, hence contributing to the increase of impedance of functionalized gold electrode. Many articles suggested alkanethiol based SAM layers, as it is the most exploited protocol for insulating the gold surface [118, 120, 138]. However, recent studies suggested the use of polytyramine and Phenol as an insulating layer [128, 129, 132], or as a patching material for SAM defects.

To measure the impedance of gold wire and planar electrode a series of cleaning, washing and depositing steps were performed. Instead of planar gold electrode, gold wire was used at the beginning to bypass the procedure of sensor fabrication. Gold wire was cleaned in sulfuric acid ($H_2SO_4$) and with oxygen plasma. Later with planar gold electrode in use, to exclude any unwanted effects of gold wire connecting the electrode surface and the contact, a passivation with SU-8 photoresist was performed. Photoresist was heated on the hot plate at 100°C for 30 minutes and additionally cross-linked for 2 minutes under UV light. After photoresist application, oxygen plasma was used for 30 sec to clean the sample. With that, metal surface was ready for depositing insulating layer of alkanethiol based SAM, polytyramine or Phenol layers. Impedance was measured in a low-pass filter configuration using 7265 DSP Lock-in amplifier by Signal Recovery. An AC source signal was applied from output stage of the lock-in amplifier on the input of low-pass configuration of 2.2kΩ resistor and deposited electrode connected to ground (Figure 5.1). Gate electrode was dipped in 1X Phosphate-buffered saline (PBS) solution and connected to the ground via passive electrode. AC bias signal with amplitude 0.2 V was applied to the low-pass filter with the frequency sweep from 100 Hz to 250 kHz.
5.1. Impedance measurements

For interpretation of electrochemical impedance spectroscopy, a Randles circuit is commonly used. It consist of electrolyte resistance $R_s$ in series with parallel connection of electrical double layer capacitor $C_{edl}$ and impedance of faradic reaction. In the basic form it consist of charge transfer resistance $R_{ct}$ and Warburg element. Commonly, electrical double layer capacitor is replaced by constant phase element (CPE). Schematic representation of Randles circuit is presented in Figure 5.2. This circuit is a simple model describing the processes at the electrochemical interface, used for parameter extraction by fitting the model to the experimental data. However, in real electrochemical systems, impedance spectra is usually more complicated, and a more complex Randles circuit is needed, to fit the data adequately. Nevertheless, basic characteristics and values of some elements can be simply extracted from EIS spectrum presented in the Nyquist plot. To gather the plot, a frequency sweep over desired range should be performed, with real and imaginary part of impedance being recorded. In Figure 5.3, the

![Figure 5.1: Schematic representation of electrochemical impedance spectroscopy measurement setup in low-pass configuration.](image1)

![Figure 5.2: Schematic representation of Randles circuit, as a simplest model describing the processes at the electrochemical interface.](image2)
EIS spectra of three Randles circuits with different element values is shown, to highlight basic characteristics.

**Figure 5.3:** A Nyquist plot of simulated, basic Randles circuit with the frequency sweep from 50 mHz to 100 kHz. Typical semicircle curve (black) represents the reference circuit with $R_s = 5 \, \text{k}\Omega$, $R_{ct} = 10 \, \text{k}\Omega$, $C_{edl} = 0.1 \, \mu\text{F}$ and $W = 5000$. Characteristics points of the curve, defined by each element can be identified as following. $R_s$ is the offset of the semicircle along real axis, where lowering the value to $R_s = 1 \, \text{k}\Omega$ manifests in semicircle shift towards lower values (red). $R_{ct}$ is represented by the diameter of the semicircle. By lowering $R_{ct}$ to 5 kΩ, the diameter decreases (green). Double layer capacitance $C_{edl}$ can be extracted from the upper most part of the semicircle. By increasing $C_{edl}$ to 10 µF, the increase and distortion of semicircle can be observed (blue). Warburg element $W$ manifests as a 45° straight line in the low frequency range.

Electrolyte resistance $R_s$ can be extracted as the point at highest frequency range. It is represented as an offset of the semicircle on real impedance axis. Further, the diameter of the semicircle can be attributed to charge transfer resistance $R_{ct}$. Warburg element, as a specific electrochemical diffusion element manifests in a 45 degree straight line in the low frequency region. Electrical double layer...
capacitor $C_{\text{edl}}$, can be extracted from the Nyquist plot, as the upper most point of the semicircle. With the most basic understanding of electrochemical spectra and Randles cell elements, a basic interpretation of Nyquist plots can be done. Developing a complex model for fitting of experimental data and extracting values of each element is unfortunately out of scope of this master thesis, hence a most basic interpretation will be used.

First impedance measurements were done with gold wires, to check the deposition of alkanethiol SAM, Tyramine and Phenol. Gold wires were partially passivated with hot glue, to exclude any changes in impedance, due to different amount of wire dipped in 1X PBS measuring solution. Cyclic voltammetry from 0.0 V to 0.9 V was used as electropolymerization process to form an insulating layer of Tyramine and Phenol. 50 mM Phenol and 50 mM Tyramine solutions were prepared in 5 mL of ethanol and 15 mL of 1X PBS. The Nyquist plot with negative imaginary part of impedance versus real part of impedance for electropolymerization of gold wire with Tyramine and Phenol is presented in Figure 5.4. Before the deposition, a cleaned bare gold wire was measured, as a base impedance used for reference. 50 cycles of cyclic voltammetry was done in Tyramine solution, followed by 50 cycles of cyclic voltammetry in Phenol solution. As expected, the impedance of gold wire increased after each deposition, indicating the passivation of metal surface. In the next stage the activation of carboxylic group of Glutaric acid, and immobilization on deposited insulating layer was done, with applying 33µL of 150 mM EDC in 1X PBS, 51µL of 100 mM NHS in 1X PBS and 25µL of 100 mM Glutaric acid in 10 mM MES solution. After approximately 1 hour, the impedance was measured again, where a drop in $R_{ct}$ value and slight increase in $C_{\text{edl}}$ was observed. Further, bovine serum albumin (BSA) as a blocking protein, was prepared in 1X PBS, and applied on the functionalized gold wire. After 16 hours of BSA solution, the impedance dropped back to similar value as the one of bare gold wire. This peculiar phenomenon was not expected, hence further measurements were carried out.
Figure 5.4: Nyquist plot of gold wire impedance measurements. The imaginary part can be approximated with the impedance of double layer capacitor, under the assumption that the $R_{ct}$ value is very high. The impedance of bare gold (black) was used as a reference for further measurements. After deposition of Phenol (50 cycles of cyclic voltammetry) the impedance increased (red). Further increase of impedance was observed with additional deposition of Tyramine (green). This was an indicator that the gold wire was passivated. After activation of carboxyl group of SAM and Glutaric acid, impedance decreased (blue). However, after leaving the wire in BSA/1X PBS solution for 16 h (orange), impedance dropped back to the impedance of bare gold wire.

Since the impedance dropped, after BSA was applied to the functionalized surface of gold wire, we focused on the effect of BSA solution. As seen on Figure 5.5, after 50 cycles of cyclic voltammetry deposition of 50 $mM$ Tyramine, the impedance of gold wire increased. Afterwards, BSA in 1X PBS was applied directly for 1 hour, which resulted in impedance drop. In attempt to increase the impedance again, 50 cycles of 50 $mM$ Tyramine followed by 50 cycles of 50 $mM$ Phenol was redeposited with cyclic voltammetry. Subsequently, sample with
5.1. Impedance measurements

high impedance was exposed to BSA solution once again. As the exposure time was short, the impedance of gold wire appeared stable. However, the initial drop of impedance after 50 cycles of Tyramine deposition, showed the instability of polymer layer.

![Nyquist plot showing the change in the impedance due to functionalization of the gold wire surface.](image)

**Figure 5.5:** Nyquist plot showing the change in the impedance due to functionalization of the gold wire surface. after the deposition of Tyramine (red) impedance substantially increased, compared to bare gold (black). In line with previous measurements, impedance dropped after the exposure to the BSA solution (green). The redeposition of Phenol and Tyramine was done, which resulted in increased impedance (blue). After the redeposition of Phenol and Tyramine, a short exposure to BSA solution did not affect the impedance (orange).

From the measurements in the Figures 5.4 and 5.5 it was obvious, that after the exposure of Tyramine and/or Phenol to the BSA solution prepared in 1X PBS, the insulating layer dissolved. Measurements presented in Figure 5.6 showed, that Tyramine dissolves or peels off when exposed to 1X PBS solution. As impedance dropped when exposed to BSA using Phenol and/or Tyramine, it can be expected that Phenol is also unstable in 1X PBS solution. Hence, neither Phenol nor
Tyramine serve as a stable, good insulating layer.

Figure 5.6: As BSA was prepared in 1X PBS solution, we investigated the effect of 1X PBS solution on Tyramine. As expected, after Tyramine deposition the impedance increased (red), indicating that the gold wire was successfully passivated. After 1.5 h in 1X PBS (green), the impedance of gold wire dropped down, almost to the value of bare gold wire (black). This shows, that the Tyramine and possibly Phenol are not stable in 1X PBS solution, and are therefore not appropriate passivation layer for gold electrodes.

As Tyramine and Phenol polymers dissolve in 1X PBS, we focused on alkanethiol based SAM layers. 5 mM MUA was prepared in ethanol, and deposited on cleaned surface of gold wire. Mostly, we were interested in the stability of layer when exposed to 1X PBS. Figure 5.7 shows the impedance response due to functionalization of gold wire with MUA. Impedance increased after MUA functionalization, and remained stable even when exposed to 1X PBS for 1 hour and overnight. Further, impedance remained stable even when sample was left in the dry air over night.
5.1. Impedance measurements

Figure 5.7: Nyquist plot, showing the change of impedance, when passivating the gold wire with MUA SAM layer. Successful passivation of gold surface with MUA is indicated in the increase of impedance (red) compared to bare gold (black). Even after the exposure of passivated wire to the 1X PBS solution (green, blue) and dry conditions (orange) the imaginary part, representing the impedance of capacitor, remains high. The shift of the impedance curves, towards lower real impedance values, may be due to the washing of the surface. With each measurement, the sample was dipped in the solution, and the excess unbound molecules at the SAM surface are washed away.

With impedance being stable over longer period of time, regardless of exposure to the 1X PBS solution or dry conditions, SAM layer based on alkanethiols proved as a good foundation insulating layer. The same functionalization scheme with MUA was applied to the planar gold electrode of the sensor. Electrode was cleaned prior to functionalization with oxygen plasma for 30 sec. Figure 5.8 presents the change in the impedance of sensor electrode with each functionalization step. Even though there is a significant drop of the real part of impedance, indicating the reduction of $R_s$, the imaginary part remains stable when exposed to BSA in 1X PBS or DI-H$_2$O. With this measurement we confirmed a good SAM
insulating base layer formed from MUA.

![Nyquist plot of impedance measurements performed on a planar gold electrode.](image)

**Figure 5.8**: Nyquist plot of impedance measurements performed on a planar gold electrode. After the passivation of gold wire with MUA was successful and stable, the same procedure was done on the actual planar gate electrode. Even though the thickness of gold is only 100 nm, the passivation with MUA (red) and exposure to BSA(green) or DI-H$_2$O(blue), yields high imaginary impedance value.

In the last stage of impedance measurements, the difference between alkanethiol based SAMs with different back bone chain lengths was investigated. Three gold electrodes were functionalized with MUA, MHA and MPA respectively. As seen in Figure 5.9, there is a significant difference between 11-chain, 6-chain and 3-chain SAM layers. MUA with longest, 11-chain backbone, exhibits the highest impedance, with imaginary part reaching above 7 kΩ, followed by MHA with 6-chain backbone, reaching above 3 kΩ. Thickness of insulating SAM layer is thereby closely related with impedance of the system, as MUA has almost twice the length of MHA. Impedance of MPA, as the shortest one with 3-chain backbone, reaches up to 1.5 kΩ, confirming the impedance-thickness relation. This measurement therefore serves as a proof of concept that thickness
5.1. Impedance measurements

greatly contributes to the impedance of the functionalized gold surface.

![Nyquist plot showing the difference in the impedance between short chain length SAM, and long chain length SAM. MPA (blue), as the thinnest insulating layer, results in a highest capacitor at gate-electrolyte interface, thus the impedance is the lowest. With thicker MHA (green) layer, the impedance increase, as the capacitor value decrease. The impedance is the highest in the case of the thickest insulating layer - MUA (red). This indicates that introducing new impermeable layers on the gate-electrolyte interface results in the change of the double layer capacitor.](image)

**Figure 5.9:** Nyquist plot showing the difference in the impedance between short chain length SAM, and long chain length SAM. MPA (blue), as the thinnest insulating layer, results in a highest capacitor at gate-electrolyte interface, thus the impedance is the lowest. With thicker MHA (green) layer, the impedance increase, as the capacitor value decrease. The impedance is the highest in the case of the thickest insulating layer - MUA (red). This indicates that introducing new impermeable layers on the gate-electrolyte interface results in the change of the double layer capacitor.

The measurements of impedance give us a good insight into the insulating layers. The electropolymerization of gold electrode with Tyramine and Phenol results in multi-layer insulation of the metal surface. However it is unstable, as both dissolve in the PBS solution. Dissolving process happens after deposited layer is exposed to the PBS for a longer time. Hence, using polytyramine and Phenol is not suitable as an insulation layer for gold electrodes, as it will make the sensor eventually unstable. Further, we tried the passivation of metal surface with alkanethiol based SAM - MUA, MHA and MPA. From the impedance measurements, it can be concluded that the passivation of surface results in impedance
increase. MUA insulating layer appears to be stable in PBS solution and DI-
H$_2$O, even if exposed for longer time (more than 12h). While the imaginary
part of impedance, indicating the capacitance change, is stable, the real part of
impedance appears to be drifting towards lower values with each measurement.
As presented in Figure 5.3, this phenomena may be due to the change of so-
lution resistance. One of the possible explanation is the insufficient washing of
the gold electrode. Therefore with each deposition of new layer, some residues
may stick and interfere at the layer surface, increasing the overall solution re-
sistance. With measuring in the 1X PBS solution, residues are slowly washed
away, hence the impedance graph is shifting towards lower values. Nevertheless,
the drifting always stops around $R_s$ of bare gold electrode. From the Nyquist
plots of impedance measurements presented, one can observe that the thickness
of the insulating SAM layer greatly contributes to the impedance of the gold
surface. Longer backbone chain SAM results in higher thickness of the insulat-
ing layer, increasing the impedance, while shorter backbone chain SAM results
in lower impedance. Hence, this measurements may serve as a proof of concept
that capacitance is strongly affected by immobilized layers on the gold electrolyte
interface.

5.2 Capacitive voltage divider

The formation of double layer capacitors at electrolyte-semiconductor and electrolyte-
gate interfaces introduces a capacitive gating system of transistor as presented
in Figure 4.1 on page 57. Two capacitors in series introduce a voltage divider,
where across larger capacitor the voltage drop is lower. The double layer capac-
itor formed at gate-electrolyte interface is changing due to the functionalization
of gate electrode surface, while the capacitor at the electrolyte-semiconductor
remains the same. Hence, the potential of the electrolyte is changing accord-
ing to functionalization of the gate electrode. Functionalization of the electrode
with different backbone chain lengths of SAMs is affecting the double layer capaci-
5.2. Capacitive voltage divider

tance, as indicated in Figure 5.9 on page 81. According to impedance results, gate electrode with thickest insulating layer formed from MUA alkanethiol based SAM introduces the lowest capacitance, while the thinnest insulating layer formed from MPA introduces the highest capacitance. Thus, the voltage drop across thicker insulating layer should be higher than the voltage drop across thinner insulating layer. In a FET without a second capacitor introduced at the gate electrode, the channel current is driven by gate potential $V_g$, while in case of EGFET with two electric double layer capacitors, the channel current is driven by the potential of electrolyte $V_{el}$ (Figure 5.10). To see, how gate electrode functionalization and consequently the change of capacitance affects the electrolyte potential, measurement of capacitive voltage divider was performed.

Figure 5.10: Schematic representation of capacitive voltage divider, where $C_1$ and $C_2$ represent double layer capacitors formed on electrolyte-gate and electrolyte-semiconductor interface. In the case of measurements, $C_2$ was replaced with discrete 1µF capacitor, used as a stable reference.

A simple schematics on Figure 5.10 is showing two capacitor in series biased with an artificial AC voltage source. Gate electrodes functionalized with SAM layer were dipped in a 1X PBS solution. Discrete 1µF capacitor used as a reference, was connected in series with gate electrode, using reference electrode (node $V_s$ in Figure 5.10). Artificial AC signal with peak-to-peak voltage $V_{pp}$ of 0.8 V alternating between 0.4 V and −0.4 V at 1 kHz was produced by Kiethley 2602B System SourceMeter, and applied to the gate electrode as presented in Figure 5.10. Using a Volcraft VC 870 Voltmeter, voltage drop was measured across gate
Table 5.1: Voltage drop across functionalized double layer interface

<table>
<thead>
<tr>
<th>SAM</th>
<th>$V_{sam}$</th>
<th>$V_{ref}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUA</td>
<td>343 ± 38mV</td>
<td>51 ± 45mV</td>
</tr>
<tr>
<td>MHA</td>
<td>279 ± 7mV</td>
<td>119 ± 9mV</td>
</tr>
<tr>
<td>MPA</td>
<td>228 ± 4mV</td>
<td>178 ± 65mV</td>
</tr>
</tbody>
</table>

The total $V_{rms}$ drop across both capacitors was consistently 0.395 V. Voltage drop across gate-electrolyte interface passivated with MUA, MHA or MPA was measured with respect to 1 μF reference capacitor. The recorded values are presented in table 5.1, where $V_{sam}$ is voltage drop across gate interface and $V_{ref}$ is voltage drop across reference capacitor. Measurement with voltmeter indicated the change of electrolyte potential, due to difference in SAM layer thickness. According to the results from table 5.1, voltage drop across MPA SAM layer was the lowest, when measured against reference discrete capacitor. As MPA SAM layer was the thinnest insulating layer, with only 3 carbon bonds backbone chain, the capacitor formed on such interface was large. Hence, the voltage drop across it was smaller compared to other SAM layers. Using MHA SAM, formed a thicker insulating layer, and the capacitance decreased, which correspond to a larger drop across this interface. Voltage drop across capacitor formed from MUA SAM, as the thickest insulating layer, was accordingly the highest. Even though the deviation of measurements was rather high especially in case of MUA layer, the difference between different SAM thicknesses is still clearly distinguishable. This results are in line with impedance measurements from section 5.1, indicating that the thickness of layer introduced at gate-electrolyte interface is affecting the double layer capacitor. As voltage drop across capacitor at functionalized interface is increasing with increasing layer thickness, the potential driving the channel current through transistor is decreasing. Hence, with biorecognition layer
of antibodies, forming complexes with targeted antigens, the capacitance should further decrease upon binding event, changing the potential and current of the transistor.

The measurements with voltmeter were not satisfactory, as the insight of signal waveform was not present. Further, the sum of voltage drop across double layer capacitor and discrete capacitor yielded higher value than total $V_{rms}$ drop in some cases. This phenomena was emphasized especially when measuring voltage drop across thinner SAM layers. In order to get a better insight into proposed capacitive voltage divider, the measurements using oscilloscope were performed. The peak-to-peak $2.6 \, V_{pp}$ AC signal was produced using 32 bit Teensy 3.2 USB development board. Since digital to analog converter (DAC) has an offset of $1.65 \, V$, $\pm 2.5 \, V$ bias supplied by Kiethley 2602B System SourceMeter was used, to shift the AC signal to 0V offset. AC output signal and the signal across double layer capacitor were monitored with oscilloscope. In the Figure 5.11, measurement setup for monitoring signal across double layer capacitor (Figure 5.11A), dipped in 1X PBS solution, and across discrete $1 \, \mu F$ reference capacitor (Figure 5.11B) connected in series through reference electrode is presented.
Results and Discussion

Figure 5.11: Schematic representation of measurement setup for monitoring AC voltage signal (A) across double layer capacitor \((C_{edl})\) and (B) across discrete 1 µF capacitor \((C_{ref})\). Since the functionalized electrode, where double layer capacitor forms, is dipped in the 1X PBS solution, the reference electrode is used to make a series connection. 1X PBS ionic solution introduces additional resistance \(R_s\) in series with \(C_{edl}\). Points for measuring bias AC signal and signal across \(C_{edl} + R_s\) or \(C_{ref}\) with probes is marked as \(P_y\) and \(P_b\), respectively.

To make a series connection between discrete capacitor and functionalized gold electrode dipped in 1X PBS solution, a reference electrode was used. Therefore, additional solution resistance \(R_s\) was introduced to the capacitor divider. The potential of voltage divider, without solution resistance \(R_s\) can be calculated as in equation 5.1. The potential of the electrolyte, when measuring in the configuration as seen in figure 5.11 can be calculated following

\[
V_{el} = \frac{Z_{ref}}{Z_{ref} + Z_{ge}} V_g = \frac{1}{\frac{1}{j\omega C_{ref}} + \frac{1 + j\omega C_{edl} R_s}{j\omega C_{edl}}},
\]

(5.1)

where \(V_g\) is the bias AC signal, \(Z_{ref}\) is impedance of reference capacitor \(C_{ref}\) and \(Z_{ge}\) is the impedance of gate electrode composed of \(C_{edl}\) and \(R_s\).

In the Figures 5.12, 5.13 and 5.14 a signal across MPA, MHA and MPA functionalized gold electrode interface and solution resistance is presented, re-
5.2. Capacitive voltage divider

respectively. With oscilloscope, we got the information on the phase shift of the signal seen on the figures. Yellow signal is showing the signal applied to the capacitor voltage divider ($P_y$ in Figure 5.11A), while blue signal is signal measured across $C_{edl}$ and $R_s$ ($P_b$ in Figure 5.11A). Based on the measurements, the

![Figure 5.12: Capture of the oscilloscope screen, monitoring the signal waveform across double layer capacitance $C_{edl}$ on the electrode modified with MPA SAM layer and solution resistance $R_s$. The peak-to-peak value of AC signal biasing voltage divider (yellow) was approximately 2.6 V. Voltage drop across $C_{edl}$ and $R_s$ (blue) yields 2.34 V peak-to-peak. High voltage drop across MPA modified electrode, according to other other results, may be due to the insufficient washing, or some defects in the layer. The phase delay, introduced due to $R_s$ present in the capacitive voltage divider, is 27°.](image)

thickness of the SAM insulating layer also affects the phase delay in presented voltage divider. The smallest phase delay is present using interface with MUA SAM layer. Based on previous results, the MUA layer has the lowest capacitance. Solution resistance introduces an imaginary part in the voltage divider equation, and consequently a phase delay. With the smallest double layer capacitor, due to thickest SAM layer, the phase delay is the lowest. Upon functionalization of gate electrode with shorter backbone chain SAM layer, like MHA and MPA,
Figure 5.13: Signal waveform of capacitive voltage divider using electrode with MHA insulating layer. The 2.6 V peak-to-peak AC signal (yellow) is biasing the voltage divider. Voltage drop across $C_{edl}$ and $R_s$ (blue) yields 2.2 V peak-to-peak. The phase delay introduced, using MHA insulating layer is 21°, which is lower than MPA layer and higher than MUA layer.

the phase delay increases, respectively. Considering the voltage drop measured with the oscilloscope, a slight inconsistency was observed with MPA layer. The possible explanation for higher voltage drop, compared to MHA and MUA insulating layers may be the insufficient washing and defects in the MPA layer. It is known, that the SAM layers composed of shorter chains have a higher disorder, than longer chain assemblies [119]. Nevertheless, oscilloscope measurements gave us a better insight into the capacitive voltage divider behavior, providing an information about phase shift. The reason for phase delay is in accordance with capacitor change due to thickness increase, like impedance measurements and voltmeter measurements. Since the signal of proposed immunosensor is the current of the EGFET transistor, the effect of SAM layer thickness was investigated also with transistor measurements.
5.3 Transistor measurements

Using transistor as a transducer has the advantage of its inherent signal amplification. Therefore, even slight changes on the surface should reflect in the change of the signal produced. The output of our EGFET based immunosensor is the drain current $I_D$, modulated by the electric field over double layer capacitor. In general, electric field is generated by the potential applied on the gate electrode. However, in our case, second double layer capacitor is formed at gate-electrolyte interface. As we proposed, this capacitor is changing due to immobilization of insulating layer on the electrode, introduction of biorecognition layer, and most importantly, the binding event of antibody-antigen complexes. Together with double layer capacitor on semiconductor-electrolyte interface, they form a capacitive voltage divider, as presented and discussed in section 5.2. The increase of the thickness of capacitor, due to immobilization and binding events,
decreases the capacitance. Hence, the potential of electrolyte $V_{el}$ drops as the maximum voltage drops across smaller capacitor in proposed voltage divider. Current of our EGFET is modulated by electrolyte potential instead of gate potential. Therefore, upon immobilization or binding event, which increase the thickness of gate-electrolyte double layer capacitor and consequently reduces the electrolyte potential, the decrease in the current is expected. Accordingly, SAM insulating layers with different thicknesses immobilized on the gate electrode, should result in different output currents of the EGFET.

Every transistor characterization consist of transfer curve and output curve. With those, multiple parameters of transistor can be extracted, such as threshold voltage ($V_{th}$), transconductance ($g_m$), On-Off ratio, possible hysteresis, linear region, saturation region and others. To show, that our device acts as a transistor, the output curve and transfer curve are presented in Figure 5.15 and 5.16, respectively.

Our transistor has a p-type behavior, due to the semiconducting channel based on the random network of CNTs. By applying negative potential on the gate electrode, the conducting channel in the semiconductor is induced under the influence of electric field. To get the output characteristic curve, the drain potential is swept with DC bias in the appropriate operating range, while the gate potential is kept constant. By conducting multiple measurements at different gate potentials, the output current curves are obtained (Figure 5.15). This characterization helps determining linear, saturation, and possibly sub-threshold regions. At low gate potential ($|V_{GS}|$), the transistor is not conducting. After surpassing the threshold voltage $V_{th}$, the transistor starts to conduct a current. While in the linear region ($|V_{DS}| < (V_G - V_{th})$), the current is linearly dependent on drain voltage $V_{DS}$, as seen in Equation 4.1. With increasing drain potential ($|V_{DS}|$), transistor reaches the saturation region ($|V_{DS}| > (V_G - V_{th})$), where the current has a quadratic dependence on the gate potential, but is no longer dependent on the drain potential (Equation 4.2). Therefore the current seen in the transistor output characteristic stabilizes after some point, even though the drain potential
5.3. Transistor measurements

Figure 5.15: Transistor output curve of our device. Drain potential ($V_{DS}$) was swept between 0.0 V and −0.8 V, at different constant gate potentials ($V_{GS}$). In general, output curve gives a good insight into the linear and saturation regions. However, in presented case, the saturation region is not visible, due to the very low operating voltages. Nevertheless, indication of saturating current can be observed at highest drain and gate potentials applied.

is increasing. In our case, we can not observe the real saturation of the current. Reason for that are very low operating voltages. Nevertheless, a saturating trend can be seen at highest drain and gate potential applied.

Transfer curve can be obtained by keeping the drain potential constant, while the gate potential is swept within the appropriate range. Drain potential ($V_{DS}$) was kept constant at −0.2 V, while the gate potential $V_{GS}$ was swept from 0.8 V to −0.8 V. Transfer characteristics obtained is presented in Figure 5.16. By conducting transfer characterization, the threshold voltage can be extracted at the point where the current rapidly increases. This is also known as the point where the transistor turns on. Steeper transfer curve after threshold voltage indicates the higher sensitivity to the gate potential. The threshold voltage of
Figure 5.16: Transfer curve of the device showing the drain ($I_D$) and gate ($I_G$) currents with respect to gate potential ($V_{GS}$). Threshold voltage of this device is at $-0.34 \text{ V}$, while the on-off ratio is around 47.

The measured device is approximately $-0.34 \text{ V}$. On-off ratio of the transistor current can be simply extracted by the ratio of highest and lowest current. The On-Off ratio in presented case is 47.

To monitor the output changes of our EGFET biosensor, an online measurement was done. Here, both electrodes, gate and drain, are biased with a DC signal. In our case, $-0.5 \text{ V}$ was applied to both terminals. By applying high bias to the EGFET, the transistor is operating in the saturation region. Hence, the transistor output current $I_D$ has a quadratic dependence on the driving potential, which is beneficial for the sensitivity of the device. DC bias was applied by Kiethley 2602B System SourceMeter, configured through Python script. Developed immunosensor was placed in a holder designed especially for transistor measurements. The holder, presented in Figure 5.17, consists of 5 wells, for gate electrodes and channel, connected with a bigger well at the top, where the measurement liquid (1X PBS) is placed. To ensure that the liquid does not spill
5.3. Transistor measurements

Figure 5.17: 3D model of the holder designed for sensor measurements. Front part has 5 wells for 4 gate electrodes and channel IDES in the middle. A bigger well is there for the liquid, which establishes electrical connection among electrodes and channel. Sensor is placed on the squared box visible on the bottom part of the holder, and pressed against top part, by screwing the bolts. In the inset, the back side of the top holder is shown, where a dent is made around each well, to place a sealing o-ring. Therefore, liquid in the well can not spill around the rest of the sensor.

Over the entire sensor, o-ring seals in the wells are pressed against sensor surface. Source, drain and four gate terminals are connected through connector pads and golden pins with the source unit.

Figure 5.18, shows the result of the online measurement, with three different SAM layers. Overall the measurement was conducted for 3 minutes, during which the connection was switched between electrodes with MUA, MHA and MPA insulating layers, respectively. In the figure, the measurement interval of each SAM layer is marked. First insulating layer, MUA, introduces the thickest double layer capacitor. Hence, the capacitor is small, and the electrolyte potential driving the current of the transistor drops. The value, at which the output current stabilized was $-8.16\mu\text{A}$, corresponding to the lowest current value measured.
Figure 5.18: Transistor online measurement, showing the current change due to different SAM layers. Measurement was ongoing for approximately 3 minutes. First the electrode with MUA (red time period) was measured, resulting in the highest current (−8.16μA). Then the electrode with MHA was connected (green time period) and the transistor current increased (−8.31μA). Further, by connecting electrode with MPA insulating layer (blue time period), the current additionally increased (−8.75μA). Signal response of 150 nA is acquired, corresponding to the change of only few carbon chains. This measurements are in accordance with previously presented measurements. Thicker insulating layer on gate-electrolyte result in lower capacitor, hence the current is lower.

By switching to the MHA layer with lower thickness, a slight increase in the current can be observed. Using electrode with MHA layer, current stabilized at −8.31μA. Further, by switching to MPA insulated electrode, introducing the thinnest double layer capacitor, the output current stabilized at the highest value, yielding −8.75μA.

Transistor measurements performed are in correlation with previously presented results. Insulating layer introduced at the gate-electrolyte interface in-
duces a change in the capacitor and electrolyte potential, consequently leading to the response of the sensor. Using SAMs with different backbone chain lengths, resulted in distinguishable change of the transistor output current. The smaller current difference, between MUA and MHA layer, is around 150 nA, which can be detected easily. Current change is induced by a really small change in the capacitor thickness, of a few carbon chains. This implies, that the sensitivity and overall performance of such immunosensor device is excellent. Using transistor with inherent current amplification is definitely beneficial for this kind of applications. Overall, the thickness of double layer capacitor clearly plays an important role in the sensor response. Further, this measurement can be seen as the simulation of stacking layers on the gate electrode. Introduction of new layer, with low dielectric constant, results in the change of transistor output current. Thus, we can expect that the antigen-antibody binding event, would produce a signal change.
Chapter 6

Conclusion

In this master thesis, the working principle of electrolyte-gated transistor based immunosensor was investigated, by means of experimental work. Design, fabrication and functionalization steps were performed, to test the viability of the proposed device.

The formation of electric double layer at the metal-electrolyte interface has been explained in details with extensive studies of Gouy-Chapman and Gouy-Chapman-Stern theories. A potential applied on the metal electrode, induces a screening charge in the electrolyte, forming a double layer capacitor. This phenomena in relation with advanced nanotechnology materials and electronic devices was proven to be extremely beneficial in biosensor technology.

The functionalization of the electrode surface induces a change in the double layer capacitor formed at the interface. It is our belief, that the impermeable layer moves screening charge further away from the electrode surface, by introducing a new dielectric material. Hence, the double layer capacitor with the thickness of a bit more than the Helmholtz layer is ”extended” for a thickness of the insulating media. We focused on a good functionalization scheme of the electrode surface, since it is very important for the performance of the immunosensor based on electrolyte-gated transistor.

Firstly, the passivation of the golden electrode surface with polymers has been
investigated. Based on reviewed literature, a few groups used polymers such as Tyramine and Phenol for the insulating layer. Further, polymers have also been used for passivation of the defects in a well known SAM layer based on alkanethiols. Adopted from the research articles, Phenol and Tyramine were deposited on the gold using cyclic voltammetry. Impedance measurements showed, that the impedance increased with electropolymerization of Phenol or Tyramine. However, the stability of such insulating layers was under question. The impedance of gold surface passivated with Phenol and/or Tyramine drastically decreased when exposed to 1X PBS solution. Based on the impedance, whole deposited layer detached from the surface, as the impedance value reached the base value of bare gold. As insulating layer should be dense and stable, offering a base for biorecognition layer, the use of Phenol and Tyramine was abandoned.

Instead of polymers, we investigated SAM layers based on alkanethiols. These are most widely used insulating layers, especially in combination with gold surfaces. The adhesion of SAM layer was monitored with impedance measurements. Formation of good and dense SAM layer resulted in a higher impedance of the electrode-electrolyte interface. Even after the exposure to 1X PBS solution and DI-H$_2$O, the impedance remained high. This serves as an indicator, that a good insulation layer can be obtained with alkanethiol SAMs. Additionally we tested the effect of different thickness on the impedance of the system. Using MPA, our shortest alkanethiol with 3 carbon chains backbone, the impedance was smaller compared to MHA alkanethiol, with 6 carbon chains backbone. MPA alkanethiol, with 11 carbon chains backbone, resulted in the highest impedance values. With those measurements, we got the feedback, that the thickness of impermeable layer has an impact on impedance of the interface and consequently on capacitor value.

Further we measured the voltage divider based on capacitor formed at the gate-electrolyte and semiconductor-electrolyte interfaces. The gate potential applied, is divided between two double layer capacitors, where the potential of electrolyte is actually modulating the current of the transistor. Verified with impedance measurements, the insulating layer, as a new dielectric material on
the gate electrode, decreases the double layer capacitor. Accordingly, the voltage drop across this capacitor is higher, compared to other double layer capacitor at semiconductor-electrolyte interface. Hence, the potential of the electrolyte is decreasing with introduction of new dielectric materials on the gate electrode. Here the voltage drop across functionalized electrode was measured using voltmeter and oscilloscope. Even though, the measurement equipment was not really suitable for our needs, a clear difference in the voltage drop across capacitors with MUA, MHA and MPA insulating layer was recorded. Additionally, signal waveform was inspected using oscilloscope, where the phase delay due to solution resistance present in voltage divider was observed. As the transistor is driven by electrolyte potential, the current as an output signal should be reduced.

Finally we showed, that the change in thickness of insulating layer affects the output current of electrolyte-gated transistor. Prior to those measurements a transistor characterization was performed with transistor transfer and output curve measurements. Based on this measurements, transistor parameters can be extracted, which is helpful for further optimization of the device. To show the effect of varying dielectric material thicknesses on transistor output current, online measurement with different SAM layers was performed. The results showed that the device responds with the lower signal even upon a small increase of the thickness. Comparing MUA and MHA insulating layers, the current dropped for 150 nA. Even though the change is not tremendous, we believe it is easily distinguishable. The current drop of 440 nA was observed switching from electrode with MHA layer to the electrode with MPA layer. This results indicate, that the proposed sensing mechanism of immunosensor based on electrolyte-gated transistor is very sensitive. This measurements showed, that the thickness of dielectric material immobilized on the gate electrode do have an impact on transistor output current. Therefore, by further immobilization of biorecognition layer, such as capture antibodies, the immunosensor with high sensitivity and selectivity can be developed. Based on investigation of working principle, the signal response of immunosensor should be obtained upon binding event of antigen-antibody com-
plexes.

In conclusion, presented work provides a proof-of-concept experimental results highlighting the effects of electrode with deposited dielectric material on double layer capacitor. Proposed change of double layer capacitor due to thickness of dielectric material was confirmed with impedance measurements, voltage divider measurements and transistor output measurements. As this is only the first step towards the working immunosensor, further steps are proposed. The immobilization methods and stability of biorecognition layer on SAM should be investigated. Next step would be, to check the sensitivity and selectivity of proposed immunosensor, using samples with targeted analyte only, as well as real, complex samples. Apart from deposited dielectric material on the electrode surface, the effect of charge of biomolecules on double layer capacitor and transistor output signal needs to be checked. The optimization of the device, by revising the sensor layout, biorecognition layer, transistor parameters and others would be the final step towards proposed immunosensor for point-of-care diagnosis.
Bibliography


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