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

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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.,

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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-