Biosensors and Bioelectronics 24 (2008) 72–77

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Impact of spacers on the hybridization efficiency of mixed self-assembled DNA/alkanethiol films

Sara Peeters[a,b,*], Tim Stakenborg[a,c], Gunter Reekmans[a], Wim Laureyn[a], Liesbet Lagae[a], Arthur Van Aerschot[d], Marc Van Ranst[b]

[a] IMEC vzw, Nano Engineered Component Science Research, B-3000 Leuven, Belgium
[b] KULeuven, Department of Medical Diagnostic Sciences, B-3000 Leuven, Belgium
[c] Veterinary Research Institute (VAR), B-1180 Brussels, Belgium
[d] KULeuven, Department of Pharmaceutical Sciences, Rega Institute, B-3000 Leuven, Belgium

ARTICLE INFO

Article history:
Received 25 October 2007
Received in revised form 26 February 2008
Accepted 17 March 2008
Available online 22 March 2008

Keywords:
Biosensor
Surface chemistry
Alkanethiol
SPR
Immobilization
DNA hybridization

ABSTRACT

The immobilization of DNA strands is an essential step in the development of any DNA biosensor. Self-assembled mixed DNA/alkanethiol films are often used for coupling DNA probes covalently to the sensor surface. Although this strategy is well accepted, the effect of introducing a spacer molecule to increase the distance between the specific DNA sequence and the surface has rarely been assessed. The major goal of this work was to evaluate a number of such spacers and to assess their impact on for example the sensitivity and the reproducibility. Besides the commonly used mercaptohexyl (C_6) spacer, a longer mercapto-undecyl (C_{11}) spacer was selected. The combination of both spacers with tri(ethylene)glycol (TEG) and hexa(ethylene)glycol (HEG) was studied as well. The effect of the different spacers on the immobilization degree as well as on the consecutive hybridization was studied using surface plasmon resonance (SPR). When using the longer C_{11} spacer the mixed DNA/alkanethiol films were found to be more densely packed. Further hybridization studies have indicated that C_{11} modified probes improve the sensitivity, the corresponding detection limit as well as the reproducibility. In addition two different immobilization pathways, i.e. flow vs. diffusion controlled, were compared with respect to the hybridization efficiency. These data suggest that a flow-assisted approach is beneficial for DNA immobilization and hybridization events. In conclusion, this work demonstrates the considerable impact of spacers on the biosensor performance but also shows the importance of a flow-assisted immobilization approach.

1. Introduction

The discovery of numerous genetic loci related to complex diseases, the rapid spreading of infectious agents, the increasing quality standards for food production and consumption and the necessity to detect genetic-modified organisms are just a few examples of what has led to complete new challenges in molecular diagnostics (Chen et al., 2004; Castillo et al., 2004; Deisingh and Thompson, 2004; Andreotti et al., 2003; Bashir, 2001). As a result, the interest and research in new and sensitive diagnostic tools, such as DNA biosensors, has increased dramatically (Soper et al., 2006; Hahn et al., 2005). Within this growing field, the immobilization of single-stranded DNA (ssDNA) probes and the subsequent hybridization with their target sequences have proven to directly impact the biosensor performance (Mannelli et al., 2005; Yao and Tan, 2004; Wang, 2000). Therefore, it is important to further study and optimize both aspects into detail.

Thiolated ssDNA oligonucleotides are commonly used to immobilize DNA onto gold substrates via self-assembly (Herne and Tarlov, 1997). This immobilization approach is most often performed in combination with alkanethiol "backfilling" molecules (e.g. 6-mercapto-1-hexanol or 11-mercapto-1-undecanol). These latter molecules reduce the overall density of the immobilized DNA layer by displacing weakly bound thiolated ssDNA probes in a time-dependent manner (Gong et al., 2006; Satjapipat et al., 2001; Steel et al., 2000; Levicky et al., 1998). This results in a better accessibility of the surface-confined probes towards their target. Despite the extensive study of this mixed DNA/alkanethiol approach, little is known about the impact of spacers on the biosensor performance. It is generally accepted that the further an immobilized molecule is away from the surface the closer it is to the solution state and the more likely it is to react freely with dissolved molecules (Ricci et al.,

* Corresponding author at: IMEC vzw, Nano Engineered Component Science Research, NEXT, Kapeldreef 75, B-3001 Leuven, Belgium. Tel.: +32 16 288516; fax: +32 16 281097.
E-mail address: araspeeters@hotmail.com (S. Peeters).

0956-5663/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2008.03.012

2007; Halperin and Buhot, 2006; Wong et al., 2005; Shchepinov et al., 1997). Therefore, in this work, the alkane chain length was varied beyond the commonly used C_6 spacer. Besides the impact of the length, the influence of adding supplementary poly(ethylene)glycol (PEG) units in the spacer was examined as well. These molecules are highly hydrophilic and ensure additional chain flexibility. At the same time a flow-assisted immobilization method was compared to a more diffusion-controlled approach.

The in-depth study on the effect of spacers was performed using a surface plasmon resonance (SPR) system (Jonsson et al., 1991). To allow a more quantitative estimation of the surface coverage, additional methods based on quartz crystal microbalance (QCM) and fluorescence were used as well (Castelino et al., 2005; Cho et al., 2004; Wang et al., 2004; Demers et al., 2000).

2. Materials and methods

2.1. Materials

6-Mercapto-1-hexanol (MCH; 97% purity), 11-mercapto-1-undecanol (MCU; 97%), dithiothreitol (DTT) and sodium chloride (NaCl) were all purchased from Sigma–Aldrich (St. Louis, Mo, USA). Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) was obtained from Merck (Rahway, NJ, USA). Ethylenediaminetetraacetic acid (EDTA) was from Fluka (Buchs, Switzerland). Ethanol was purchased from Honeywell (Brussels, Belgium). All other products were supplied by Air products (Brussels, Belgium), unless mentioned otherwise.

2.2. DNA probe sequences and spacers

The 5′ thiol-modified 25-base pair capture probe (25 bp) was selected from the shiga toxin type 2 gene (*stx2*) of enterohaemorrhagic *Escherichia coli* (Genbank accession number: AF461171) (Fraser et al., 2004). This probe, its complement (25 cp) and a non-specific probe (25 np) have the following sequence:

25 bp: 5′-TTCACAGGTACTGGATTTGATTGTG-3′
25 cp: 5′-CACAATCAAATCCAGTACCTGTGAA-3′
25 np: 5′-GTGCTCAGTTGACAGGAATGACTGT-3′

A mercaptohexyl (C_6) or mercapto-undecyl (C_{11}) spacer was used to link a thiol moiety at the 5′ end of a specific DNA sequence via a phosphodiester bond.

In case of the C_6 spacer, the linkage is commonly performed starting from a symmetric disulfide. Hereto, one hydroxyl end is protected via the classic dimethoxytrityl ether and the other end is functionalized as a phosphoramidite allowing automatic assembly on column. Subsequently, the C_6 spacer can be attached via a phosphodiester bond to supplementary hydrophilic PEG units under the form of triethylene glycol (TEG) or hexaethylene glycol (HEG). These PEG moieties are being introduced like regular phosphoramidites after monoprotection of the symmetric diol and the subsequent phosphitylation. All thiolated 25 bp probes bearing a C_6 spacer were purchased from Eurogentec (Wavre, Belgium). A 5′-thiolated probe with C_6-TEG spacer and a 3′Alexa594 fluo label was ordered at DNA Technology A/S (Aarhus, Denmark).

In case of the C_{11} spacer a new synthesis strategy based on the double phosphitylation of the symmetric disulfide was used (Van Aerschot and Rozenski, 2006). This approach is preferable as the use of a symmetric diol is a low yielding reaction. First the disulfide is coupled to solid-supported oligonucleotides. By cleaving this coupled disulfide during deprotection the desired oligonucleotide constructs are obtained. Likewise upon reaction of two oligonucleotide molecules with a single spacer, the dithiothreitol in the deprotection cocktail (1:1 mixture of 30% aq. ammonia:40% aq. methylamine) should provide the desired product. Indeed, after coupling the in-house synthesized bis-(11-hydroxyundecyl)-disulfide to solid-supported oligonucleotides it was cleaved during deprotection resulting in the desired thiolated 25 bp with C_{11} spacer. Similar as for the C_6 spacer, this longer C_{11} spacer was supplemented with TEG and HEG moieties. Since the attachment of the TEG and HEG units generates additional phosphodiester bonds, a new spacer, combining both the lipophilic and hydrophilic part in one molecule, was prepared. Hereto, the 20-hydroxy-12,15,18-trioxa-eicosylsulfide was synthesized, dimerised to its disulfide and subjected to a double phosphitylation reaction. However, no correct product could be isolated. As a consequence, the thiolated 25 bp probes with C_{11}-TEG and C_{11}-HEG spacers were constructed alike the C_6-modified probes.

Both the complete experimental preparation and the obtained mass spectrometric data of the C_{11} spacer prepared via double phosphitylation reaction are provided in the Supplementary Information. The mass spectrometric analysis of the oligonucleotides is included as well. All six spacers used within the scope of this paper, are schematically represented in Fig. 1 .

2.3. Comparative study of the different spacers using SPR

The gold substrates were prepared by depositing 2 nm Ti and 50 nm Au on glass using electron beam evaporation. Before use, they were cleaned for 15 min using a homemade UV/O_3 device containing an ozone producing Mercury Grid Lamp (BHK Inc., Claremont, CA, USA). Afterwards, they were mounted onto a plastic support and docked into the SPR instrument (Biacore™ 2000, GE Healthcare, UK) according to the instructions of the manufacturer. The temperature during the SPR experiments was kept at 25 °C.

For immobilization, 1 μM of the thiolated 25 bp probe was dissolved in immobilization buffer (1M KH_2PO_4; pH 3.8) and injected during 1 h at a constant flow rate of 5 μl/min. Following immobilization, the surface was rinsed with running buffer (1M NaCl, 10 mM Tris–HCl, 2 mM EDTA, pH 7.0) and incubated for 30 min with either 1 mM of MCH or MCU, corresponding to the length of the incorporated C_6 or C_{11} spacer. Before starting the hybridization experiments the surface was rinsed again with running buffer.

All hybridizations were carried out at a flow rate of 5 μl/min. To accurately investigate the effect of the spacers, different concentrations of 25 cp were hybridized for 10 min to the immobilized thiolated 25 bp probes. Concentrations ranging from 10 to 320 nM and from 0.625 to 320 nM were used for the C_6 and C_{11} probes, respectively. For each spacer three independent experiments were run. In order to hybridize the whole concentration range on the same mixed DNA/alkanethiol film, each hybridization cycle was followed by the regeneration of the surface using 2.5 mM HCl during 5 min. This regeneration procedure was followed by a rinsing step with running buffer. Fig. 2a shows a schematic representation of the whole experimental process as described above. All SPR signals (in RU) vs. concentration of 25 cp (in nM) were plotted using a dose–response curve fit. The differences in signal expressed in resonance units or RU, before and after each step, were calculated from the sensograms and were used to estimate the degree of immobilization (Fig. 2b) and hybridization (Fig. 2c). A SPR signal of 1000 RU was reported to correspond to 100 ng/cm^2, as estimated with a radio labeling-based calibration method (Bunimovich et al., 2006). The surface density of the immobilized 25 bp probes and the number of hybridized DNA molecules were calculated using this conversion factor. To estimate the sensitivity these curves were partially fitted linearly. The corresponding slope is taken as a mea-

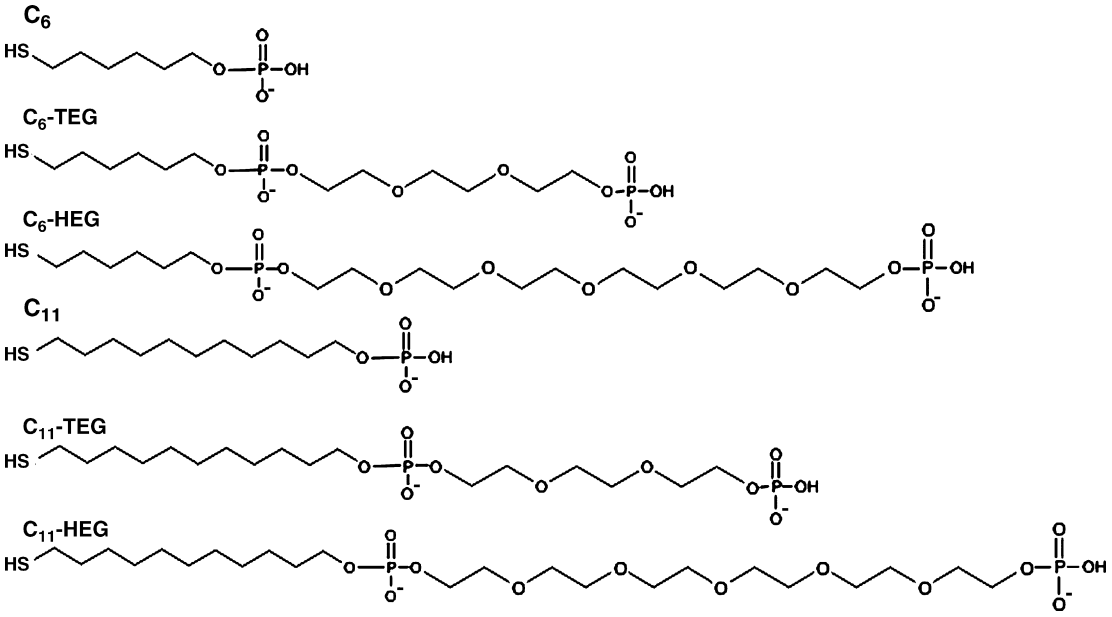

Fig. 1. Chemical structure of the different spacers coupled to the 5′ thiol moiety of the 25 bp probe.

sure for the sensitivity of the immobilized DNA film. The detection limit (DL) in nM can be calculated using following formula:

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

where N is the noise (RU), I is the intercept (RU) and S is the sensitivity (RU/nM). The noise level was calculated as the standard deviation on a ten fold blank injection on each mixed DNA/alkanethiol film. The intercept is given as the value where the linearly fitted dose–response curve, crosses the Y-axis, whereas S is considered to be the slope as described above (De Palma et al., 2007). The dynamic range was determined starting from the DL to the concentration at which no RU differences between two successive measurement points were observed (i.e. from DL to saturation).

2.4. Study of the surface density using QCM and fluorescence

The QCM gold substrates were prepared and cleaned as described above. Before being measured, they were mounted into the QCM (Q-sense D300, Sweden) liquid cell with an o-ring surface area of 1 cm^2. Thiolated 25 bp probes with 3′Alexa594 label and C_6-TEG spacer (1 μM) were injected in the axial flow chamber under gravity driven flow for 1 h followed by MCH backfilling (1 mM) for 30 min. Using the Sauerbrey equation the frequency changes (Δf in Hz) obtained via QCM were recalculated to the adsorbed mass (Δm in ng/cm^2) (Sauerbrey, 1959). After studying the immobilization with QCM, the samples were demounted and immersed in a 0.5 M dithiothreitol (DTT) solution for 1 h to ensure the selective detachment of the fluorescently labeled 25 bp probe from the gold surface. The corresponding fluorescence was measured using

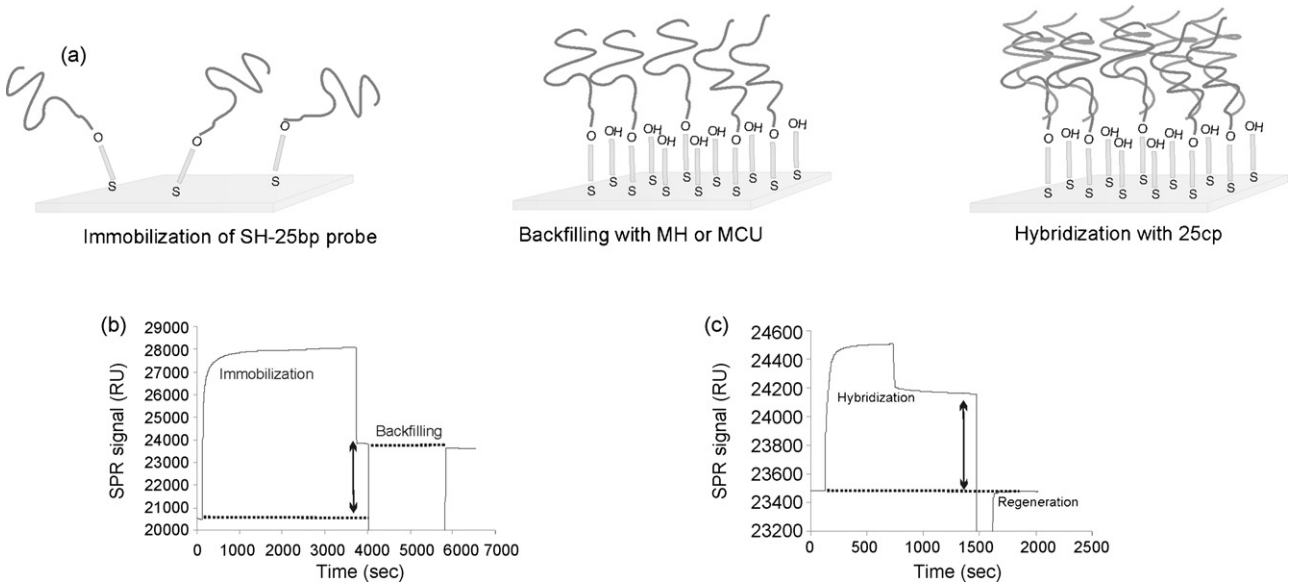

Fig. 2. Schematic representation of the DNA/alkanethiol functionalization approach used in this study (a). A representative sensogram of the immobilization/backfilling (b) and the hybridization (c) is shown as well.

a spectrophotometer (RF-5301PC, Shimadzu, Japan) and converted to a surface coverage (i.e. molecules/cm^2) based on a previously prepared standard curve.

2.5. Flow-assisted vs. diffusion-controlled immobilization of DNA probes

To study the influence of flow on the consecutive hybridization reactions, two different immobilization pathways were evaluated and compared. For the flow-assisted approach, samples were cleaned, functionalized with the thiolated 25 bp probes and backfilled as described above. For the diffusion-controlled approach the immobilization was performed overnight at 25 °C under continuous shaking using an Eppendorf thermomixer (450 rpm, Thermomixer R, Eppendorf, Germany). Afterwards, the samples were rinsed with distilled water, dried under a flow of N_2 and docked in the SPR device. For both approaches the subsequent backfilling and hybridization of the 25 cp were performed within the SPR device as described before. To compare the difference between both approaches two different spacers (i.e. C_6 and C_{11}) were selected. Again three independent experiments were run to assess the reproducibility.

3. Results

3.1. Comparative study of the different spacers using SPR

The immobilization grade was carefully evaluated for the thiolated 25 bp probes modified with the six different spacers (Table 1). All surfaces functionalized with probes containing the C_{11} spacer (including TEG and HEG) had a clearly higher surface density compared to surfaces immobilized with probes containing the shorter C_6 spacer.

The hybridization efficiency was calculated for each spacer based on the surface density and maximum hybridization signals (i.e. 320 nM) (Table 1). The C_6-TEG spacer was found to show the highest hybridization efficiency (i.e. 31.0%), whereas the C_6 spacer (i.e. 23.5%) and C_{11}-HEG spacer (i.e. 23.3%) showed the lowest efficiencies. Since these efficiencies only take into account the maximum hybridization signals, it is impossible to fully assess the impact of spacers on the biosensor performance. Therefore, the sensitivity, detection limit, dynamic range and reproducibility were also determined using the concentration-dependent hybridization curves as shown in Fig. 3. The obtained results for all these parameters are summarized in Table 2.

3.1.1. Sensitivity

The lowest sensitivity was observed for the C_6 spacer (i.e. 3.6 RU/nM). When introducing supplementary PEG units the sensitivity more than doubled. Moreover, a tremendous increase (>25 times) was observed when using the longer C_{11} spacer. This was also very clear from the C_{11} dose–response hybridization curves that clearly shifted to lower concentrations (Fig. 3b). In contrast to the C_6 spacer, the sensitivity decreased when PEG units were attached (Table 2).

Table 1
Overview of the surface density, the maximum hybridization level of the 25 cp and the corresponding hybridization efficiency as calculated for the six different spacers

Spacer	Surface density ($\times 10^{13}$ molecules/cm^2)	Hybridization ($\times 10^{12}$ molecules/cm^2)	Hybridization efficiency (%)
C_6	2.0 ± 0.02	4.8 ± 0.19	23.5
C_6-TEG	1.3 ± 0.30	4.2 ± 0.27	31.0
C_6-HEG	1.7 ± 0.06	4.8 ± 0.09	28.6
C_{11}	2.3 ± 0.02	5.8 ± 0.02	25.3
C_{11}-TEG	2.0 ± 0.15	5.4 ± 0.13	26.9
C_{11}-HEG	2.7 ± 0.03	6.3 ± 0.03	23.3

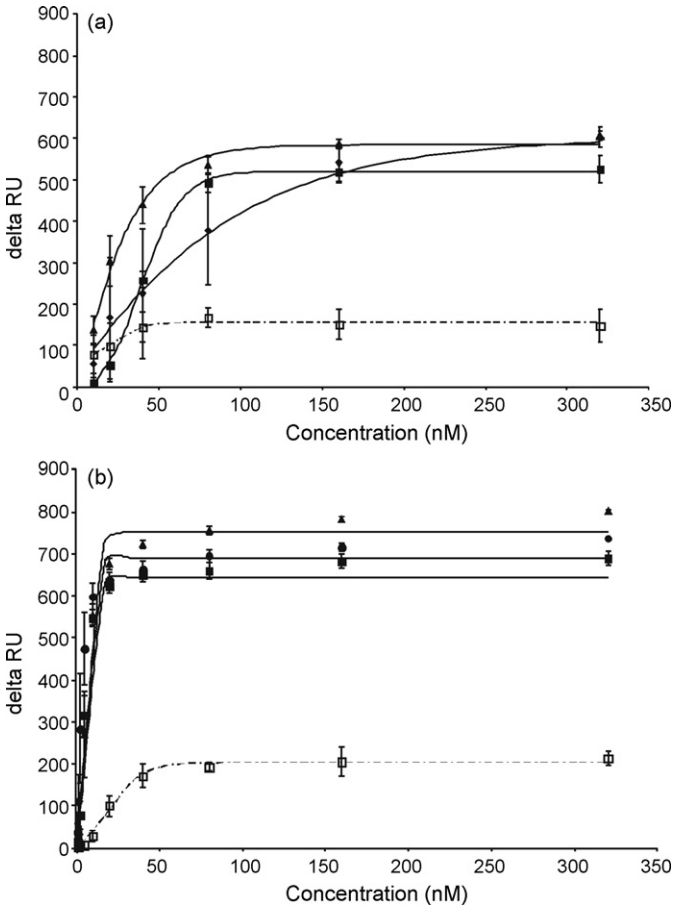

Fig. 3. Dose–response hybridization curves after immobilization of probes with C_6 spacers ((a) C_6, ♦; C_6-TEG, ■; and C_6-HEG, ▲); and C11 spacers ((b) C_{11}, ♦; C_{11}, TEG, ■; and C_{11}-HEG, ▲) are expressed as the SPR response (RU) in function of the concentration of 25 cp (nM). The solid lines represent the hybridization curves after a flow-assisted immobilization, while the dashed line represents a diffusion-controlled immobilization of the probes. The error bars represent the standard deviation on three independent experiments.

3.1.2. Detection limit

Besides the low sensitivity, the C_6 spacer showed to have the highest and thus the least desired detection limit. The lowest detection limit (i.e. 0.3 nM) was obtained for the C_{11} spacer. As noted for the sensitivity, the detection limit improved when TEG and HEG

Table 2
Summary of the sensitivity, the detection limit and the dynamic range, calculated for both the flow-assisted immobilization of thiolated 25 bp probes and the diffusion-controlled immobilization pathway (indicated by 'no flow')

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_6-TEG	7.1 ± 0.9	11.5 ± 2.9	11.5–80
C_6-HEG	9.6 ± 0.6	5.7 ± 1.3	5.7–80
C_{11}	99.2 ± 13.7	0.3 ± 0.2	0.3–20
C_{11}-TEG	70.6 ± 6.7	1.2 ± 0.3	1.2–20
C_{11}-HEG	69.0 ± 3.5	2.3 ± 0.7	2.3–20
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.

Table 3
QCM responses and corresponding surface densities calculated via the Sauerbrey equation and via fluorescence

QCM response ($\Delta f/3$ in Hz)	Surface density ($\times 10^{13}$ molecules/cm^2)	Fluorescence ($\times 10^{13}$ molecules/cm^2)
48.9	6.5	0.98
44.0	5.8	0.99
42.0	5.6	1.2

units were introduced to the C_6 spacer. Also for the C_{11} spacer modified with PEG moieties an improvement was observed, albeit less pronounced (Table 2).

3.1.3. Dynamic range

The dynamic range of each spacer was determined based on the hybridization curves. The broadest dynamic range (~2 decades) was observed for probes modified with the C_{11} spacer. The dynamic range for all other probes was found to be much narrower, especially for probes with C_6, C_6-TEG and C_6-HEG spacer (Table 2).

3.1.4. Reproducibility and specificity

The short C_6 spacer was found to be the least reproducible, particularly in the linear range (Fig. 3a). When attaching supplementary PEG units to this spacer, the reproducibility between individual experiments improved over the whole concentration range. The introduction of a C_{11} spacer was shown to be even more effective. When attaching TEG or HEG moieties to this spacer, the reproducibility hardly seemed to improve any further (Fig. 3b). Hybridization of the 25 np sequence on all six thiolated 25 bp probes modified with the different spacers resulted in no or very low hybridization signals (0–5 RU) (data not shown).

3.2. Study of the surface density using QCM and fluorescence

To determine the amount of immobilized probes in an alternative method and to estimate the extent of discrepancy between the different techniques (i.e. SPR, QCM and fluorescence) the thiolated 25 bp probe with 3′Alexa594 label and C_6-TEG spacer was immobilized on a gold substrate. The corresponding immobilization curve is provided in the Supplementary Information. Table 3 summarizes for each technique the results of three independent experiments. By recalculation of the QCM data using the Sauerbrey equation an almost six times higher surface density was observed compared to SPR. The surface densities determined by fluorescence showed a high similarity with the densities obtained via SPR (i.e. $1.1 \pm 0.1 \times 10^{13}$ molecules/cm^2).

3.3. Study of the immobilization approach: flow vs. diffusion controlled

In order to assess the impact of using an integrated flow system, such as the one included in the SPR instrument, two different immobilization approaches (i.e. flow vs. diffusion) were compared. When immobilizing the probes in a diffusion-controlled manner the observed differences between the C_6 and C_{11} spacer decreased. For the C_6 spacer a sensitivity of 2.25 RU/nM and a corresponding DL of 12.9 nM were noted. Hybridization on the C_{11} spacer resulted in a sensitivity of 4.7 RU/nM and a DL of 8.4 nM. These results are summarized in Table 2. For both spacers the dynamic range was negatively affected and the maximum hybridization signal dropped for more than 70% (Fig. 3). Despite the negative impact of the diffusion-controlled immobilization on most parameters, the reproducibility particularly within the linear range improved (data not shown).

4. Discussion

In this study, thiolated DNA probes with different spacers were immobilized and compared mainly using SPR as a characterization technique. When comparing the length (C_6 vs. C_{11}) of the spacer molecule, a significant increase in surface density for thiolated 25 bp probes having a C_{11} spacer was observed. These higher surface densities are most likely the result of the increase in the van der Waals forces. This will result in more ordered DNA films with most probes orientated perpendicular to the surface (Porter et al., 1987). In contrast, thiolated 25 bp probes with the shorter C_6 spacer may bind non-specifically on the gold surface leading to a strand orientation parallel to the surface (Wolf et al., 2004). Addition of a backfilling molecule (i.e. MCH or MCU) will reorganize the immobilized DNA layer by displacing some of the probes (Herne and Tarlov, 1997). It is apparent that the surface density will be affected by this conformational change.

Besides SPR, the surface densities were characterized using fluorescence and QCM. As proven by our QCM data, as well as by others, the use of the Sauerbrey equation led to an overestimation of the actual surface density (Voinova et al., 2002; Höök et al., 2001). This overestimation can be explained by assuming that hydration layers are being formed around the immobilized DNA strands (Su et al., 2005; Cho et al., 2004; Ha et al., 2004). This hydration effect was found to be sensitive to the buffer conditions used (Cho et al., 2004). For buffers with a low pH the overestimation was found to be more pronounced as the immobilized DNA layer likely becomes less rigid, changing its viscoelestic properties (Cho et al., 2004). Within this report the thiolated DNA probes were immobilized using a 1 M KH_2PO_4 buffer at pH 3.8. Consequently, QCM data used to study DNA immobilization and hybridization events should be interpreted with care and further profound studies through combined frequency and dissipation monitoring (i.e. QCM-D) are desired (Höök et al., 2001; Okahata et al., 1998).

As the hybridization of the 25 cp is directly related to the surface density, the impact of the spacers on the hybridization efficiency was assessed as well. The overall low hybridization efficiencies (23–31%), as reported in this study and observed by others (Gong et al., 2006; Steel et al., 2000), are likely caused by the high surface densities. High surface densities are expected to introduce steric effects that will reduce the accessibility of the immobilized probes (Ricci et al., 2007). Consequently, the introduction of longer alkane chain spacers will lead to lower hybridization efficiencies. Despite the limited hybridization efficiencies, the sensitivities and detection limits obtained with thiolated 25 bp probes bearing solely the longer C_{11} spacer were shown to be superior over those having the shorter spacers. Indeed, the chance of a low concentrated target DNA strand to bind with its surface immobilized complement increases at higher probe densities. However, as mentioned before, high surface densities will still promote steric hindrance. To increase the accessibility and flexibility of the immobilized DNA probes, spacers containing PEG molecules may be introduced (Biswal and Gast, 2003). In our case, the linking of PEG moieties to the C_{11} spacer negatively affected both the sensitivity and the detection limit, while the opposite was observed for the shorter C_6 spacer. Apparently, there is a trade-off between the beneficial effect of the alkane chain length and the introduction of PEG units. In addition, the individual impact of these two parameters is difficult to assess as the surface density differs for each of the six studied probes. Nonetheless, both modifications, i.e. the length of the alkane chain and the presence of PEG units are important as they determine the quality of the immobilized DNA/alkanethiol film to a great extent. In addition, PEG units are expected to improve the specificity as well. When performing assays in more complex biological media the combination of mixtures of thiolated DNA and

alkanethiol "backfilling" molecules (e.g. MCH or MU) was shown not to be sufficient (Lee et al., 2007). Therefore, spacers attached to PEG units, as studied in this report, are expected to further reduce these non-specific interactions (Unsworth et al., 2005; Saerens et al., 2005; Ostuni et al., 2001; Chapman et al., 2000).

Besides possessing high sensitivities and low detection limits, DNA biosensors need to be highly reproducible and stable as well. The data obtained within this study show an enormous increase in reproducibility when using thiolated 25 bp probes with C_{11} spacers (i.e. C_{11}, C_{11}-TEG and C_{11}-HEG). In addition, sensors immobilized with the longer C_{11} spacers have shown improved stability over the shorter C_6 spacer (Lai et al., 2006). This indicates that both factors are strongly related to the length of the spacer and the associated increase in van der Waals forces.

The comparison between the two immobilization pathways (i.e. flow vs. diffusion) confirmed the need for an integrated flow-system. Solid-phase based immobilization and hybridization reactions, like performed within this report, are poorly understood and rather complex (Erickson et al., 2003). It is assumed that flow-systems are beneficial as they accelerate both reactions by the introduction of convection. This was already shown by others as well (Kim et al., 2006). When using diffusion-controlled systems fewer probes will take part in the immobilization and depletion zones are expected to be introduced during the subsequent hybridization process.

5. Conclusions

In this work, the impact of six different spacers used to link a thiol moiety to the 5′ end of a specific DNA sequence, was studied with respect to the immobilization degree and hybridization performance. To assess the effect of these spacers on the biosensor performance, different parameters such as the sensitivity, the detection limit and the reproducibility were determined using SPR. To our knowledge, this is the first report presenting such a study. From the obtained results it was shown that varying the spacer can be advantageous for the design of sensitive and reliable DNA biosensors. The alkane chain length was found to be an important factor. The sensitivities and detection limits significantly improved when using probes with longer C_{11} spacers (i.e. C_{11}, C_{11}-TEG and C_{11}-HEG). The effect of introducing PEG units, on the other hand, was probably masked by the high overall surface densities. PEG moieties may, however, still be beneficial for real-world applications because of their high protein repelling capacities. Although the C_6 spacer is generally used to link thiols to specific DNA sequences, the findings presented in this report show that longer alkane chain spacers are highly recommended especially for applications where the detection of low target concentrations is desired. Furthermore, we have demonstrated the clear impact of the immobilization approach (flow vs. static) on the biosensor performance.

Acknowledgments

The authors would like to thank all current and former members of the NEXT/NS research group for their valuable scientific input, Dr. A. Standaert for his help with the statistical analysis of the data and Dr. F. Frederix for synthesizing the C_{11} precursor molecule. Sara Peeters is grateful to the Institute for the Promotion and Innovation through Science and Technology (IWT-Vlaanderen) for their funding. This work was also partially financed by a grant of the Belgian Federal Service of Public Health, Food Chain Safety and Environment (Grant no. S-6167) and by the Commission of the European Communities in the context of IST project 027652 MASCOT and IST-NMP project 016817 SmartHEALTH.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.03.012.

References

Andreotti, P.E., Ludwig, G.V., Peruski, A.H., Tuite, J.J., Morse, S.S., Peruski, L.F., 2003. Biotechniques 35, 850–859.
Bashir, R., 2001. Superlattices and Microstructures 29, 1–16.
Biswal, S.L., Gast, A.P., 2003. Physical Review E 68, 021402-1–021402-9.
Bunimovich, Y.L., Shin, Y.S., Yeo, W.S., Amori, M., Kwong, G., Heath, J.R., 2006. Journal of the American Chemical Society 128, 16323–16331.
Castelino, K., Kannan, B., Majumdar, A., 2005. Langmuir 21, 1956–1961.
Castillo, J., Gaspar, S., Leth, S., Niculescu, M., Mortari, A., Bontidean, I., Soukharev, V., Dorneanu, S.A., Ryabov, A.D., Csoregi, E., 2004. Sensors and Actuators B-Chemical 102, 179–194.
Chapman, R.G., Ostuni, E., Yan, L., Whitesides, G.M., 2000. Langmuir 16, 6927–6936.
Chen, J.R., Miao, Y.Q., He, N.Y., Wu, X.H., Li, S.J., 2004. Biotechnology Advances 22, 505–518.
Cho, Y.K., Kim, S., Kim, Y.A., Lim, H.K., Lee, K., Yoon, D.S., Lim, G., Pak, Y.E., Ha, T.H., Kim, K., 2004. Journal of Colloid and Interface Science 278, 44–52.
De Palma, R., Reekmans, G., Laureyn, W., Borghs, G., Maes, G., 2007. Analytical Chemistry 79, 7540–7548.
Deisingh, A.K., Thompson, M., 2004. Canadian Journal of Microbiology 50, 69–77.
Demers, L.M., Mirkin, C.A., Mucic, R.C., Reynolds, R.A., Letsinger, R.L., Elghanian, R., Viswanadham, G., 2000. Analytical Chemistry 72, 5535–5541.
Erickson, D., Li, D., Krull, U.J., 2003. Analytical Biochemistry 317, 186–200.
Fraser, M.E., Fujinaga, M., Cherney, M.M., Melton-Celsa, A.R., Twiddy, E.M., O'Brien, A.D., James, M.N.G., 2004. Journal of Biological Chemistry 279, 27511–27517.
Gong, P., Lee, C.H., Gamble, J.L., Castner, D.G., Grainger, D.W., 2006. Analytical Chemistry 78, 3326–3334.
Ha, T.H., Kim, S., Lim, G., Kim, K., 2004. Biosensors & Bioelectronics 20, 378–389.
Hahn, S., Mergenthaler, S., Zimmermann, B., Holzgreve, W., 2005. Bioelectrochemistry 67, 151–154.
Halperin, A., Buhot, A., 2006. Langmuir 22, 11290–11304.
Herne, T.M., Tarlov, M.J., 1997. Journal of the Chemical Society 119, 8916–8920.
Höök, F., Ray, A., Nordén, B., Kasemo, B., 2001. Langmuir, 8305–8312.
Jonsson, U., Fagerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Lofas, S., Persson, B., Roos, H., Ronnberg, I., Sjolander, S., Stenberg, E., Stahlberg, R., Urbaniczky, C., Ostlin, H., Malmqvist, M., 1991. Biotechniques 11, 620–627.
Kim, H.S., Marafie, A., Jia, X.Y., Zoval, J.V., Madou, J., 2006. Sensors and Actuators B-Chemical 113, 281–289.
Lai, R.Y., Seferos, D.S., Heeger, A.J., Bazan, G.C., Plaxco, K.W., 2006. Langmuir 22, 10796–10800.
Lee, C.Y., Nguyen, T.P., Grainger, D.W., Gamble, L.J., Castner, D.G., 2007. Analytical Chemistry 79, 4390–4400.
Levicky, R., Herne, T.M., Tarlov, M.J., Satija, S.K., 1998. Journal of the American Chemical Society 120, 9787–9792.
Mannelli, I., Minunni, M., Tombelli, S., Wang, R., Spiriti, M., Mascini, M., 2005. Bioelectrochemistry 66, 129–138.
Okahata, Y., Kawese, M., Niikura, K., Ohtake, F., Furusawa, H., Ebara, Y., 1998. Analytical Chemistry 70, 1288–1296.
Ostuni, E., Chapman, R.G., Holmlin, R.E., Takayama, S., Whitesides, G.M., 2001. Langmuir 17, 5605–5620.
Porter, M.D., Bright, T.B., Allara, D.L., Chidsey, C.E., 1987. Journal of the American Chemical Society 109, 3559–3567.
Ricci, F., Lai, R.Y., Heeger, A.J., Plaxco, K.W., Sumner, J.J., 2007. Langmuir 23, 6827–6834.
Saerens, D., Frederix, F., Reekmans, G., Conrath, K., Jans, K., Brys, L., Huang, L., Bosmans, E., Maes, G., Borghs, G., Muyldermans, S., 2005. Analytical Chemistry 77, 7547–7555.
Satjapipat, M., Sanedrin, R., Zhou, F.M., 2001. Langmuir 17, 7637–7644.
Sauerbrey, G., 1959. Zeitschrift für Physik 155, 206–222.
Shchepinov, M.S., Case-Green, S.C., Southern, E.M., 1997. Nucleic Acids Research 25, 1155–1161.
Soper, S.A., Brown, K., Ellington, A., Frazier, B., Garcia-Manero, G., Gau, V., Gutman, S.I., Hayes, D.F., Korte, B., Landers, J.L., Larson, D., Ligler, F., Majumdar, A., Mascini, M., Nolte, D., Rosenzweig, Z., Wang, J., Wilson, D., 2006. Biosensors & Bioelectronics 21, 1932–1942.
Steel, A.B., Levicky, R.L., Herne, T.M., Tarlov, M.J., 2000. Biophysical Journal 79, 975–981.
Su, X., Wu, Y.J., Knoll, W., 2005. Biosensors & Bioelectronics 15, 719–726.
Unsworth, L.D., Sheardown, H., Brash, J.L., 2005. Langmuir 21, 1036–1041.
Van Aerschot, A., Rozenski, J., 2006. Journal of the American Society for Mass Spectrometry 17, 1396–1400.
Voinova, M.V., Jonson, M., Kasemo, B., 2002. Biosensors & Bioelectronics 17, 835–841.
Wang, J., 2000. Nucleic Acids Research 28, 3011–3016.
Wang, R., Muninni, M., Tombelli, S., Mascini, M., 2004. Biosensors & Bioelectronics 20, 598–605.
Wolf, L.K., Gao, Y., Georgiadis, R.M., 2004. Langmuir 20, 3357–3361.
Wong, E.L., Chow, E., Gooding, J., 2005. Langmuir 21, 6957–6965.
Yao, G., Tan, W., 2004. Analytical Biochemistry 331, 216–223.