Chapter 11

DNA Sensors Based on Mixed Self-Assembled DNA/Alkanethiol Films

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Abstract

When designing DNA biosensors, the immobilization of specific DNA probes is one of the most essential parts. Unfortunately, many of the existing strategies (e.g., adsorption, complexation, and entrapment) can only be used on standard microscope slides, while for more tailored surfaces alternative strategies are still required. In the case of gold surfaces, the self-assembly of mixed DNA/alkanethiol films is a very common approach to directly couple single-stranded DNA probes. The quality of these mixed films greatly depends on different parameters including the sensitivity and the detection limit. We have shown a positive relation between the length of the used carbon spacer and the general performance of the DNA biosensor. In this chapter an extended protocol for the controlled immobilization and subsequent hybridization of DNA is described.

Key words: DNA biosensor, surface chemistry, alkanethiol, SPR, DNA immobilization, DNA hybridization.

1. Introduction

For most biosensor applications the efficient and reproducible coupling of DNA is one of the most essential parts. However, the coupling depends to a great extent on the surface type. The leading methods for glass slides to synthesize DNA probes on site are still not fully adapted to other surfaces and often alternative DNA-coupling strategies are required (1, 2). In this chapter we will focus on the immobilization of single-stranded DNA

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fragments on gold surfaces for which thiolated DNA probes in combination with alkanethiols remain the method of choice. The method is usually performed in two steps. In a first step, thiolated DNA molecules will directly couple to the surface by means of self-assembly (3), while in the second step alkanethiol backfilling will improve the final order and the stability of the formed DNA-layers. This combination has proven to positively affect subsequent hybridization steps (4, 5). The length of the carbon spacer of both DNA probes and backfilling molecules plays an important role herein. By the use of an 11-carbon long spacer (i.e., mercapto-undecyl or C_{11}), instead of the generally used 6-carbon long spacer (i.e., mercaptohexyl or C_6), we have confirmed the positive relation between the spacer length and the general biosensor performance (6). To validate this relation, parameters such as the sensitivity and the detection limit need to be determined. These parameters can easily be calculated based on the immobilization and hybridization steps, monitored in real-time with SPR (7). Since commercial SPR devices make use of an integrated flow system (i.e., IFC microfluidic chip), they allow the comparison of a flow-mediated immobilization approach to a diffusion-controlled approach. Hence, the impact of the immobilization approach on the biosensor performance can be estimated (*see* **Section 3.4**). In this chapter, a general protocol for the reproducible immobilization of mixed DNA/alkanethiol films and the following hybridization steps is described. An alternative protocol for the diffusion-controlled immobilization is included as well.

2. Materials

2.1. Buffers and Solutions

1. A 5′-thiolated DNA probe (25 nt) with C_6 (Eurogentec, Belgium) or C_{11} spacer (*see* **Note 1**). The probes with C_{11} spacer were synthesized by Dr. Van Aerschot (Rega Institute, Belgium) (8). The spacers (*see* **Fig. 11.1**) are inserted to link the thiol moiety to the 5′-end of the probe.
2. A complementary (25 cp) and non-complementary (25 np) probe (*see* **Note 1**).
3. Backfilling molecules: 6-Mercapto-1-hexanol (MCH, Sigma, USA) and 11-mercapto-1-undecanol (MCU, Sigma, USA).
4. Immobilization buffer: 1 M KH_2PO_4, pH 3.8 (*see* **Note 2**).
5. Hybridization buffer or running buffer: 1 M NaCl, 10 mM Tris–HCl, 2 mM EDTA, pH 7.0.
6. Regeneration solution: 2.5 mM HCl or 20 mM NaOH.

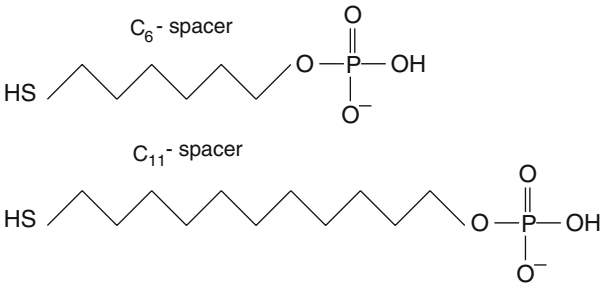

Fig. 11.1. Chemical structure of the short traditional C_6 and the longer C_{11} spacer. These two spacers are coupled to the 5′thiol moiety to separate the 25nt probe from the surface (See 2.1 buffers and solutions).

2.2. Devices

1. Unmounted gold-coated surfaces and separate chip supports (SIA Kit Gold, GE Healthcare, UK) (*see* **Note 3**).
2. A homemade UV/O_3 device (*see* **Fig. 11.2**) containing a mercury grid lamp to clean the SPR samples (*see* **Note 4**).
3. 24-Well microplate containers for the diffusion-controlled immobilization (BD Falcon™, BD Biosciences, USA).
4. Microplate shaker (Thermomixer, Eppendorf, Germany) for homogenous immobilization.
5. SPR system (Biacore™ 2000, GE Healthcare, UK) for real-time monitoring.

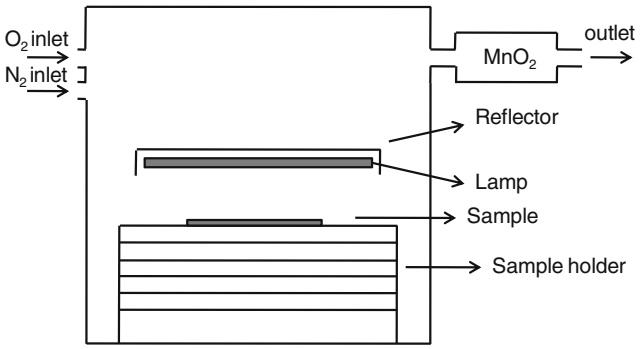

Fig. 11.2. Schematic illustration of the IMEC homemade UV/O_3 cleaning device.

3. Methods

3.1. Sample Preparation

For reproducible immobilizations, the quality and the cleanliness of the gold-coated surfaces are essential (9). The surface cleaning, handling (i.e., mounting of the gold surface on the chip support

and insertion of the chip in the SPR device) as well as the device settings used are described.

1. Clean the gold surfaces by UV/O_3 for 15 min.
2. In the case of a flow-assisted immobilization, mount the gold surface on a chip support and insert the chip in the SPR system according to the instructions of the manufacturer. For the diffusion-controlled approach (*See* **Section 3.4**), functionalize the gold surface with the thiolated DNA before mounting it on the chip support.
3. Set the temperature (25°C) and the flow rate (5 μL/min) constant during the entire SPR run. Before immobilization of thiolated 25 nt DNA probes, inject running buffer to set a baseline (*See* **Note 5**) (See **Fig. 11.3**) .

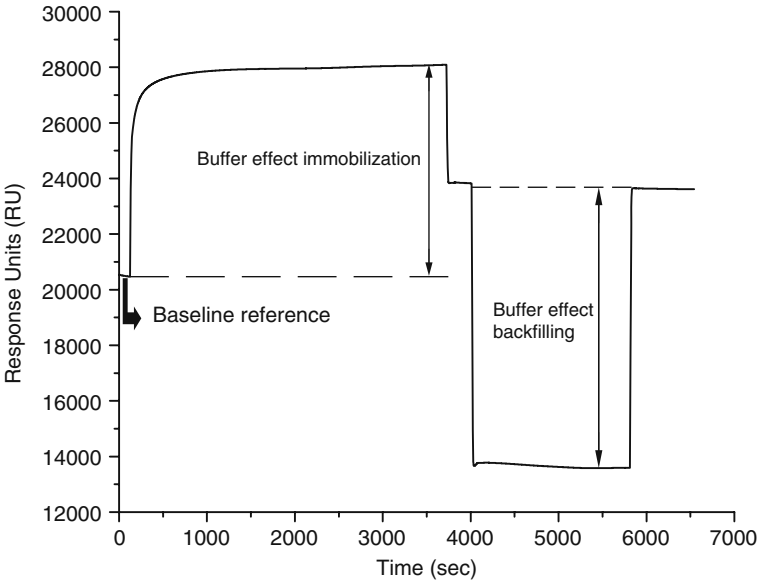

Fig. 11.3. By using buffers with different refractive indices, with respect to the running buffer, buffer effects appear. In case of the immobilization this is shown as an increase in signal whereas for the backfilling a decrease in signal is observed.

3.2. Determination and Optimization of the Probe Concentration

The amount of immobilized probes is a crucial element for further hybridization steps. By testing and comparing different concentrations of thiolated DNA during immobilization, an optimal probe concentration can be found. Based on these results a standard probe concentration can be determined for use in future tests.

1. Prepare a 1:10 dilution series in immobilization buffer, ranging from 1 fM to 10 μM, of thiolated DNA probes with C_6 spacer and test all these concentrations individually.
2. Inject the thiolated probe solution for 1 h.

3. Rinse the surface with running buffer (i.e., ExtraClean step in the Biacore software) to wash away all unbound probe and to set a new baseline.
4. Dissolve 1 mM MCH backfilling in water, sonicate for 15 min, and inject this solution over the sample for 30 min. After the backfilling insert again an ExtraClean step.
5. Determine the changes in resonance units (RU) after both the injection of the thiolated probe and the MCH backfilling step as shown in **Fig. 11.4a**.
6. Prepare different concentrations of 25 cp, ranging between 10 and 320 nM, in hybridization buffer and inject each concentration randomly in the system for 10 min.
7. After each hybridization step inject 2.5 mM of HCl for 2 min to regenerate the surface. Alternatively, 20 mM NaOH can be used (*see* **Note 6**).
8. Determine the RU changes after each hybridization step. The relation between the used probe concentration and the hybridization is presented in **Fig. 11.4b** (*see* **Note 7**).

3.3. Immobilization and Hybridization of DNA on Gold: General Protocol

Based on the probe optimization as described above, a general immobilization protocol has been designed. This protocol can function as a starting point during experiments for which a controlled immobilization of thiolated DNA is required. By following this protocol various parameters, affecting the biosensor performance, can be calculated. As such, the relation between the spacer length and the biosensor behavior was determined. In addition to the immobilization, the protocol describes the subsequent hybridization steps on the mixed DNA/alkanethiol films.

1. Prepare 1 μM of thiolated probe, either with C_6 or with C_{11} spacer, in immobilization buffer and inject this solution for 1 h in the system.
2. Rinse the surface following the ExtraClean procedure.
3. Dissolve 1 mM MCH in water or 1 mM MCU in a mixture of 90:5 water/ethanol and sonicate (*see* **Note 8**). Use MCH for surfaces prepared with the C_6 spacer and MCU for surfaces prepared with the C_{11} spacer. Inject the backfilling solution for 30 min.
4. Rinse the surface following the ExtraClean procedure.
5. Determine the RU changes after deposition of the mixed DNA/alkanethiol film and calculate the immobilization degree using following conversion factor: 1,000 RU $\approx$ 100 ng/cm^2 (10) (*see* **Table 11.1**).
6. Prepare different concentrations of 25 cp in hybridization buffer. For the short C_6 spacer concentrations between 10 and 320 nM are used (*see* **Section 3.2**; step 6), whereas for

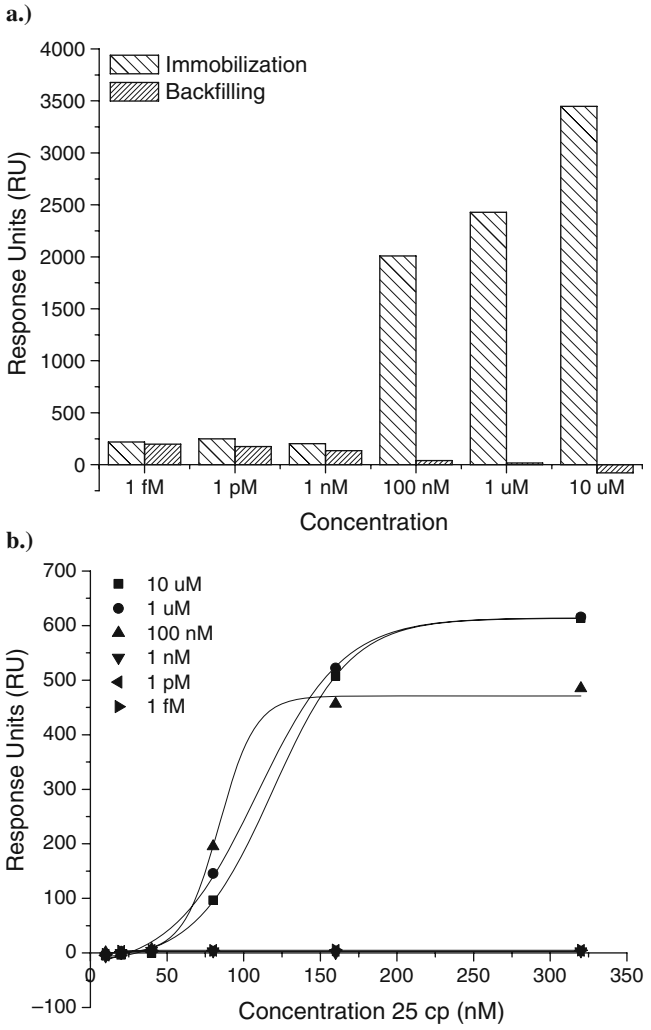

Fig. 11.4. (**a**) Comparison of the RU changes obtained after 1 h of immobilization for different concentrations of thiolated 25 nt probe (i.e., 1 fM–10 μM) and after post-treatment of the surface with 1 mM MCH backfilling. Based on their molar mass, only a limited exchange between the probes and the backfilling takes place for concentrations >1 nM of DNA (i.e., 100 nM, 1 μM, and 10 μM). When the lower probe concentrations are used a clear RU increase (i.e., increase in mass) is seen after backfilling. This clearly indicates that the immobilized DNA layer is less dense. (**b**) Hybridization of 25 cp on the different DNA layers with positive signals for the three highest concentrations, i.e., 100 nM, 1 μM, and 10 μM.

the C_{11} spacer concentrations between 0.625 and 320 nM are used. For the 25 np only prepare a concentration of 320 nM.

7. Inject each concentration of 25 cp randomly on the mixed DNA/alkanethiol layer for 10 min and regenerate the surface after each hybridization step using 2.5 mM HCl

Table 11.1
Calculated immobilization and hybridization degrees at saturation for the short C_6 and long C_{11} spacer. The hybridization of the 25 np is negligible and therefore not included

Spacer	Immobilization ($\times 10^{13}$ molecules/cm^2)	Hybridization ($\times 10^{12}$ molecules/cm^2)
C_6	2.0 ± 0.2	4.7 ± 0.19
C_{11}	2.3 ± 0.02	5.8 ± 0.02

(*see* **Section 3.2**; step 7). Test the non-complementary 25 np fragment in a separate step.

8. Determine the RU changes after hybridization of both the 25 cp and 25 np and calculate the hybridization degree at saturation (*see* **Note 9**) as explained for the immobilization. *See* **Table 11.1** for the obtained values.
9. Linearly fit the dose–response hybridization curves using the Origin Data Analysis software (Linear Fit, Origin v 6.0, OriginLab, USA) and determine the sensitivity, the detection limit, and the dynamic range (*see* **Note 10**). **Figure 11.5** gives an example of such a hybridization curve whereas **Table 11.2** contains the calculated values.

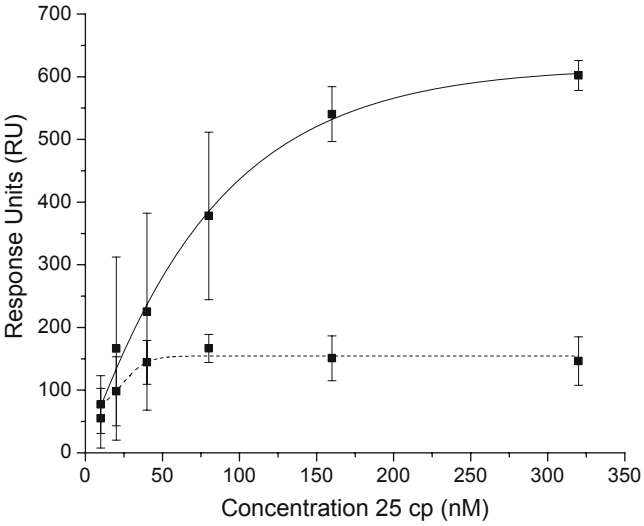

Fig. 11.5. Example of fitted dose–response hybridization curves using thiolated probes with C_6 spacer for the flow-assisted (*solid line*) and the diffusion-controlled immobilization approaches (*dashed line*). This figure clearly shows the impact of the immobilization approach.

Table 11.2
Summary of the sensitivity, the detection limit, and the dynamic range, calculated for both the flow-assisted and diffusion-controlled (indicated by no flow) immobilization of thiolated 25 nt probes.

Spacer	Sensitivity[a] (RU/nM)	Detection limit[a] (nM)	Dynamic range (nM)
C_6	3.6 ± 2.1	21.6 ± 1.9	21.6–160
C_{11}	99.2 ± 13.7	0.3 ± 0.2	0.3–20
C_6, no flow	2.3 ± 0.9	12.9 ± 31.0	12.9–40
C_{11}, no flow	4.7 ± 0.2	8.4 ± 0.71	8.4–40

[a]The standard deviations were determined based on the average of the individual experiments.

3.4. Diffusion-Controlled Immobilization

Besides the length of the carbon spacer, the immobilization approach itself can have a great impact on the biosensor performance. Since this aspect can easily be tested using the SPR device a protocol for the diffusion-controlled immobilization is given below. By comparing hybridization on the surfaces, prepared according to the two described protocols (i.e., flow-assisted and diffusion-controlled), the effect of the immobilization approach can be estimated as is done here for again both spacers.

1. Prepare 1 μM thiolated probe, with either a C_6 or a C_{11} spacer, in immobilization buffer.
2. Incubate the gold-coated surfaces overnight at 25°C in 500 μL of the DNA solution while continuously shaking.
3. Rinse the DNA-functionalized surfaces thoroughly with water, dry them under a flow of nitrogen, and finally mount them on the chip support.
4. From this point on, follow steps 3 to 9 as described in **Section 3.3**.

4. Notes

1. As an example we have studied a 25-nucleotide (nt) long fragment of an *Escherichia coli stx2* gene (AF461171). However, the protocol is also valid when using other sequences with different lengths. The exact sequences used in this chapter are:
   25 nt (5′-TTCACAGGTACTGGATTTGATTGTG),
   25 cp (5′-CACAATCAAATCCAGTACCTGTGAA),
   25 np (5′-GTGCTCAGTTGACAGGAATGACTGT).

2. All buffer solutions (i.e., immobilization, hybridization, and running buffer) were stored at 4°C and were degassed and filtered before use.
3. The combination of unmounted gold surfaces and separate chip supports allows the easy assembly of the SPR sensor chips after cleaning and/or coating of the surface.
4. **Figure 11.2** includes a schematic illustration of our homemade UV/O_3 device. The device has an O_2 inlet to elevate the amount of O_2 in the chamber as well as a N_2 inlet to flush away the O_3 (after cleaning). In addition manganese(IV)dioxide (MnO_2) is used to reduce the toxic O_3 to O_2 before releasing it into the atmosphere. The used mercury grid lamp (BHK Inc., USA) emits both the wavelengths 185.0 and 253.7 nm which allow the following reactions, responsible for the sample cleaning, to occur:

$$O_2 + O^* \xrightarrow{185\text{nm}} O_3$$
$$O_3 \xrightarrow{253.7\text{nm}} O_2 + O^*$$

   During this photosensitized cleaning mechanism, the contaminant molecules are excited and/or dissociated by absorption of short-wavelength UV light. Simultaneously, atomic oxygen is generated when O_2 is dissociated by the absorption of UV with a wavelength of 185 nm and when O_3 is dissociated by the absorption of the 253.7 nm and longer wavelengths of radiation. The products of the excitation of contaminant molecules react with atomic oxygen to form simpler volatile molecules such as CO_2, H_2O, N_2. This combination of short-wavelength UV light and O_3, as used in our homemade device, has shown to produce clean surfaces about 200–2,000 times faster than UV light or O_3 alone (11).
5. SPR is sensitive to changes in refractive index causing the SPR angle to change, for instance as a result of using different buffers. To limit these buffer effects, when hybridizing different concentrations of 25 cp on the same chip and to calculate RU changes the hybridization buffer is chosen as running buffer. The buffer effects thus only appear during all other SPR steps (i.e., immobilization, backfilling, and regeneration). **Figure 11.3** clearly demonstrates the buffer effects seen during immobilization and backfilling.
6. The regeneration of the DNA surfaces is studied by hybridization of the 25 cp followed by a regeneration step with either 2.5 mM HCl or 20 mM NaOH. Based on earlier results (not shown), only a negligible decrease (i.e., on

average 2% for each cycle) in hybridization efficiency was observed for both regeneration solutions. As a result different hybridization experiments can be performed on the same sample.

7. A probe concentration of 1 μM was chosen as optimal probe concentration since maximum hybridization signals were observed using this concentration. In addition, hybridization performed on these DNA layers was more reproducible than using lower concentrations.
8. Because the use of >70% ethanol is not compatible with the integrated fluidic system (IFC) of the Biacore™ 2000 device, the MCU backfilling solution is first dissolved in a minimal amount of ethanol. The final solution is a water/ethanol mixture.
9. As saturation appears for hybridization at 320 nM of 25 cp, the amount of hybridized probes is only calculated for this concentration.
10. For each concentration of 25 cp and 25 np the corresponding RU changes are plotted in a dose–response curve. The slope of these hybridization curves is taken as the sensitivity (S), whereas the detection limit (DL) can be calculated using following formula:

$$\mathrm{DL} = \frac{\left|(3\,N) - I\right|}{S}$$

with N the noise and I the intercept in RU.

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