BIOASSEMBLER

Integrating bio-inspired assembly into semiconductor manufacturing technology for biosensors

D4.1 Chemistry for DNA on wafers

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Revision History

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1.0	Review and comments	24.8.2023	Mark Somoza / Univie	Second draft version
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2.0	Revised report	9.7.2024	Mark Somoza / Univie	Per reviewer's request, added explanatory context and references.

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1. Introduction

Traditional nucleic acid synthesis begins with a column containing controlled-pore glass (CPG) beads modified with long, cleavable spacer arms. Typical columns also include the 3'-hydroxyl nucleosides coupled to the spacer arm's end. The 5' hydroxyl moiety of the nucleotide phosphoramidites is usually protected with either a dimethoxytrityl (DMT) or a monomethoxytrityl (MMT) group. Before the subsequent phosphoramidite can be added through a coupling reaction, this protective group must be removed or "deblocked." The beads are exposed to a deblocking solution containing trichloroacetic acid (TCA) or dichloroacetic acid (DCA) in dichloromethane to achieve this. The removal of the DMT reveals a 5' hydroxyl moiety, which can then react with a new phosphoramidite in a subsequent extension process.

Photolithographic synthesis works chemically in much the same way as this standard solid-phase DNA synthesis. The main difference is that a photolabile protecting group is used instead of an acid-labile protecting group. This has the advantage that the synthesis can be controlled by light and allows the synthesis of complex arrays of nucleic acids on a planar surface. Standard photolabile groups are of the nitrophenylpropyloxycarbonyl (NPPOC) family, or derivatives thereof, which have high photolysis efficiency and a reasonably high quantum yield. There are currently three good options for NPPOC derivatives, NPPOC itself, Bz-NPPOC, and SPh-NPPOC. The fundamental photochemistry of the three groups is the same. However, the Bz-NPPOC and SPh-NPPOC have improved quantum efficiency for photocleavage or have a higher extinction coefficient, thus allowing to perform faster syntheses[1]. The structures of these groups are shown in Figure 1 as protecting groups on a dT phosphoramidite.

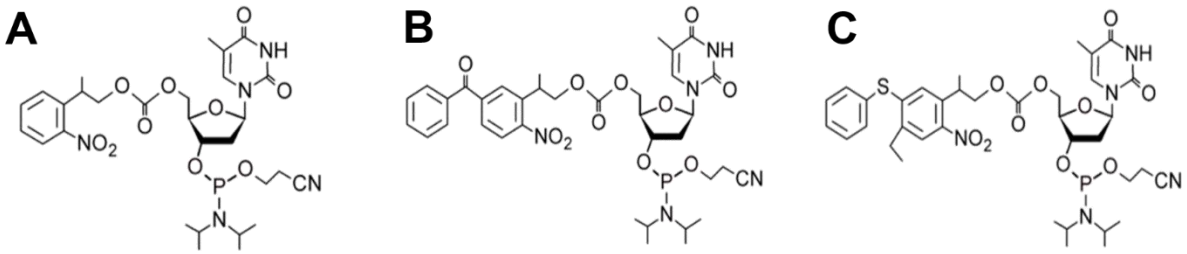

Figure 1 - Structure of (A) NPPOC, (B) Bz-NPPOC, and (C) SPh-NPPOC as 5'-OH protecting groups on dT phosphoramidites.

Before synthesis, the surface is functionalized to provide functional groups for the first coupling reaction. Silane chemistry[2-3] is the most common approach for glass; however, other functionalization can be adopted depending on the surface material[4-5]. Functionalization procedures that result in hydroxy-functionalized surfaces are typically used, but syntheses on surfaces with amino and epoxy groups are also efficient[6]. The first coupling reaction is not spatially selective. However, it generates an initial layer of nucleotides with 5' photocleavable moieties that enable subsequent spatially

controlled synthesis via selective photoremoval of the photolabile group. The steps necessary to add more nucleotides to the sequence are described below.

1) **Photodeprotection:** The photolabile protecting group is removed by irradiation in the presence of a base using light of the appropriate wavelength, generally 365 nm. We typically use imidazole as this organic base, but other bases such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), diisopropylethylamine, *N*-methylmorpholine, *N*-methylimidazole, and piperidine are also suitable additives to the deprotecting step[7].

2) **Coupling:** In this step, an electrophilic phosphoramidite is coupled to nucleophilic hydroxyl groups from the last incorporated monomer. The coupling reaction itself is extremely fast. However, the reaction rate is greatly accelerated using an activator that transforms the phosphoramidite into a highly electrophilic species with an excellent leaving group. Examples of commercially available activators are dicyanoimidazole (DCI), 5-ethylthio-1H-tetrazole (ETT), and 5-benzylthio-1H-tetrazole (BTT)[8].

3) **Oxidation:** The unstable phosphite triester linkages (P-III) resulting from the coupling reaction are oxidized to a stable phosphate triester (P-V). This is typically carried out using iodine in the presence of water and pyridine. In photolithographic synthesis, contrary to solid phase synthesis, it is optional to carry out oxidation in each cycle since the photodeprotection step does not affect the phosphite triester. This allows the optimization of oxidation, reducing both the consumption of reagents and the introduction of water that can decrease the synthesis yield. Although iodine-based oxidants are the standard for DNA synthesis, non-aqueous oxidants such as (1S)-(+)-(10-camphorsulfonyl)-oxaziridine (CSO)[9] are also commercially available.

4) **Capping:** When necessary, capping of hydroxyl functionalities that did not react in the coupling step can be done with either acetylating agents or by another phosphoramidite designed to block further coupling reactions[10].

Figure 2 outlines the chemical cycle for the photolithographic synthesis of DNA. Steps (1) to (4) are repeated until the target sequences have been obtained. After synthesis, removing the chemical protective groups in the exocyclic amines and the cyanoethyl group from the phosphate is necessary. The conditions used to carry out this deprotection depend on the type of substrate and mainly on the protective groups present in the phosphoramidites. Efficient deprotection is typically achieved by immersing the glass slides for 2 hours in a 1:1 mixture of ethanol and ethylenediamine for synthesis with phosphoramidites with tert-butylphenoxyacetyl (tac) protecting group.

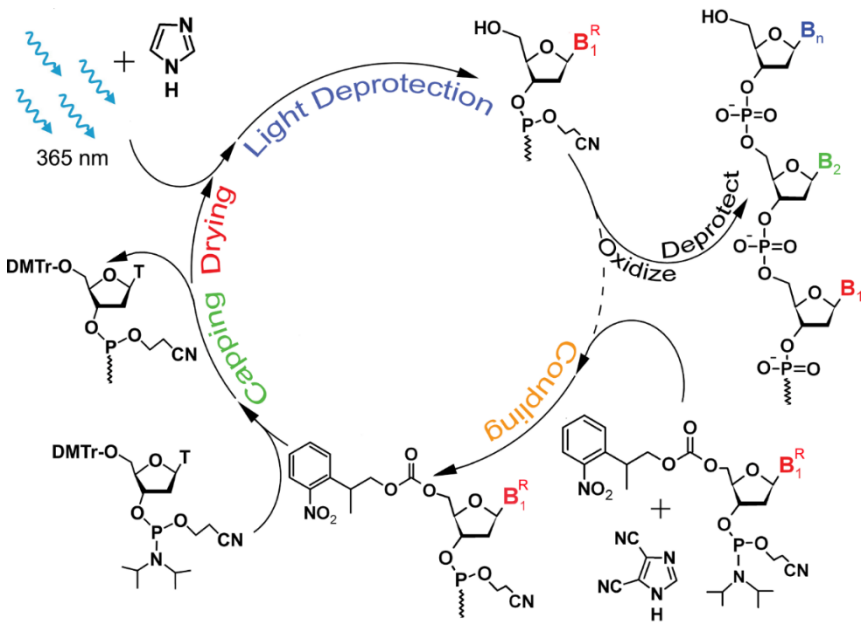

Figure 2 - Photolithographic synthesis cycle for DNA.

Following the principles mentioned above, the photolithographic synthesis of DNA on glass is a straightforward process that can be executed through methodologies that have been previously established and optimized at UniVie[11-12]. The challenge in the context of Bioassembler is to adapt these established procedures for synthesis on wafers. In this regard, two aspects hold particular significance. Firstly, the strategy employed for functionalizing the surface to facilitate the addition of the initial nucleotide must be defined. It is also necessary to ascertain the compatibility of all involved procedures and reagents in the synthesis and deprotection steps with the materials on the sensor surface. This is the objective of task 4.1, with which this deliverable is associated.

This deliverable provides information on the protocols for surface functionalization and synthesis of DNA on wafers. We did the tests using "wafer-like" substrates, which are pieces of wafers modified with different materials to mimic the sensors' surface. These substrates, which have the correct dimensions for the synthesis system currently available in UniVie, were designed and manufactured by VTT.

2. Results

A photograph of the substrates used to test the DNA synthesis on wafers is shown in Figure 3 (A). The wafer piece with dimensions of 75 × 25 mm^2 contains 40 nm thick aluminum in 400 x 400 μm^2 rectangular arrays separated by 400 µm wide gaps. A 40 nm layer of SiO_2 was deposited on top using plasma-enhanced chemical vapor deposition (PECVD). This top layer prevents the dissolution of aluminum during hybridization reactions carried out at high salt concentrations and, at the same time,

facilitates the functionalization of the surface through silanization. The functionalization was done using N-(3-triethoxysilylpropyl)-4-hydroxybutyramide (Figure 3 (B)), resulting in hydroxy-functionalized surfaces.

The silicon substrates were drilled and assembled in the synthesis cell along with a regular glass slide, both separated by a 50 µm thick polytetrafluorethylenee (PTFE) gasket as shown in Figure 1 (C) and arrays consisting of a single sequence (QC25: 5' GTCATCATCATGAACCACCCTGGTC-3') were synthesized over the entire synthesis area (area for all the mirrors on: 1 cm × 1.4 cm). After deprotection in a 1:1 mixture of ethanol and ethylenediamine, the arrays were hybridized for 2 hours at 42 °C to the complementary Cy3-labeled oligonucleotide.

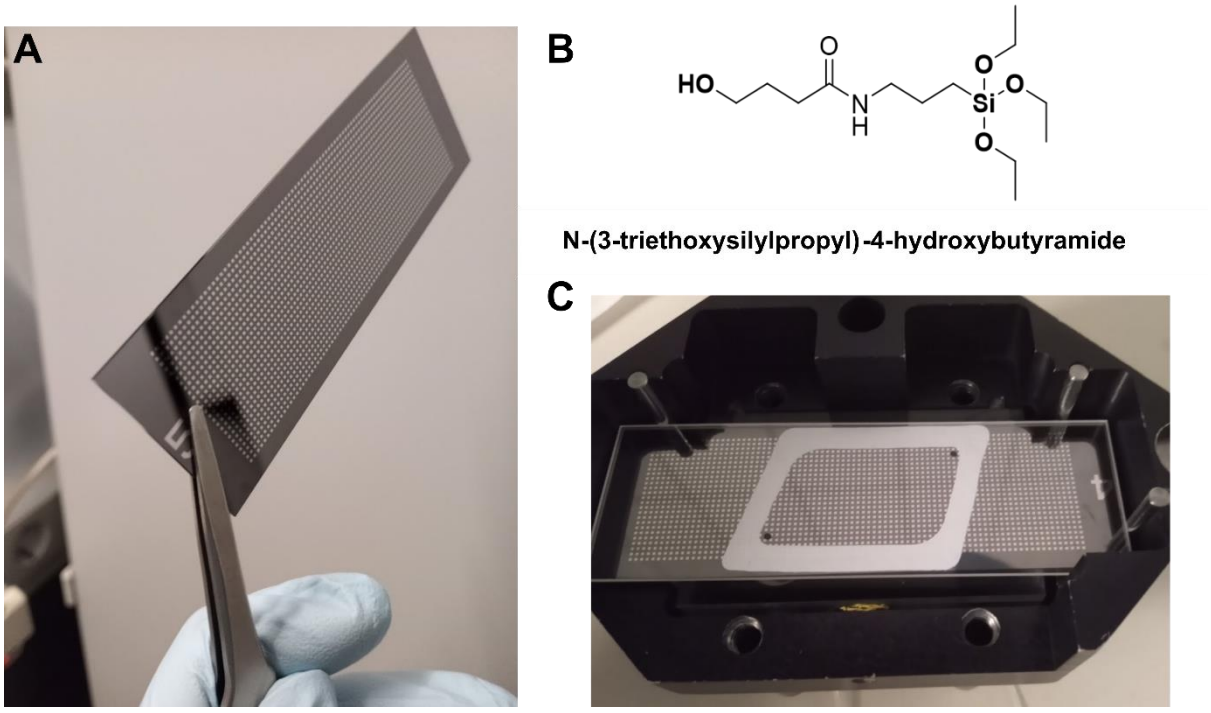

Figure 3 - (A) Photograph of a substrate used to assess the DNA photolithographic synthesis on wafers. (B) Structure of N-(3-triethoxysilylpropyl)-4-hydroxybutyramide silane used to functionalize the surface of wafer pieces coated with SiO_2 through PECVD. (C) Photograph of the assembled synthesis cell showing the drilled wafer piece under a non-drilled glass slide. The top and bottom slides are separated by a thin PTFE gasket, delimiting the surface area in contact with the reagents used in the synthesis.

Using the conditions summarized in Table 1, we synthesized DNA on the wafer-like substrates without observing any oxidation or corrosion of the underlying aluminum layer. Considering the drying and washing times, introducing a new nucleotide takes approximately 1.8 minutes per cycle. However, for further improvements, the photodeprotection time can be significantly reduced using SPh-NPPOC phosphoramidites without altering other synthesis parameters. Compared with other synthetic approaches, photolithographic synthesis is the shortest due the fast removal time of modern

photolabile protecting groups in contrast to the removal of the acid-labile DMTr protecting group used otherwise. In particular, DMTr removal requires approximately 60 seconds, whereas SPh-NPPOC removal is performed with 6 seconds of exposure with an easily achievable 100 mw/cm^2 exposure with 365 nm light. This time can be shortened to, e.g. 3 seconds, using a radiant intensity of 200 mw/cm^2. Additionally, exposure to the strongly acidic conditions required for DMTr removal requires approximately 15 seconds of oxidation before DMTr removal step in order to convert the phosphite to the more stable phosphate, whereas in photochemical synthesis, oxidation can be intermittent or even omitted until the very end, saving and additional circa 15 seconds per cycle[8, 11]. Therefore, the photolithographic approach can achieve per cycle times approximately 60 seconds faster than any known alternative approach.

Figure 4 displays a scan of the array (A), along with a plot of the average fluorescence intensities for the regions with different modifications (wafer surface modified with Al-SiO_2 or just with SiO_2) (B).

Table 1. Reagents and conditions used to synthesize a 25-mer sequence on the wafer-like substrates.

Step	Reagents	Time (s)
Coupling	0.03 M 5' Bz-NPPOC phosphoramidites 0.025 M DCI activator	15
Photodeprotection	1% imidazole in dimethyl sulfoxide	42 s (at 71 mW/cm^2)
Oxidation	0.02 M I_2 in water/pyridine/tetrahydrofuran mixture	3

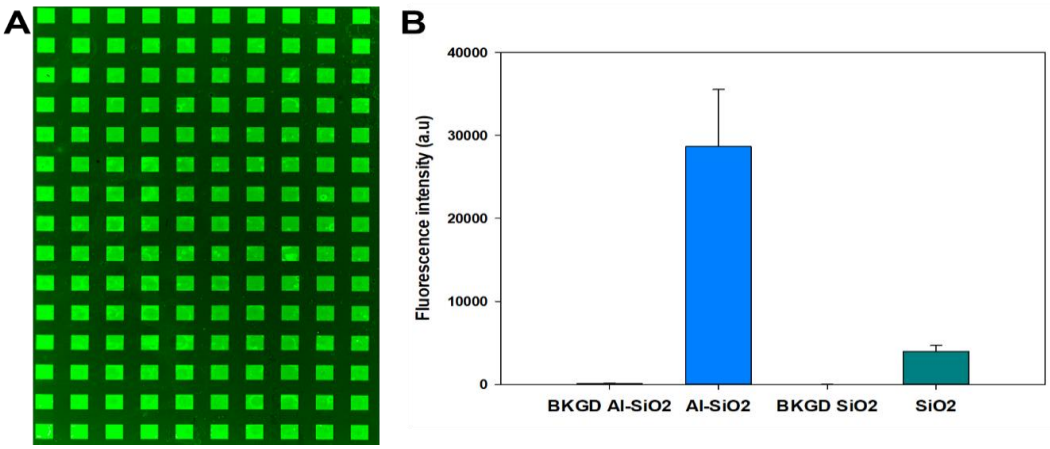

Figure 4 - (A) Fluorescent scan image of a microarray consisting of QC25 oligomer synthesized the entire synthesis area (according to the size of the mirrors) of the wafer-like substrates. The brighter squares correspond to the surface modified with aluminum and SiO_2. (B) Average hybridization-based fluorescent intensity for the wafer regions with different modifications, i.e., wafer surface modified with Al-SiO_2 or SiO_2.

Using an I_2/pyridine mixture as oxidizer reagents is incompatible with syntheses in substrates containing exposed metals such as gold, as it can etch the metal from the surface. This can be seen in Figure 5 (A), which shows a photograph of a glass slide covered with gold after synthesis with I_2/pyridine. All the gold in the area in contact with the reagents was removed after 30 cycles of oxidation with I_2/pyridine. Considering that it may be necessary to use non-passivated gold on the sensors' surface for connectors and pads, we tested non-aqueous CSO as an alternative oxidizer [13]. Figure 5 (A) also shows a photograph of the same type of slide after a 30 cycles synthesis with CSO, where it can be clearly seen that there is no damage to the surface. Also relevant is that using CSO, it is possible to synthesize arrays on gold with similar features to those done with glass, as observed in Figure 5 (B and C), for simultaneously synthesized gold and glass-based microarrays consisting of a single sequence (QC25).

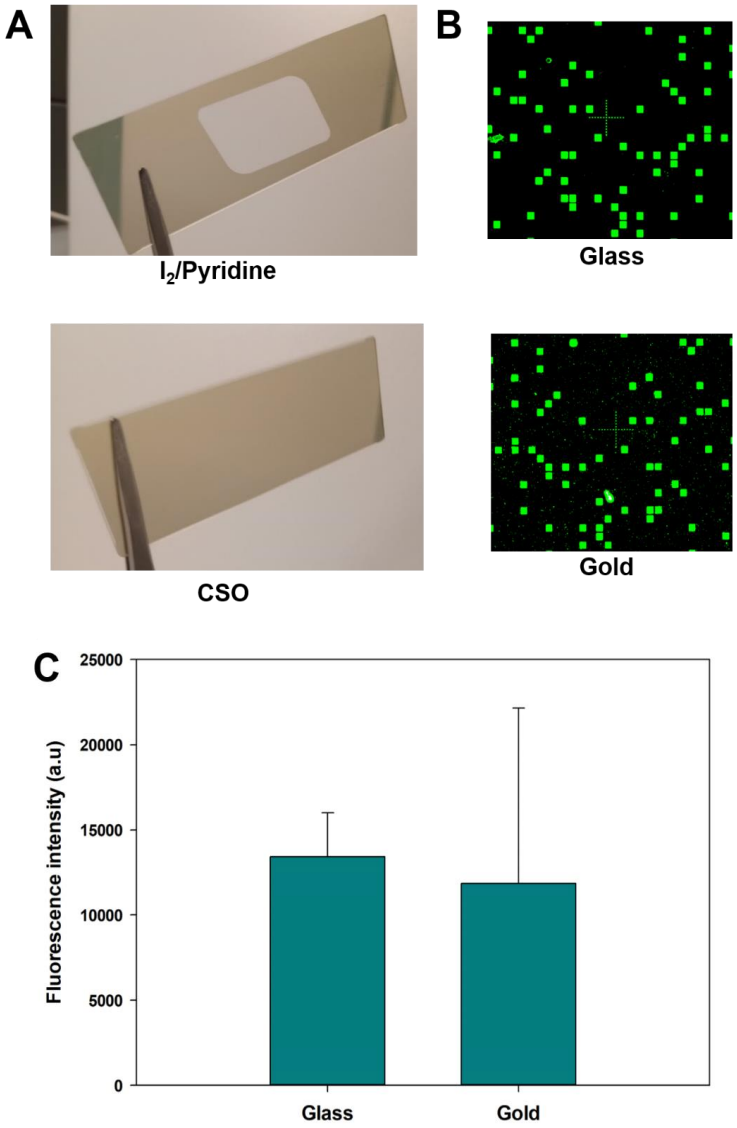

Figure 5 - (A) Photographs of gold-coated slides after DNA synthesis using I_2/Pyridine or CSO as oxidizer reagent. (B) Fluorescent scan image of a microarray consisting of QC25 oligomer synthesized in glass or gold surfaces using CSO as the oxidizer. (C) Average hybridization-based fluorescence intensities after background subtraction for QC25 synthesized on glass and gold-coated glass substrates.

3. Conclusions and outlook

In summary, we have described the functionalization and synthesis protocols for the direct photolithographic DNA synthesis on wafer surfaces. The compatibility of all stages - functionalization, synthesis, and deprotection - with the materials we intended to use in the sensors has been confirmed. Synthesis times of approximately 1.8 minutes per nucleotide were observed under tested conditions, with the potential for reduction using specific phosphoramidites. Future efforts will focus on

optimizing the procedure to decrease synthesis times and reagent usage during wafer-scale synthesis. One approach involves refining the oxidation step to a single occurrence at the end of the synthesis. Simultaneously, we will explore alternative silanization reagents to enhance the stability of surface-bound DNA. This is especially pertinent for extended hybridizations with DNA-tagged binders.

4. References

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