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Oxime Bond Formation for the Covalent Attachment of Oligonucleotides on Glass Support

Eric Defrancq,[a,*] Antoine Hoang,[b] Françoise Vinet[b] and Pascal Dumy[a]

[a]*LEDSS, UMR CNRS 5616, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France*
[b]*LETI-CEA-Grenoble 17, rue des Martyrs, 38054 Grenoble Cedex 9, France*

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Abstract—The chemical attachment of oligonucleotides on glass slides has been achieved using oxime bond formation. This method has been shown very efficient by comparison with the attachment of amino-oligonucleotides via reductive amination.

During the last decade, the DNA micro-array technology has emerged as a powerful tool for gene discovery and expression, detection of mutations or polymorphisms and mapping.[1] DNA micro-array analysis involves the hybridisation of the labelled nucleic acid target with support bound oligonucleotides (probes), followed by detection of the label via an imaging or a scanning process. Two major strategies for the preparation of DNA micro-arrays have been developed. The first one is the direct on-surface synthesis (in situ synthesis),[2] the second involves immobilisation of prefabricated oligonucleotides on the support.[3] In situ synthesis represents the most efficient way for the preparation of high-density oligonucleotide micro-arrays. However, this process shows drawbacks in terms of flexibility. For example, the sequence length is limited to short oligonucleotides because the failed sequences cannot be removed from the solid support and so accumulate after each round of coupling. The second method, called deposition method allows the incorporation of modified bases in the oligonucleotide or the loading of long DNA sequences. This latter method has thus become the most widely used way for creating low to medium density DNA arrays.

A number of methods have been described for the covalent binding of oligonucleotides on solid support, most of them using glass slides. Thiolated or disulphide-modified oligonucleotides have been grafted on amino-silanised glass slides using heterobifunctional cross linker.[4] The most popular modified oligonucleotides are indeed the 5′-amino functionalised oligonucleotides which have been anchored to aldehyde-activated glass,[5] or to glass surfaces modified with epoxide.[6] Silanised oligonucleotides have also been prepared and immobilised on glass.[7] Limitation of the sensitivity of the analysis has been attributed in first approximation to the insufficient loading of the oligonucleotide on glass surfaces. Therefore, some strategies to enhance the loading have been investigated by using dendritic linker systems.[8] Development of a more efficient reaction between the solid support and the oligonucleotides to be anchored, represents a further way to enhance the loading.

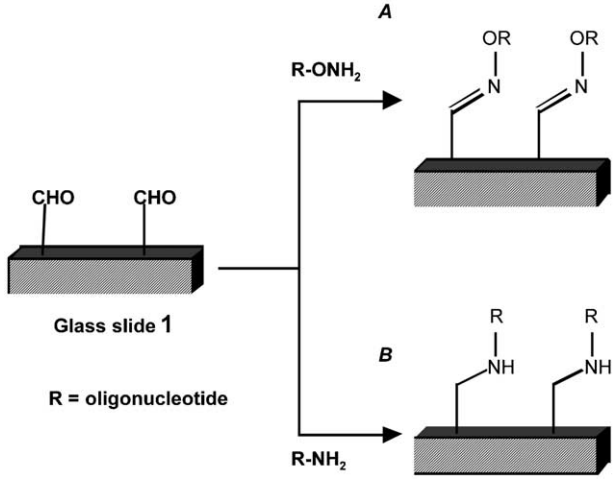

Figure 1. Covalent binding of oligonucleotides on aldehydic glass support **1** using (A) oxime bond formation, (B) reductive amination strategy.

*Corresponding author. Tel.: +33-476-514433; fax: +33-476-514946; e-mail: eric.defrancq@ujf-grenoble.fr

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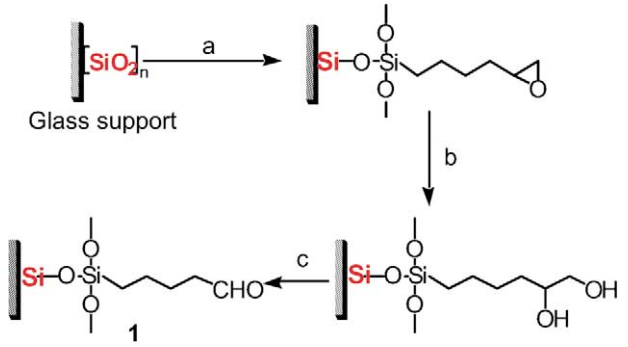
In this context, we have investigated the use of the oxime linkage for the covalent binding of oligonucleotides to glass support. A similar strategy has been recently described for covalent immobilisation on gold support or polymer particles.[9] The oxime bond formation has been used efficiently for the chemical ligation of peptides[10] as well as for the conjugation of peptides with carbohydrates[11] and oligonucleotides.[12] A major advantage of this ligation technique is that it requires neither a coupling reagent nor chemical manipulations except mixing of the two components, namely an oxyamine and an aldehyde derivative. In this paper, preliminary results concerning the chemical attachment of 5′-oxyamino oligonucleotides on aldehyde functionalised glass slides via oxime bond formation are described. The efficiency of the loading is compared with glass slides obtained by the attachment of the corresponding 5′-amino-oligonucleotide using reductive amination (Fig. 1). The superiority of the oxime approach is further emphasised by performing the deposition in a capillary tube, which can represent a simple model for microsystem devices.[13]

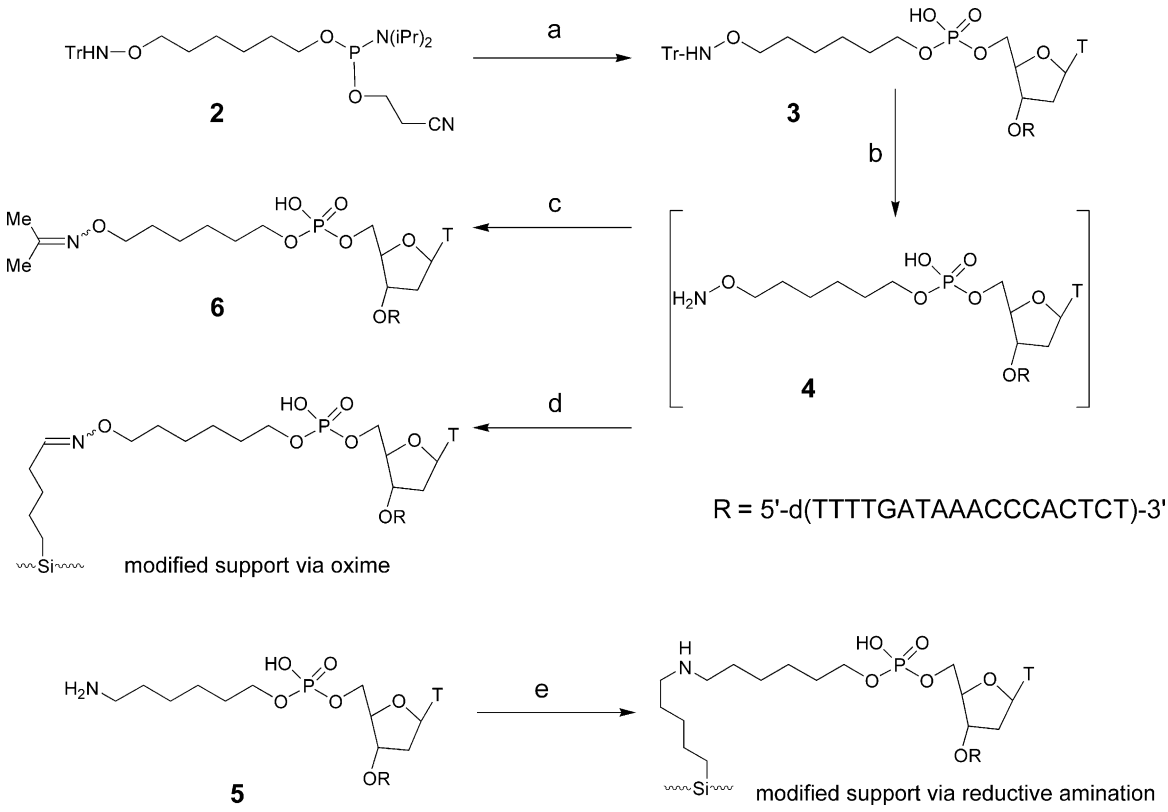
The aldehydic glass slides are commercially available. However, we have developed a novel procedure for the functionalisation of glass slides, as well as for capillary tubes, by an aldehydic group.[14] The strategy is briefly described in Scheme 1. An epoxide function was anchored on the glass support by silanisation with commercial triethoxy(4-oxiranylbutyl)silane. Subsequent hydrolysis of the epoxide function in basic conditions afforded the vicinal diol, which was then converted to the aldehyde by sodium periodate oxidation. The aldehydic function on the solid support **1** was evidenced by multireflexion IR (MIR method) by the presence of the carbonyl band at 1720 cm^{-1}. The roughness of the silane's layer on the surface was measured at about 5–6 nm by AFM method (Atomic Force Microscopy).

In a recent paper, we described a convenient synthetic route to aminooxy containing oligonucleotides by incorporating a trityl protected aminooxy group using the phosphoramidite **2** as depicted in Scheme 2.[12a] The oligonucleotide **3** (5′-d(XTTTTTGATAAACCCACTCT)-3′ in which X represents the 5′-trityl protected oxyamino linker) was thus prepared according this strategy using standard β-cyanoethylphosphoramidite chemistry by incorporating the phosphoramidite **2** at the final step of the automated DNA synthesis. Purification by reverse phase HPLC afforded the oligonucleotide **3** which was characterised by ES-MS analysis.[15] The trityl protection was then removed by treating **3** with a 80% aqueous acetic acid solution overnight at room temperature. The yield of the acidic deprotection was quantitative as shown by HPLC analysis. However, due to the high reactivity of the oxyamino group, the oligonucleotide **4** containing the free oxyamine was not isolated. The oligonucleotide **4** was characterised using ES-MS analysis by the oxime adduct **6** formed immediately after addition of acetone.[15] The 80% aqueous acetic acid solution, containing the crude oligonucleotide **4**, was then spotted directly on the aldehydic slides **1** to afford the surface bound oligonucleotides. Different concentrations (10, 5 and 1 μM) of the 5′-tailored oxyamino oligonucleotide **4** were spotted manually (130 nL/spot) on the aldehydic glass slide **1**. The slide was then kept in a humidified chamber for 4 h at 20 °C. The same protocol was applied for the attachment of the 5′-amino oligonucleotide **5** (5′-d(XTTTTTGATAAACCCACTCT)-3′ in which X represents the 5′-amino linker) for comparison but in this case, sodium borohydride must be added to reduce the Schiff base formed.



Scheme 1. Preparation of the aldehyde functionalised glass slides **1**. Reagents and conditions: (a) 3.5 M aq NaOH/EtOH (1/1), 2 h then 5 mM triethoxy(4-oxiranylbutyl)silane in toluene, 80 °C, overnight; (b) 0.2 N aq HCl, 3 h; (c) 0.1 N aq $NaIO_4$, 1 h.

The success of the two attachment strategies was tested by the ability of the surface-bound oligonucleotide to hybridise with the complementary strand **7** labelled with a fluorescent probe (5′-d(XAGAGTGGGTTTATCAAAAA)-3′ in which X represents the Cy3™ labelled linker). After hybridisation and washing steps, the glass slide was scanned using a fluorescent scanner. In this manner, the difference in signal intensities directly reflected the proportional difference in covalent attachment efficiency for each oligonucleotide **4** and **5**. Figure 2 shows a representative scanned image from the hybridisation experiments. The first line represents the control using the unmodified oligonucleotide 5′-d(TTTTTGATAAACCCACTCT)-3′. In this case, no covalent bonding of the oligonucleotide on the glass slide should take place. Thus, the very weak fluorescence intensity observed represents the fluorescence background due to the unspecific binding of the fluorescent complementary strand **7** on the aldehydic glass slide. In the other hand, the use of the 5′-oxyamino oligonucleotide **4** and the 5′-amino oligonucleotide **5** leads to the appearance of fluorescent spots. Comparison of the intensities of fluorescence of lines 2, 4, 6 and 3, 5, 7, respectively, confirms as expected that the amount of anchored oligonucleotide increase with increasing oligonucleotide concentration. More interestingly, a higher intensity of fluorescence is observed for the oligonucleotide **4** in comparison with the oligonucleotide **5** at each concentration. As an example, the intensity of fluorescence for the spots of the lowest concentration (1 μM) of oligonucleotides **4** and **5** (lines 7 and 6, respectively) is quite the same as the control (line 1) for **5** whereas for **4** the intensity is still at the maximum. These results clearly show that a higher amount of oligonucleotide was



Scheme 2. Synthesis of the 5′-oxyamino oligonucleotide **4** from the phosphoramidite **2** and reaction of the 5′-modified oligonucleotides **4** and **5** with the aldehydic glass slides **1**. Reagents and conditions: (a) automated DNA synthesis then NH_4OH 28%, 55 °C, 24 h; (b) 80% aq AcOH, rt, overnight; (c) acetone/H_2O, rt; (d) spotting (130 nL) on the aldehydic glass slide **1**; (e) spotting (130 nL) on the aldehydic glass slide **1** in presence of $NaBH_4$.

bound on the glass slide in case of **4** versus **5** and thus demonstrate the efficiency of the oxime bond formation for covalent binding of oligonucleotides on glass support.

The strategy via oxime bond formation was then used for the attachment of oligonucleotides inside a capillary tube. A glass capillary tube (internal diameter: 100 μm) was functionalised with aldehydic groups using the same protocol as for the glass slide **1**. The aldehydic functionalised tubes were then connected to an aspirator pump (flow rate: 5 μL/min) and dipped into an aqueous solution containing the 5′-oxyamino oligonucleotide **4** or the 5′-amino oligonucleotide **5** for 2 h (C = 10 μM). After washing the tubes by aspiration of water, the hybridisation experiment was performed by dipping the capillary tubes in a solution containing the complementary Cy3™ fluorescently labelled oligonucleotide **7**. After several washings with water to remove the non-hybridised complementary strand, the capillary tubes were scanned using a fluorescent scanner (Fig. 3). A control was performed by dipping the capillary tube, after the attachment of 5′-oxyamino-oligonucleotide **4**, into a solution containing a non-complementary Cy3™ fluorescent strand. In this case, only the 'natural' fluorescence of glass is observed at the border of the tube (capillary tube C in Fig. 3).

Figure 3 clearly shows a great difference between the strategy of attachment using the oligonucleotides **4** and

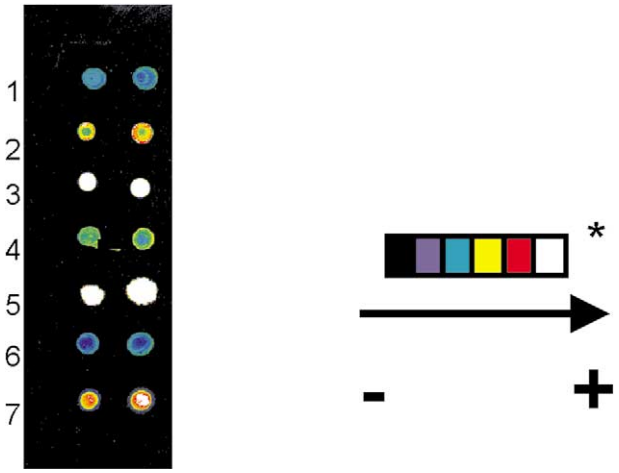


Figure 2. Scanned image of hybridisation experiments with the complementary strand labelled with Cy3™ (C = 0.2 μM). Line 1: deposition of unmodified oligonucleotide (10 μM), lines 2, 4 and 6: covalent attachment of 5′-amino oligonucleotide **5** (concentration is 10, 5 and 1 μM, respectively), lines 3, 5 and 7: covalent attachment of 5′-oxyamino oligonucleotide **4** (concentration is 10, 5 and 1 μM, respectively). Spotted volume = 0.13 μL.* black = 0 a.u. (arbitrary units), blue = 10 a.u., green = 20 a.u., yellow = 30 a.u., red = 50 a.u., white = 65 a.u. (saturation).

5. For **5** (capillary tube A in Fig. 3), the fluorescence is located at the border of the tube as for the control while with the 5′-oxyamino oligonucleotide **4** an intense fluorescence is observed inside the tube (capillary tube B). The chemical attachment of the 5′-amino-oligo-

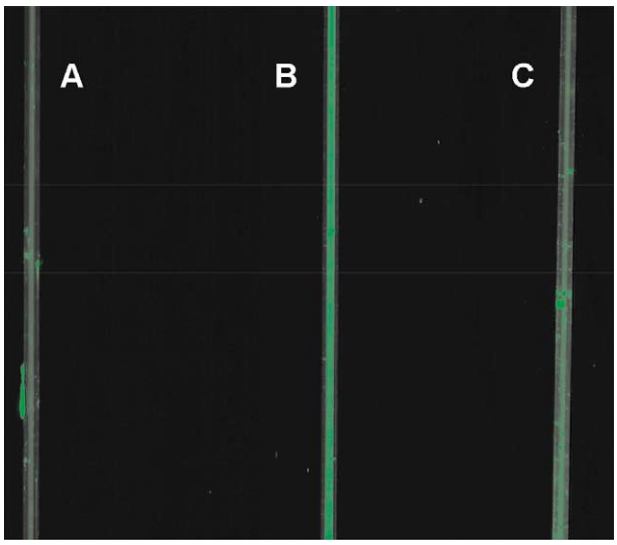


Figure 3. Scanning image of the capillary tube after covalent attachment of the 5′-amino oligonucleotide **5** (A) or the 5′-oxyamino-oligonucleotide **4** (B) and hybridisation with the Cy3™ fluorescent complementary strand. (C) Control: attachment of the 5′-oxyamino-oligonucleotide **4** and hybridisation with a non-complementary Cy3™ fluorescent strand.

nucleotide **5** appears to be inefficient in the 'closed environment' of the capillary tube and thus only the 'natural fluorescence' of glass is observed after hybridisation experiments.

In conclusion, we have demonstrated the efficiency of oxime bond formation for the chemical attachment of oligonucleotides on glass support. A greater quantity of oligonucleotides is anchored on the glass support as compared with the reductive amination strategy. This result is emphasised using a capillary tube in which no anchoring of 5′-amino oligonucleotides can be detected applying the reductive amination strategy. Moreover, no extra agent, such as a reductive or a coupling reagent, is required for performing the oxime formation with the aldehyde functionalised glass support. These data all together demonstrate that oxime mediated surface modification is a promising method that may prove useful in the next future for the ready preparation of new devices for DNA arrays.

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