DNA Immobilization

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Potential-Assisted DNA Immobilization as a Prerequisite for Fast and Controlled Formation of DNA Monolayers

*Daliborka Jambrec, Magdalena Gebala, Fabio La Mantia, and Wolfgang Schuhmann**

Abstract: *Highly reproducible and fast potential-assisted immobilization of single-stranded (ss)DNA on gold surfaces is achieved by applying a pulse-type potential modulation. The desired DNA coverage can be obtained in a highly reproducible way within minutes. Understanding the underlying processes occurring during potential-assisted ssDNA immobilization is crucial. We propose a model that considers the role of ions surrounding the DNA strands, the distance dependence of the applied potentials within the electrolyte solution, and most importantly the shift of the potential of zero charge during the immobilization due to the surface modification with DNA. The control of the surface coverage of ssDNA as well as the achieved speed and high reproducibility are seen as prerequisites for improved DNA-based bioassays.*

Detection of DNA hybridization is the basis of the majority of DNA-based biosensors and bioassays with multiple applications in medical diagnostics, forensics, genomics, and environmental research.[1] Hybridization detection using electrochemical detection schemes has become increasingly important[2] since electrochemical DNA hybridization assays allow label-free approaches with high sensitivity based on electrochemical amplification steps.[3]

Electrochemical impedance spectroscopy (EIS) enables label-free DNA hybridization detection and moreover the in-depth investigation of processes occurring at the electrified interface by sequentially following each step of the build-up of the DNA assay.[4] The first step in the construction of a DNA-assay is the immobilization of the probe DNA (pDNA) on a gold electrode surface through the formation of Au–sulfur bonds. Subsequently, the pDNA-modified electrode is further modified with an alkylthiol, typically mercaptohexanol (MCH), to remove unspecifically bound DNA and to ensure a more upright orientation of the DNA strands by filling the free space on the gold surface. Finally, the ssDNA/MCH-modified electrode is used to bind the complementary target DNA (tDNA) in the hybridization process.[5]

The formation of the pDNA layer, that is, the amount and accessibility of the specific pDNA on the electrode surface greatly influences the later hybridization process. Thus, well-defined, reproducible, and controlled pDNA-modified surfaces are a prerequisite for the development of optimized DNA sensors.[5,6]

The behavior of DNA in the vicinity of electrified interfaces can be manipulated by means of the potential applied to the electrode surface.[7,8] However, this process is still not fully understood. Bartlett and Sosnowski[8] contributed to the understanding of potential-assisted hybridization and denaturation of DNA by studying effects of the applied potential on, for example, the pH value and temperature changes. Effects of potential pulsing on the kinetics of DNA hybridization and the contribution of unfavorable electrostatic interactions with the immobilized DNA layer were also discussed previously.[9] Regarding the influence of the electric field on already immobilized DNA, potentials more positive than the potential of zero charge (pzc) are reported to induce the grafted ssDNA strands to bend towards the surface, while negative potentials are supposed to favor an upright orientation of the immobilized ssDNA strands.[10] Similarly, for the immobilization process itself it was reported that ssDNA strands are attracted or repelled during the immobilization supported by the application of potentials, supposedly due to charge interactions with the negatively charged ssDNA strands. However, the potential values for the pulse-deposition protocol were experimentally found or in the best case referring to the pzc of the bare electrode (pzc(bare)).[11]

We show that the impact of electrostatic attraction/repulsion alone is far too simple to explain the processes taking place at DNA-modified electrified interfaces. We propose a model for the potential-assisted immobilization of pDNA on gold electrode surfaces that considers the role of ions in the vicinity of the electrified interface as well as the DNA strands, the distance over which applied potentials have an impact on ssDNA strands in the vicinity of the electrified interface, and most importantly the shift of the pzc of the electrode invoked by the surface modification with pDNA strands. This shift of the pzc requires a careful selection of the applied pulse potentials to obtain control of the pDNA immobilization process. The model provides insights that make it possible to establish a reproducible potential-assisted pDNA immobilization protocol that is much more efficient and much faster than the commonly applied incubation

[*] D. Jambrec, Prof. Dr. W. Schuhmann
Analytical Chemistry—Center for Electrochemical Sciences (CES), Ruhr-Universität Bochum
Universitätsstrasse 150, 44780 Bochum (Germany)
E-mail: wolfgang.schuhmann@rub.de
Dr. M. Gebala
Stanford School of Medicine
Biochemistry; Beckman Center (USA)
Dr. F. La Mantia
Semiconductor and Energy Conversion—Center for Electrochemical Sciences (CES); Ruhr-Universität Bochum
Universitätsstrasse 150, 44780 Bochum (Germany)

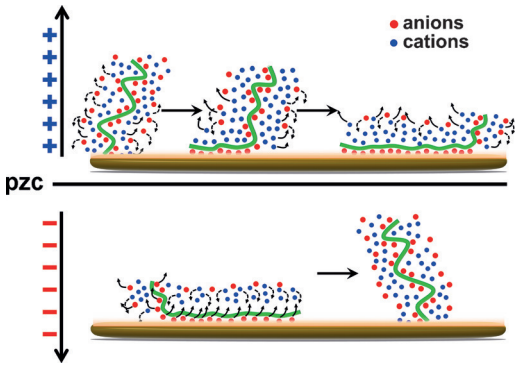
Supporting information (Experimental section, acceleration of MCH immobilization on the electrode surface, determination of the pzc, hybridization yields, reproducibility)and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/anie.201506672.

method. Even though ssDNA-modified surfaces are the foundation for the envisaged applications, the mechanism of ssDNA immobilization has not been not fully elucidated. DNA immobilization is a complex process depending on many parameters such as ionic strength, strand length, and the valence of the ions screening the charge of the DNA strands.[12] By introducing varying applied potentials, this process becomes even more complex. While it is known that certain potentials (with respect to the pzc) induce the bending or repelling of DNA at the electrified interfaces, the reasoning that the DNA itself is attracted by the positively charged electrode (or repelled by a negatively charged electrode) is in our opinion not justified.

With the aim of high pDNA coverage and fast kinetics, pDNA immobilization is often performed in solutions of high ionic strength. However, the Debye length, which describes the decay of the applied potential in the electrolyte solution in front of the electrode, substantially decreases with increasing ionic strength. Therefore, under conditions of high ionic strength a significant potential drop occurs in the immediate proximity of the electrode.[13] Evidently, only a small fraction of a DNA strand in the vicinity of an electrified interface can be affected by the potential. DNA is a highly negatively charged polyelectrolyte that strongly interacts with ions resulting in a charge compensation, the so-called DNA screening as first described by the ion condensation theory introduced by Manning.[14] In the case of salts composed of monovalent ions the charge at a DNA strand is screened by condensed counterions accumulating around the DNA strand and additionally by ions in a second sphere.[15] Therefore, due to the absence of any effective net charge, a DNA strand cannot be directly affected by the applied potential as generally suggested. This raises the question of whether also uncharged molecules can be influenced by suitable applied potential pulse sequences. Surprisingly, applying potential pulses to a gold Au electrode in the presence of MCH substantially accelerates the formation of a compact MCH monolayer (Supporting Information (SI); Section S2).

Although the effect of varying applied potentials may be different for MCH and DNA strands, these findings imply that the applied potential pulses neither attract nor repel DNA strands directly, but rather affect the ions in the vicinity of the electrified interface. Evidently, during the charging of the electrochemical double layer in front of the electrode, ions have to rearrange both in the inner and outer Helmholtz plane as well as in the diffuse layer. Thus, when the electrode potential is switched to negative values with respect to pzc, cations move towards the electrified interface while anions move towards bulk and vice versa. Evidently, this effect exceeds the Debye length in front of the electrified interface. Switching between these two situations creates a "stirring effect" that moves molecules present in close proximity to the electrode surface including their condensed ion cloud or in the case of uncharged molecules their solvation shell (Scheme 1).

Due to the short distance from the electrode surface over which the applied potentials have an effect, a small fraction of a DNA strand is pulled down to the charged electrode surface. This brings the remaining part of the DNA strand, which was located outside of the influence of the charge at the electrified interface, closer to the electrode surface. Ultimately, repetition of this process results in a sequential attraction of the complete DNA strand to the electrode surface during a single positive potential pulse, which is only few milliseconds long. This zipper-type attraction of a complete DNA strand towards the electrode surface is followed by a jump to a potential which is negative relative to the pzc. Once an appropriate negative potential is applied, anions in the vicinity of the electrode are repelled along with the net-negatively charged DNA backbone that was left unscreened and the DNA strand is lifted up to an upright orientation, thus creating space for new strands to approach the surface.



Scheme 1. Proposed model of the potential-assisted DNA immobilization process. Switching to potentials positive relative to pzc invoked the movement of anions towards the electrode surface and cations towards the bulk, leading to a stirring effect. A small fraction of the cations surrounding the DNA in close proximity to the electrode are repelled, concomitantly decreasing charge screening of the DNA strands. A small part of the DNA strand is pulled towards the surface simultaneously bringing the rest of the DNA strand even closer to the electrode. This results in a stepwise and sequential attraction of the whole DNA strand until it lies flat on the electrode surface. Negative potentials repel negative ions in the vicinity of the electrode together with the slightly negatively charged DNA, and the DNA moves to an upright orientation. Repeated switching between positive and negative potentials causes a stirring effect, which traps new DNA strands thus accelerating the immobilization kinetics.

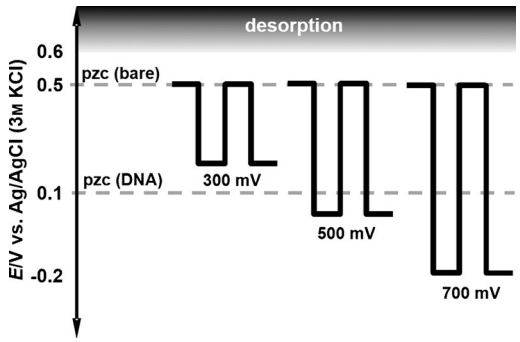
Due to the attraction of the complete DNA strand to the Au surface, at sufficiently long potential pulse times regardless the orientation of the DNA strand, the anchoring groups (a thiol or disulfide) are always pulled to the Au surface which facilitates the formation of the gold–sulfur bond. Repetitive switching between positive and negative potentials increases the amount of immobilized DNA. This suggests that potential pulsing evokes a stirring effect caused by ion movements facilitating the approach of DNA strands in close vicinity to the Au surface.

The proposed model explains the role and the importance of switching between positive and negative potentials with respect to the pzc during the potential-assisted DNA immobilization. However, the important question concerns the value of the pzc during DNA immobilization. To the best of our knowledge, previous studies suggesting the manipulation of DNA by application of a potential refer to the pzc of the bare electrode and the applied potentials were chosen with

respect to this value. However, it is known that the pzc is very sensitive to ion adsorption and surface modification.[16] Moreover, the pzc of polycrystalline Au is very sensitive to surface impurities.[17] Hence, we determined the pzc of the bare and of a DNA-modified electrode (pzc(DNA)) in the same solution in which DNA immobilization was performed.

According to the Gouy–Chapman–Stern model of the double layer, the differential capacitance (C_d) consists of two contributions, that is, the capacitance of the compact layer (C_H) and the capacitance of the diffuse double layer (C_{ddl}). While C_H depends neither on the ionic strength nor on the potential, C_{ddl} depends on both under certain conditions. At very low ionic strength and with the potential approaching pzc, C_d is mainly determined by C_{ddl} and has a minimal value at pzc (see SI; Section S3 for the detailed description of the determination of the pzc). We show that in the presence of DNA the pzc shifts towards more negative potentials, as predicted by the Gouy–Chapman–Stern model, due to the presence of specifically bound anions. Therefore, in order to predefine optimal potentials for DNA immobilization it is of high importance to take this shift of the pzc into account. The pzc shifts from 0.5 V for pzc(bare) to 0.1 V for pzc(DNA).

To unequivocally demonstrate the importance of selecting appropriate immobilization potentials of adequate amplitude and by this to satisfy one of the conditions that our model demands we compare three potential pulse profiles. Using the same upper potential (+0.5 V corresponding to pzc(bare)) and varying the lower potential, potential pulse sequences with three potential differences (ΔE) of 0.3, 0.5, and 0.7 V were defined. In Scheme 2 the potential differences are



Scheme 2. Selected potential pulse profiles with respect to pzc(bare) and pzc(DNA): $\Delta E = 0.3$ V (pulse profile +0.5/+0.2 V), $\Delta E = 0.5$ V (pulse profile +0.5/0 V), and $\Delta E = 0.7$ V (pulse profile +0.5/−0.2 V).

shown with respect to pzc(bare) and pzc(DNA). For $\Delta E =$ 0.3 V (pulse profile: +0.5/+0.2 V) both potentials are always positive with respect to pzc(DNA). For $\Delta E = 0.5$ V (pulse profile: +0.5/0 V) pulsing occurs between positive and slightly negative potentials and for $\Delta E = 0.7$ V (pulse profile: +0.5/−0.2 V) both positive and negative potentials exhibit roughly similar amplitude with respect to pzc(DNA).

If the applied potentials are both positive with respect to pzc(DNA) using the pulse profile +0.5/+0.2 V, after an initial immobilization the pDNA strands remain lying down on the electrode surface. Upon initial immobilization of only a small amount of DNA strands, the pzc of the electrode shifts down from pzc(bare) to pzc(DNA) preventing ion stirring and hence facilitating DNA immobilization. The electrode surface is physically blocked by the initially immobilized pDNA strands and access of new DNA strands is impeded.

This explains the poor immobilization efficiency for this potential pulse profile expressed as only a small increase in the charge transfer resistance (R_{ct}) of the pDNA/MCH electrode compared to that of an electrode modified only with MCH (Figure 1, ○). R_{ct} was derived from faradaic electrochemical impedance spectra in the presence of equi-

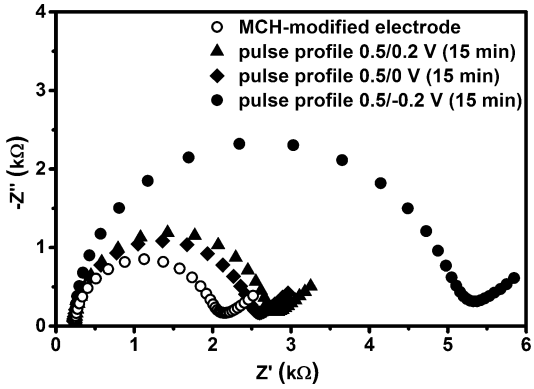


Figure 1. Comparison of the effect of the applied potential pulse profiles on R_{ct} using EIS. Nyquist plots show representative R_{ct} values for a MCH-modified electrode (○) and pDNA/MCH-modified electrodes obtained using different potential pulse profiles: $\Delta E = 0.3$ V (pulse profile: +0.5/+0.2 V; ▲); $\Delta E = 0.5$ V (pulse profile: +0.5/0 V; ◆), and $\Delta E = 0.7$ V (pulse profile: +0.5/−0.2 V; ●). Experimental conditions: pDNA immobilization: 10 mM phosphate buffer (PB), 450 mM K_2SO_4 containing 1 μM pDNA; MCH immobilization: overnight incubation in 10 mM MCH solution containing 10 mM PB, 20 mM K_2SO_4; EIS: 10 mM PB, 20 mM K_2SO_4 with equimolar concentrations of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ (5 mM); DC potential: +220 mV vs. Ag/AgCl/3 M KCl; AC perturbation of 10 mV_{pp} amplitude. Frequency range from 30 kHz to 10 mHz.

molar concentrations of $[Fe(CN)_6]^{3-/4-}$. A similar result is obtained for the pulse profile +0.5/0 V (Figure 1, ◆). Although ΔE is higher, the applied negative potential is apparently not sufficiently low to repel the initially bound pDNA strands. Therefore, the access of new strands to the electrode is impeded and poor immobilization yields are observed.

Further increase of the potential difference to $\Delta E = 0.7$ V leads to a significant improvement of the immobilization yield for pDNA (Figure 1, ●) due to the increased ion fluxes imposed during potential changes and the concomitantly improved stirring effect. It should be noted that although the potential drop is more pronounced at higher applied potentials, the absolute potential value is still higher.

DNA grafting by potential-assisted immobilization occurs by a sequential, zipper-like attraction of a DNA strand towards the electrified interface at potentials positive relative to pzc(DNA). Therefore, to achieve the highest immobilization efficiency the duration at which a certain potential is applied needs to be long enough to allow the trapping of the

complete DNA strand, including the anchoring group regardless of the orientation of a DNA strand. Rant et al.[18] reported that only about 250 μs is needed for the transition of a grafted DNA strand from the reclined to the upright orientation. Hence, to satisfy the second condition of our model and to find the optimal potential duration for DNA grafting and achieving the highest immobilization efficiency, we investigated the impact of different pulse durations on DNA immobilization efficiency (Figure 2). pDNA immobilization

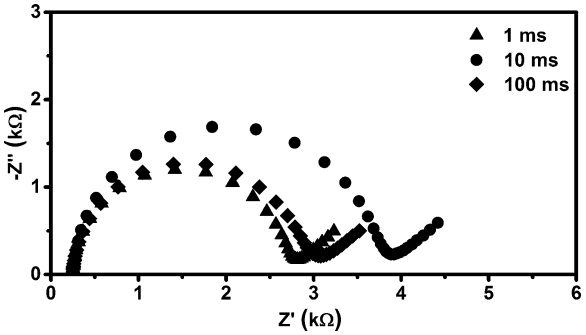


Figure 2. Effect of the potential pulse duration on the immobilization efficiency. EIS measurements were conducted using pDNA/MCH-modified electrodes. Immobilization was performed for 5 min using the +0.5/−0.2 V pulse profile with pulse durations of 1 (▲), 10 (●), and 100 ms (◆). All conditions are the same as in Figure 1.

was performed for 5 min using the +0.5/−0.2 V pulse profile and varying the pulse duration (1, 10, and 100 ms).

Decreasing the pulse time increases the number of cycles during the overall immobilization time of 5 min from 1500 potential pulse cycles for 100 ms to 15000 for 10 ms and 150000 for 1 ms. The highest pDNA immobilization yield, that is, the highest value for R_{ct} is observed for a pulse time of 10 ms, a time that is apparently long enough for the complete DNA strands to be pulled down, hence allowing the formation of the Au–S bond and simultaneously short enough to allow for a high enough number of potential pulse cycles.

After optimizing the potential-assisted immobilization method we compared it to the commonly used incubation immobilization method. Using the incubation method for pDNA immobilization, the grafting of additional pDNA strands becomes energetically unfavorable with an increasing amount of pDNA already immobilized at an electrode surface due to the increasing electrostatic repulsion between the strands. Hence, the incubation method requires between a couple of hours to several days depending on the envisaged DNA coverage. The proposed potential-assisted immobilization method overcomes this limitation and significantly accelerates the immobilization process by the pulse-type modulation of the potential applied to the electrode surface during the immobilization due to the ion-stirring effect.

Using the +0.5/−0.2 V pulse profile (pulse duration 10 ms) a surface coverage of $(6.85 \pm 0.47) \times 10^{12}$ molecules cm^{-2} was obtained in only 15 min (Figure 3), which is known to be within the optimal range of pDNA coverage for applications such as in DNA sensors.[19,20] Lower

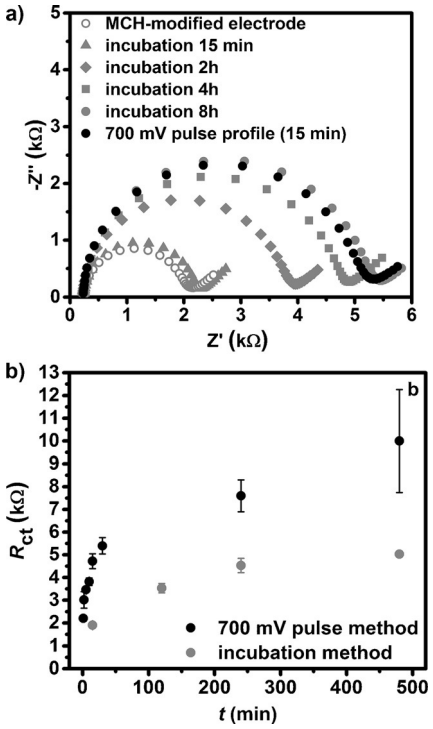


Figure 3. Comparison of pDNA immobilization by the potential-assisted and incubation methods. a) Nyquist plots with representative R_{ct} values gained for a MCH-modified electrode (○) and pDNA/MCH-modified electrodes obtained by incubation for 15 min (▲), 2 h (◆), 4 h (■), and 8 h (●) and by application of the +0.5/−0.2 V pulse profile (pulse duration 10 ms) for 15 min (●). Same conditions as in Figure 1. b) Comparison of the pDNA immobilization rates using the incubation method (●) and the +0.5/−0.2 V pulse profile (pulse duration 10 ms; ●).

coverages decrease the sensitivity of DNA hybridization sensors and higher coverages are considered to be detrimental for hybridization. A dependence of the ratio of R_{ct} of the hybridized and the pDNA/MCH-modified surface on the pulse immobilization time is shown in SI, Section S4. Using the incubation method, only a negligible R_{ct} increase for pDNA/MCH after 15 min is observed with respect to the MCH-modified electrode taken as a measure for the pDNA immobilization yield (Figure 3a, ▲). Immobilization for 2 h results in $(4.65 \pm 0.26) \times 10^{12}$ molecules cm^{-2} (Figure 3a, ◆); this coverage is 47% lower than that obtained after 15 min immobilization using the potential-assisted method. Prolonging the incubation time leads to a significant decrease of the pDNA immobilization rate due to the increased electrostatic repulsion between the pDNA strands (Figure 3b). Hence, pDNA immobilization by incubation requires at least 4–8 h to reach a similar pDNA surface coverage as that obtained by 15 min potential-assisted immobilization (Figure 3). Potential-assisted pDNA immobilization decreases the time for reaching optimal pDNA surface coverage by a factor of about 30. Furthermore, we show that the proposed method also leads to much higher pDNA coverages, as presented in Figure 3b. High DNA coverage may be valuable for applications such as the investigation of DNA repair proteins.[21]

The potential-assisted pDNA immobilization protocol leads to a highly reproducible immobilization with the ability to pre-select the envisaged pDNA surface coverage by choosing the number of applied potential pulses. The R_{ct} value after pDNA immobilization which is related to the surface coverage is obtained with a standard deviation of below 5% (SI, Section S5).

We believe that understanding of the behavior of pDNA in front of an electrified interface is a fundamental prerequisite to a highly reproducible and fast pDNA immobilization strategy. Evidently, the changing properties of the electrified interface itself induced during the course of the immobilization process, that is, the change of the pzc with the presence of immobilized DNA, is of great importance for selecting the required pulse potentials. Moreover, the attraction/repulsion of the polyelectrolyte DNA itself by the electric field in front of the electrified interface is very unlikely due to the short Debye length at high ionic strength. We therefore propose that ion movement and the concomitant stirring effect in the electrochemical double layer is the reason behind the improved immobilization kinetics. The proposed potential-assisted immobilization strategy using rationally chosen pulse potentials may become a new standard pDNA immobilization method to obtain highly reproducible DNA-modified surfaces with the desired surface coverage in a very short time. This is a prerequisite for the development of highly sensitive and reproducible DNA hybridization assays with electrochemical read-out.

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