ELECTROKINETIC METHOD FOR DETECTION OF DNA MOLECULES

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Abstract. *In this study, we demonstrate the possibility of the Si-glass microchip capturing DNA samples by functionalizing the surface with PNA molecules as the essential bioreceptive element. Measurements are carried out based on electrokinetic principles, where the measuring element is the streaming current appearing on the surface through the flow of the liquid in a capillary. The obtained measurements demonstrate an optimal concentration of 31.25µM PLL-Thiol that covers the active surface of the microchip to the maximum that allows the capture of samples used continuously without loss. Furthermore, the capture of DNA is observed by the multiple changes of the signal by ~5mV, which allows us, based on the results, to continue the research to obtain a biosensor suitable for detecting genetic mutations.*

Keywords*: zeta potential, streaming current, biosensors, surface functionalization, PNA*

Introduction

Over the years, the monitoring process of vital parameters in various fields has been of great importance, leading to the development of small analytical devices known as biosensors. Furthermore, with the development of nanotechnologies and regarding the current trends, which go towards the minimization of diagnostic devices and the integration of several actions in a single device ("lab-on-a-chip") [1] with the minimization of the impact of the human factor, the biosensor represents a great perspective, and widely studied for its integration into human life, facilitating the possibility of individual monitoring of both personal health and the environment.

The enormous amount of genetic information brought by genome-wide sequencing has increased the need for simple, rapid, inexpensive, high-throughput, miniaturized, and mass-produced analytical devices to participate in the growing molecular diagnostic market, thus fulfilling the essential criteria for decentralized DNA testing. Genome sequencing has enabled the detection, respectively, of inherited point mutations that cause disease and human pathogens by their specific nucleic acid sequences. Drug screening, differential gene expression monitoring, and forensic analysis have also benefited from ongoing research in biosensor technology. Such analytical devices, known as biosensors, convert a biochemical reaction or interaction into an analytical signal that can be further amplified, processed, and recorded. DNA biosensors consist of an immobilized DNA chain to detect the complementary sequence through DNA-DNA hybridization. In this study, a bioreceptor element will be used Peptide Nucleic Acid (PNA).

Material and methods

The work setup integrates devices to regulate and monitor the measurement process.

The nitrogen gas pressure in the analyte solution reservoir is pulsatile controlled through a pressure regulator (Elveflow OB1). For a measurement, the optimal pressure values are set at 1.5 and 3 bar, a pulse width of 30 seconds to perform a two-point leakage current measurement. These pressure ranges are proven to obtain a clean signal, according to Gevari M.T. et al. [2]. The pressure

flow is measured and transmitted to a computer using a flow sensor (Elveflow, MSF3) connected to the outlet of the pressure regulator and the inlet of the solution tube. Hydraulically forced through tubing (polyether ketone material) and microfluidic connections (Darwin Microfluidics, France) into the microchip channels, the solution creates a streaming current. The streaming current is measured using 2 Pt tube electrodes connected to the outlet and inlet of the microchannels. The resulting streaming current pulses are measured and recorded on a computer using a Keithley pico ammeter (Model #2636A).

The measured streaming current was used to calculate the surface zeta potential using the Ec.(1):

$$\zeta^* = \frac{\eta}{\varepsilon\varepsilon_0} \frac{\Delta I_S}{\Delta P} \frac{L}{A}$$

where η and $\varepsilon\varepsilon_0$ – viscosity and permittivity of the buffer
L and A – the length and cross-sectional area of the microchannels
ΔI_S – streaming current difference
ΔP- pressure difference

To allow the microchip to be connected to the rig, the polyether ether ketone (PEEK) platform was custom-made. It presents an octagonal block in the middle of which the microchip is fixed with the help of 2 plastic plates. Holes with the diameter of the microchannels are drilled inside the PEEK block to provide its entry and exit. Rubber rings (Apple Rubber Inc., USA) with a diameter of 1 mm are used to avoid liquid leakage when the microchip is in contact with the block.

The microchip used has a three-layer aluminum-silicon-dioxide-glass structure created by lithography, the active surface being the SiO_2 layer. The manufacturing method is fully described by Gevari M. T. et al. [2]. The microchip is 12 mm × 12 mm in size, with channel dimensions of 10 μm × 25 μm in cross-section and 3 mm in length.

Surface functionalization

The surface functionalization was carried out using the method used by Movilli J. et al. Described in ref. [5] in which Peptide Nucleic Acid (PNA) is an artificially synthesized polymer similar to DNA or RNA, with the difference that PNA is composed of repeating units of N-(2-aminoethyl) -glycine linked by peptide bonds compared to DNA and RNA, which are composed of deoxyribose and ribose, respectively, is used as a bioreceptor element.

Microchip cleaning

To start the surface functionalization procedure, the RCA1 cleaning of the microchip is carried out. RCA1 is a procedure to remove organic residues, oxidize the silicon, and leave a thin oxide on the surface of the wafer; in this case, it restores the SiO2 layer, which allows multiple uses of the microchip. Decontamination works based on sequential oxidative desorption and complexation with H_2O_2-NH_4OH-H_2O. All handling is performed in a laminar flow hood for personal protection.

A 120 ml beaker of water is placed on the digital magnetic stirrer plate. The water in the container is heated to a temperature of 90º C. Then, in another 50 ml laboratory beaker, are mixed the solutions of deionized water (H_2O) pre-filtered through a nylon filter with a pore size of 0.45 μm, a solution with a concentration of 27% ammonium hydroxide (NH_4OH), and 30% hydrogen peroxide solution (H_2O_2), in a ratio of 5:1:1, respectively. The used microchip is immersed in the mixture, and the container is inserted into the 120 ml glass in the hood. The procedure is done for 30 min at 90º C, which periodically needs to be mixed to better penetrate the cleaning solution inside the microchannels. Then the microchip must be immersed in filtered deionized water to remove the residue from the cleaning solution.

PLL- Thiol layer. The first layer on the negatively charged surface is deposited PLL-thiol (Nanosoft Polymers, USA) by the static method. Initially, the optimal concentration of the PLL-thiol solution was determined. The procedure proceeds as follows, 50 mg of PLL-thiol powder was dissolved in 1 ml of deionized water. After which the stock concentration was dissolved in concentrations of 15.6μM (proportion of 5μl PLL-thiol stock solution and 495 μl deionized water),

31.25μM (10 μl PLL-thiol and 490 μl deionized water) and 62.6μM (15 μl PLL-thiol and 485 μl). Next, the dissolved solution of PLL-thiol is mixed well by vortexing for 5 min and connected to the pump (Elveflow OB1). Before the inducer of the solution, filtered deionized water is introduced into the channels to create an environment similar to that of the solution, after which the surface functionalization procedure is carried out for 30 min at a constant pressure of 2 bar.

PNA layer. The capture of the PNA sample is carried out through the disulfide bonds created following the coupling of two thiol groups. To achieve this bond, the thiol groups must contain free sulfur atoms, which we have in the PNA-thiol manufactured in the Uppsala University laboratory. The PLL-thiol from the manufacturer includes the strong -SH bond, where a redox reaction with a strong acid or base can remove the H+ atom. The manufacturer recommends using trifluoroacetic acid (TFA, Sigma-Aldrich, SE), a strong acid with pH = -1, for thiol deprotection. In a 1 ml Eppendorf tube, 5μl TFA (≥99%) and 495μl filtered deionized water were mixed, after which 1μl of the dissolved solution was mixed with 12μl PLL-thiol solution and left in the dark for 30 min for the reaction to take place redox. After which, the obtained solution is dissolved in 288 μl of filtered deionized water and injected for 30 min into the microchannels of the microchip.

The next step in surface functionalization is capturing the PNA-thiol sample via the disulfide bond. 9 μL of ANP-thiol with the sequence ATCATCAACCAGG and 291 μL of filtered deionized water were mixed in a 1 ml Eppendorf tube and mixed well by vortexing. For 30 minutes, the solution obtained under a pressure of 2 bar was introduced into the microchannels of the microchip.

DNA samples capture. The capture of the DNA sample is based on the complementary sequence of PNA through hydrogen bonding. DNA with the sequence TAGTAGTTGTGGTC concentration 226.39 nmol in gel form (Eurofins Genomics, SE) is dissolved in 500μL deionized water. In a 1ml Eppendorf tube, 1μL obtained solution and 452 μL of 1XPBS solution were mixed. Next, the microchannels are washed with 0.1XPBS to create a solution medium. After that, the final solution was introduced into the ready microchannels for sample capture for 40 min with a pressure of 1 bar.

Results and discussion

For the given project, the concentration of PPL-thiol was established for the maximum coverage of the surface with a layer of molecules according to the steps and proportions maintained in the previous chapter. The full coverage ensures the maximum potential of the microchip and allows the maximum DNA molecules to be captured. This subsection aims to find the optimal concentration considering the amount of the substance and the time to stabilize the molecules as quickly as possible on the surface. In the graph below (figure 1a), we can follow the behavior of PLL-thiol molecules on the surface for 40 minutes at different concentrations. They were analyzed from the minimum concentration of 15.6μmol, constantly increasing by 50% (figure 1a). This manipulation was performed to find the maximum positive signal of the zeta potential. As can be seen, 15.6 and 62.6 μmol do not correspond to the criteria, which consist of reduced reaction time and minimal use of materials, namely 15.6 μmol does not reach the maximum signal, which characterizes the incomplete coverage of the surface, and 62.6 μmol is more difficult to stabilize over time and is the double erosion of the material. The maximum signal of the zeta potential was determined to be in the range from -5 to -10mV, which we have at the concentration of 31.25μmol, corresponding to the desired characteristics regarding the time and quantity of the material.

The manufacturer recommended using Trifluoroacetic acid to capture the receptive element of the biosensor. Due to the strength of the acid, it was dissolved in water to a concentration of 60% and pH = - 0.88. As a result of the inclusion of dissolved acid in the microchannels of the microchip, the PLL-thiol layer detached from the surface, which can be seen in figure 1b, where the zeta potential signal became more negative compared to the blue signal, which is very close to 0 in the presence of molecules of PLL-thiol. The detachment of the layer is explained by the fact that the isoelectric point of SiO_2 is 2.5, taking into account the pH of the acid used, which is much lower than the isoelectric point, the surface becomes positive compared to the given solution, because of this the PLL-thiol molecules were attracted by the acid solution as a stronger magnet and thrown out of the system with the solution.

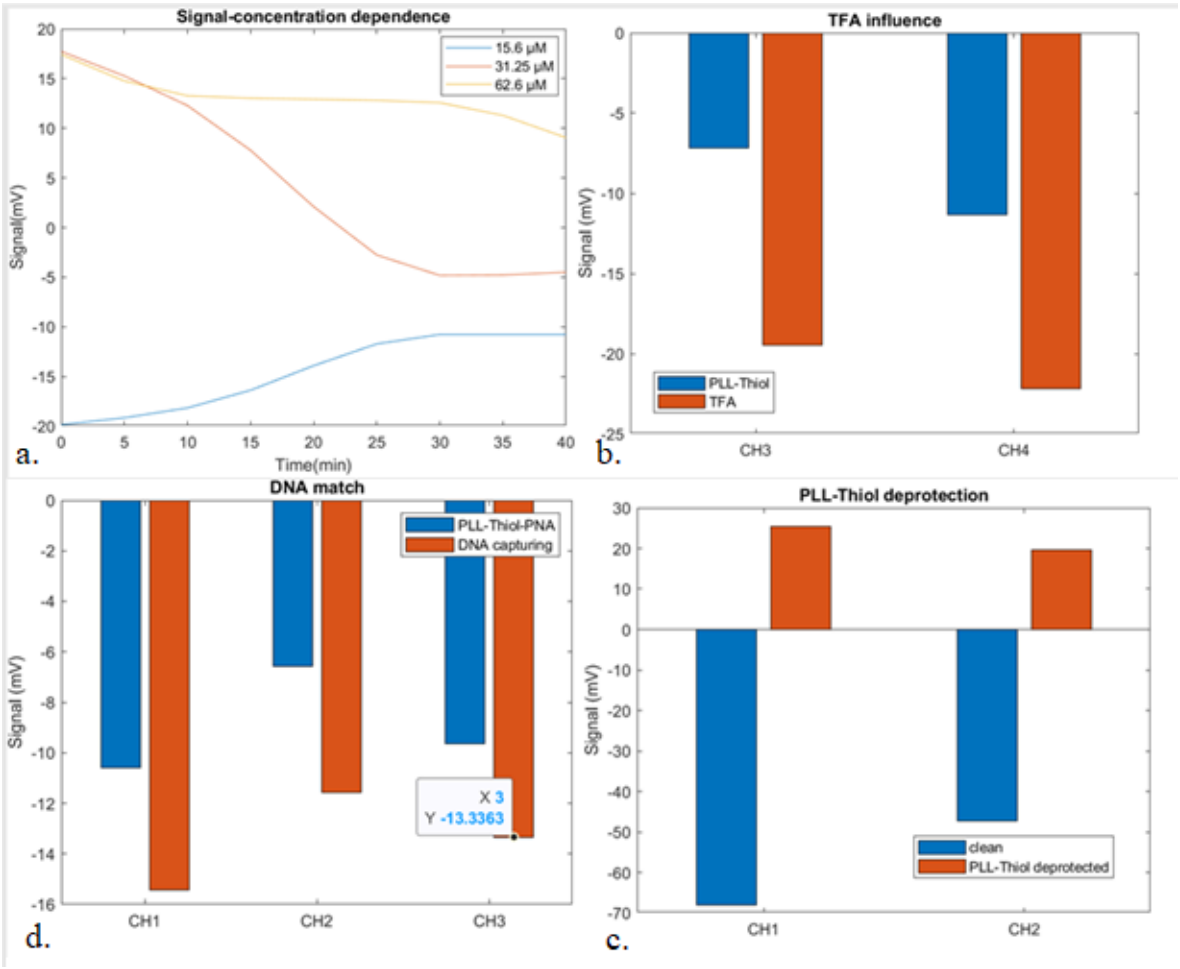

Figure 1. a. Time-dependent signal stability in different concentrations. b. Influence of TFA concentration 60% on the surface. c. Zeta potential after thiol deprotection. d. Zeta potential after capturing the DNA samples

The problem of deprotecting the Thiol group had to be solved by keeping the concentrations and decreasing the pH of the solution. In this way, it was decided that the deprotection of the molecules should be carried out until attachment to the surface. According to the method described in the Materials and Methods chapter, we get a pH of 3.4 calculated and ~4 measured with the help of the pH meter. According to figure 1c, we can see that the PLL-thiol molecules, after deprotection of the thiol molecules, were successfully attached to the surface, which is characterized by the value of the zeta potential indicated by the red color of the signal, which at the moment has a positive value compared to the signal obtained in figure 1b after the deprotection of the molecules directly on the surface.

PNA molecules are neutral due to their structure, so the zeta potential after capturing them according to the signal is observed with minor deviations that are not clearly observed. At the same time, we can't be sure that the disulfide bond formation procedure took place. Therefore, to demonstrate that the deprotection of the PLL-thiol molecules was successfully achieved and the disulfate bond was formed, the manipulations, as mentioned earlier, were repeated, after which complementary DNA molecules were injected. The results are represented in figure 1d. Since DNA molecules are large molecules and compared to PNA, they have a negative charge. Therefore, according to the graph, the signal change is clearly observed after sample capture.

Conclusion

This work elaborated on the surface modification method to obtain a biosensor capable of capturing DNA samples. First, the surface was activated through wet chemistry to attract the PLL-Thiol molecules. Then through the disulfide bond, the bioreceptive element was captured that would trigger the surface to capture the target molecules. Measuring the zeta potential before DNA capture and after that, the detection signal of the one in work is ~5mV.

In conclusion, we have successfully obtained a surface ready to capture DNA molecules simply and quickly.

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