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Parameters Controlling the One-Step Derivatization of Controlled Pore Glass with a Diol for Solid-Phase Synthesis of 3′-Modified Oligonucleotides

Alain Laurent* and Carole Chaix*

BioMérieux, Parc Polytec, 5 rue des berges, 38000 Grenoble Cedex 09, France, and Unité Mixte CNRS/bioMérieux, IFR 128 Biosciences Lyon Gerland, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69364 Lyon Cedex 07, France

Abstract:

The one-step adsorption of oligoethylene glycol (OEG) affords an efficient derivatization of controlled pore glass (CPG) to perform oligonucleotide (ODN) solid-phase synthesis. This strategy leads to the synthesis of high-quality ODN-3′-OEG. We have investigated various parameters that could influence the adsorption process to improve our understanding of the diol-anchoring mechanism. Diol concentration, solvent, temperature, and reaction time have been studied. Similarly, desorption of ODN-3′-OEG was realized successfully in both weakly basic or neutral conditions. We assumed that the adsorption mechanism involves hydrogen bond-type interactions between diol hydroxyls and silanols from the silica surface.

1. Introduction

Oligonucleotides are powerful molecular biology tools currently used in many research fields, such as gene function and regulation studies or gene therapy. These applications imply an increasing need for modified oligonucleotides, which could provide more specific properties. In the antisense approach, oligonucleotides protected on their 3′-position by a poly(ethylene glycol) (PEG) were reported to be of great interest. The 3′ protection increased ODN stability in biological fluids. To introduce an additional PEG moiety directly during ODN solid-phase synthesis, various pre-derivatized CPG supports have been described in the literature.[1] The strategies used for CPG derivatization require multistep syntheses that are often highly complex and end up increasing the overall cost of solid-supports. Base-cleavable linker arms, like succinyl, oxalyl or phthalyl derivatives, are generally introduced between the reporter molecule and the amino-derivatized support, such as LCAA-CPG, to allow release of the 3′-modified oligonucleotide during ammonia treatment. For hydroxyalkyl functionalization, only a few supports are currently available on the market, providing little choice of modification.

In a previous paper, we described an alternative and versatile method for the synthesis of 3′-diol functionalized ODN, by a one-step derivatization of bare CPG.[2] In relation to the diol immobilized on the silica surface, ODN synthesis

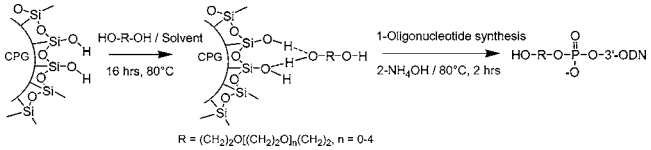

Figure 1. **The CPG one-step derivatization strategy for the synthesis of ODN-3′-diol.**

followed by the deprotection reaction in ammonia resulted in the corresponding ODN-3′-O-PO_2-O-R-OH (R = alkyl, alkoxy, poly(alkoxy)) of high quality, allowing use of a wide choice of different sized diols for ODN functionalization (Figure 1). With regards to the literature about the diol-anchoring mechanism on a silica surface, we have hypothesized that immobilization probably resulted from hydrogen bond-type interactions.[3,4] This specific reaction was mainly observed with primary alcohol and induced an oriented anchoring of the diols through hydrogen bonds with silanols.

In this article, we investigate the different parameters (diol concentration, nature of the solvent used for dilution, temperature, adsorption time) influencing the diol-anchoring process on silica; additionally, we also study the conditions of ODN release in solution after synthesis. Results reported herein confirm the versatility and good reproducibility of the method. This simple procedure of CPG derivatization with diols opens interesting perspectives for low cost solid-supports in automated synthesis. Furthermore, the versatility of the method enables the synthesis of oligonucleotides with a wide variety of 3′-end modifications or functionalities.

2. Experimental Section

2.1. Materials and General Techniques. Reverse phase HPLC analyses were performed on a Waters Alliance 2795 system using a XTERRA C18 MS 2.5 μm column 4.6 mm $\times$ 50 mm (Waters), with an acetonitrile gradient from 3 to 7% (method A) or from 4.5 to 8.5% (method B) in a triethylamonium acetate buffer (50 mM), for 30 min at 60 °C and at 1 mL/min. Part of this crude product was subjected to HPLC purification for mass analysis. Mass spectra for oligonucleotides were performed on a Bruker MALDI-TOF system using 3-hydroxy picolinic acid as a matrix for oligonucleotides.

* To whom correspondence should be addressed. E-mail: carole.chaix@ens-lyon.fr or alain.laurent@eu.biomerieux.com. Telephone: 33 (0)4 72 72 83 60. Fax: 33 (0)4 72 72 85 33.

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2.2. Diol Adsorption on CPG. CPG 2000 Å (100 mg, Fluka) was incubated in 1 mL of diol solution, in an Eppendorf vial. Various diol solutions were investigated to study the main parameters involved in the adsorption process as follows:

- In section 3.1.1., solutions with diol concentrations from 0.1 to 7.5 M in the different solvents (Diglyme, DMF, methanol, DMSO, water) were used for the adsorption reaction onto CPG. All reactions were performed during 16 h at 80 °C.

- In section 3.1.2., 1 mL of TEG solution at 3.7 M in diglyme was used for the study, and the reaction time was kept constant (16 h). In this study, different temperatures were investigated, i.e., 25 °C, 40 °C, 80 °C and 120 °C.

- In section 3.1.3., 1 mL of DEG solution at 4 M in diglyme and 1 mL of HEG (pure diol) were used for the experiment. For both reactions, the time and the temperature were kept constant, i.e., 16 h and 80 °C, respectively.

- In section 3.1.4., 1 mL of TEG solution at 3.7 M in diglyme was used for all experiments. The temperature of the reaction was kept constant. Different reaction times were performed (from 1 h to 16 h), and diol adsorption efficiency was revealed by the amount of oligonucleotide recovered from the solid-phase synthesis.

After reaction, the CPG was filtered, washed carefully with the solvent used for diol dilution (i.e., diglyme, DMF, ...), and then washed with acetone prior to drying in a speed vac at 80 °C for 30 min. Then ~20 mg of CPG were poured into SNAP FIT Column (Applied Biosystems) to perform $(GACT)_n$ sequence synthesis. ODN syntheses were performed on an EXPEDITE 8900 DNA synthesizer (Applied Biosystems), using standard phosphoramidite chemistry at 1 μmol scale. The deprotection step was realized using 500 μL of a concentrated solution of ammonia either at 80 °C for 2 h or at 25 °C for 16 h with no apparent difference. The ammonia solution supernatant was evaporated, and the dry residue was quantified twice by UV spectrometry after solubilization in water. Measurements were made on a 96-well Spectramax 190 (Molecular Devices) spectrophotometer at 260 nm. This value allowed us to calculate the nmol of ODN/mg of CPG. Concerning oligonucleotide syntheses, the average nucleotide coupling yields per cycle were calculated from HPLC profiles using the method described in a previous study.[2]

3. Results and Discussion

3.1. Adsorption Reaction Studies. To better understand the diol adsorption mechanism on controlled pore glass, we have attempted to identify the different parameters controlling the adsorption process. All the studies were performed with one of the following oligoethylene glycols: diethylene glycol (DEG), triethylene glycol (TEG) or hexaethylene glycol (HEG). These compounds afforded an efficient and reproducible loading yield using this adsorption process.

3.1.1. Influence of Diol Concentration and Solvent Used for Diol Dilution on the Adsorption Process. In a previous paper, we described experiments on diol adsorption onto CPG that were directly performed with pure alcohol solutions reacting with CPG under gentle stirring.[2] Although this loading protocol was efficient, it was diol consuming, especially when functionalizing higher amounts of CPG. Furthermore, this method did not allow the adsorption of solid diol. We then considered the diol adsorption process in different solvents. All experiments were performed with TEG at a constant temperature (80 °C) and constant reaction time (16 h). Three aprotic and polar solvents (DMF, DMSO and diglyme) and two protic solvents (methanol and water) were used to solvate the TEG at different concentrations (from pure diol solution at 7.5 M to dilution to 0.1 M in solvent). The final volume of the reaction was constant (1 mL). After the diol adsorption reaction, the synthesis of a GACTGACT ODN sequence was systematically performed on the CPG supports. The graph in Figure 2 shows the amounts of ODN-3′-TEG recovered from the different CPG after ammonia treatment. For each condition experimented in this study leading to notable ODN amounts after synthesis, the HPLC analysis of resulting crude material confirmed the high purity of the oligonucleotide synthesized from the different adsorption conditions (as illustrated by HPLC chromatograms in Figure 3).

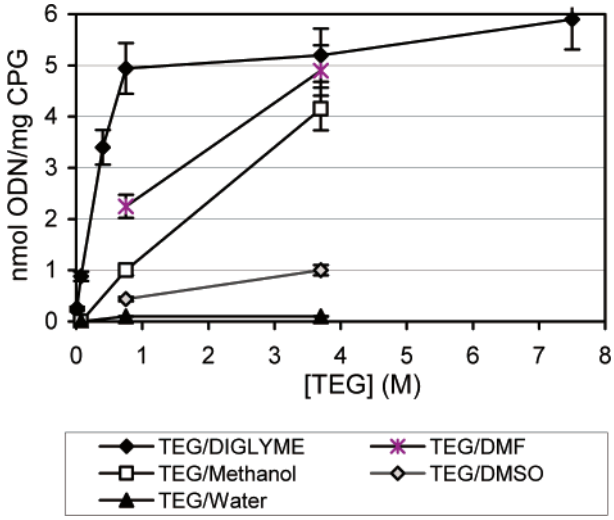

Figure 2. **TEG adsorption yields onto CPG after dilution in different solvents (reaction at 80 °C for 16 h); [TEG] = 7.5 M corresponds to 1 mL of pure TEG solution.**

First, it can be observed that the best solvent for TEG dilution was the 2-methoxyethyl ether (diglyme), as it appeared to be inert enough not to interfere in the diol adsorption process. Dilutions up to 9-fold in this solvent (0.8 M TEG) allowed conservation of a good TEG loading efficiency. At this last concentration of TEG, 5 nmol of ODN per mg of CPG were synthesized. This amount was comparable to that obtained after loading of the pure diol (6 nmol of ODN per mg of CPG). We can approximately calculate that at the TEG concentration of 0.8 M, around 30 equivalents of TEG per silanol were introduced in reaction, when considering for calculation the value of 4.6 silanols per nm^2 (data from literature[4]) and the specific surface area of CPG (2000Å) of 9.2 m^2/gr. This concentration appeared to be the minimum required to reach good diol adsorption efficiency. Indeed, grafting drastically decreased when using lower concentrations of diol in diglyme. Second, experiments

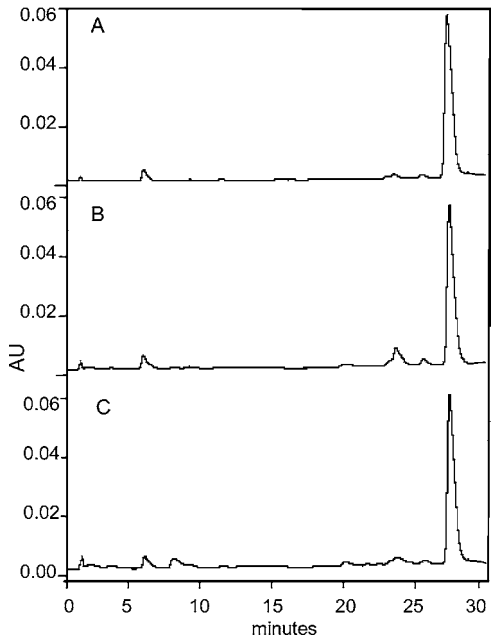

Figure 3. **HPLC analyses of ODN crude materials obtained from the following TEG loading conditions: 3.7 M TEG/ diglyme (A), 3.7 M TEG/ DMF (B), 3.7 M TEG/DMSO (C). Note: Percentages of full length product calculated from the integration of HPLC peak areas are: 88% on chromatogram A, 80% on chromatogram B, 77% on chromatogram C.**

performed in dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) were not as efficient as those performed in diglyme. The higher polarity of these aprotic solvents (dielectric constants ϵ of: (i) DMSO = 49, (ii) DMF = 37, (iii) diglyme = ~7.2) appeared to disturb the diol adsorption mechanism on silica. One property of DMSO used as solvent is that it breaks hydrogen bonds between molecules.[5] The low adsorption rate obtained in this solvent also supports the hypothesis of a mechanism involving surface hydrogen bonds between diols and silanols. Nevertheless, the experiment performed with 3.7 M of TEG in DMF appeared to give satisfactory result as diol adsorption was closer to the value obtained in diglyme than in DMSO. This result is important as DMF provides efficient solubility power for a large range of molecules, especially polar molecules, which are insoluble in organic solvents. Third, experiments were performed in methanol, a polar protic solvent (ϵ = 32). Results also revealed a lower diol-anchoring level in comparison to experiments in diglyme. Nevertheless, at 3.7 M of TEG in methanol, the loading appeared to be relevant, subsequently leading to the synthesis of good quality ODN-3′-TEG as determined by HPLC analysis (data not shown). This result signifies that the use of methanol can also be considered for adsorption on CPG of polar molecules that are insoluble in diglyme. Finally, experiments carried out in water were not successful. This solvent totally inhibited the diol adsorption (ϵ H_2O = 80).

The dielectric constant of solvents seems to be directly correlated to adsorption efficiency. Considering the results obtained on the influence of solvent type on the adsorption process, we used diglyme as the preferred solvent for the other studies described hereafter.

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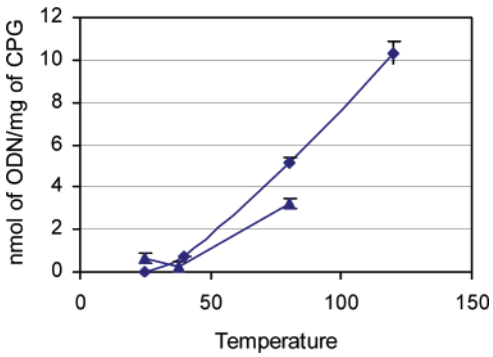

Figure 4. **The amount of ODN-3′-TEG recovered from syntheses on CPG loaded at different temperatures (experimental conditions: 16 hours' reaction at diol concentration 3.7 M in diglyme); ◆: on native CPG; ▲: on dried CPG.**

3.1.2. Influence of Temperature on the Adsorption Process. Temperature is one of the main parameters controlling hydrogen bonding. To demonstrate the direct relation that exists between this parameter and the adsorption mechanism, we studied its influence on diol adsorption efficiency.

For all the experiments of this study, TEG was used at 3.7 M in diglyme and the adsorption reaction time was constant (16 h). This study was carried out on two different kinds of CPG, i.e., native CPG from Fluka and CPG dried overnight in an oven at 150 °C + 8 h at 200 °C (dried CPG). First, it is important to note that in both cases, a relatively high temperature (≥80 °C) was necessary to obtain good adsorption efficiency (Figure 4). A diol functionalization leading to the synthesis of 10.2 nmol of ODN per mg of CPG was reached with a reaction at 120 °C. One hypothesis put forward to explain this behavior is the high viscosity of the diol solution at room temperature, which could reduce the liquid diffusion onto the CPG surface and consequently, adsorption. Heating the medium to 80 °C decreased the viscosity and probably enhanced diffusion of the diol solution inside the pores of the material. On the other hand, we can also suppose that a high temperature is necessary to promote the exchange between physically adsorbed water and the surrounding diol molecules.

Experiments with dried CPG were performed in order to show the influence of physically adsorbed water on the adsorption mechanism. Results in Figure 4 indicate that TEG loading yields obtained in this case were lower than those obtained on native CPG. To explain this difference, we hypothesized that heating CPG for 16 h at 150 °C and for a further 8 h at 200 °C prior to TEG adsorption, probably eliminated water physically adsorbed on the surface and this dehydration might have induced structural changes on the silica surface. This hypothesis was confirmed by a thermogravimetric analysis of CPG. After heating silica to a temperature of 200 °C, a loss of 0.8% of global mass was measured. This corresponds to the elimination of physically adsorbed water. However, when temperature was increased to 300 °C, an additional mass loss of 0.4% was observed that may correspond to molecular rearrangement occurring on the surface. A potential loss of some silanol functions on

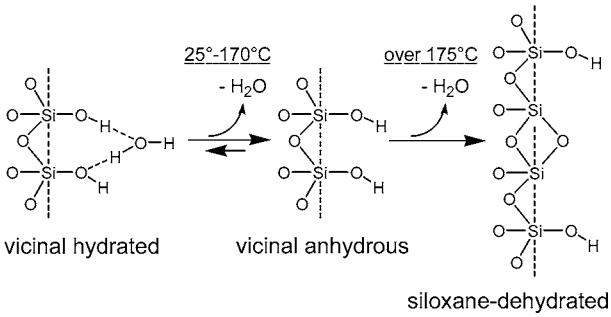

Figure 5. **Postulated types of hydroxyl groups on CPG surface, from Iler et al.[4]**

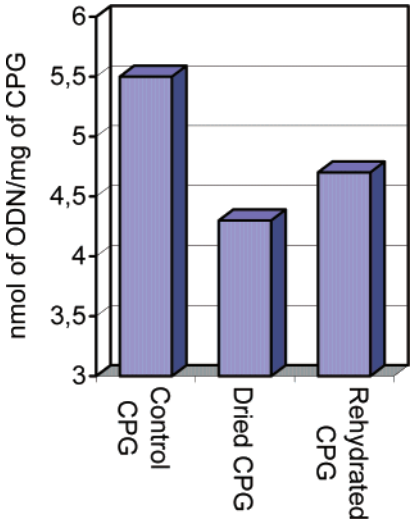

Figure 6. **The amount of ODN-3′-TEG recovered from both dried and rehydrated CPG (16 hours' adsorption reaction at diol concentration 3.7 M in diglyme).**

the surface to the benefit of siloxane bridge formation during drying could explain the lower diol functionalization yields observed when using dried CPG. This advanced dehydration process, illustrated in Figure 5, is supported by data in the literature.[4]

Aiming to recover the initial diol loading of native undried CPG (5.5 nmol/mg), we attempted to rehydrate CPG that had been dried in an oven at 170 °C for 16 h. The rehydration protocol was simply to leave the material for 48 h in ambient atmosphere and temperature conditions. By controlling CPG weight just after drying and again after rehydration on the bench, we observed that 70% of the mass lost during drying was recovered by this simple rehydration method. Oligonucleotide syntheses were then performed on both dried and rehydrated solid-phases. The amounts of oligonucleotide material recovered from the different CPG are indicated in Figure 6. As expected, heating CPG to 170 °C for 16 h induced a decrease in diol loading compared to the experiment's control CPG (standard adsorption reaction at 80°C for 16 h). Our attempt to recover the initial loading on dried CPG by simple rehydration of the surface was not conclusive. The benefit of rehydration appeared to be partial, affording an increase from 4.3 nmol to 4.7 nmol of oligonucleotide material per mg of support synthesized on dried and rehydrated CPG, respectively. Once again, this result could be explained by the partial loss of silanol functions on the

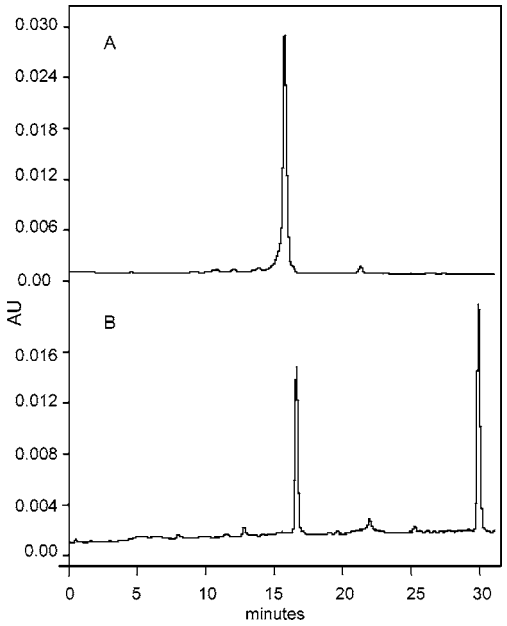

Figure 7. **HPLC analyses of crude ODN-3′-DEG (A) and a mixture of ODN-3′-DEG and ODN-3′-HEG (B). Note: MALDI-TOF MS analysis in negative mode of ODN-3′-DEG (M−H) = 2574.8 g/mol (theoretical: 2577.7 g/mol); ODN-3′-HEG (M−H) = 2754.1 g/mol (theoretical: 2753.7 g/mol).**

surface during drying, leading to a chemical modification of the solid surface that was not reversible by simple rehydration in an ambient atmosphere.

In conclusion, the study confirmed the importance of both physically adsorbed water and silanol functions on the CPG surface for the anchoring mechanism. The hypothesis of diol loading by a process involving hydrogen bond-type interactions is also reinforced by the results obtained.

3.1.3. Diol Exchange at 80 °C. This experiment was performed with the aim of investigating the reversibility of the adsorption reaction at temperature. CPG was first loaded with diethylene glycol (DEG) at a concentration of 4 M (in diglyme) for 16 h at 80 °C. Then, to investigate the possible exchange of DEG with another diol, the DEG solution was removed and the CPG was rinsed and dried. Then, a second adsorption reaction was performed on this support with pure hexaethylene glycol (HEG) for 16 h at 80 °C. Oligonucleotide synthesis was then performed on the two supports and after standard ammonia treatment, ODN crude materials were recovered from the CPG and analysed by HPLC. Chromatograms are depicted in Figure 7. First of all, it is important to note that the second loading of the DEG-CPG by HEG only increased the total amount of ODN recovered after synthesis by 25%, as 4 nmol of ODN/mg of CPG and 5 nmol of ODN/mg of CPG were obtained from DEG-CPG and DEG-CPG reloaded with HEG, respectively. Looking at the chromatograms in Figure 7B, we confirmed that a mixture of ODN-3′-DEG and ODN-3′-HEG was obtained from the CPG that had been loaded twice (by DEG and HEG). Peak areas observed on HPLC indicated a ratio of 55% of ODN-3′-HEG in the mixture. This amount does not simply correspond to the additional 25% loading initially measured by UV quantification of ODN crude material. This high percentage of ODN-3′-HEG confirmed that a partial exchange of DEG

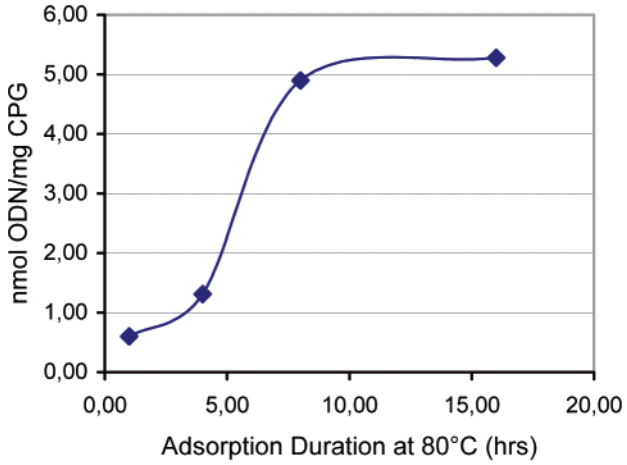

Figure 8. **The effect of TEG adsorption reaction time on the amount of ODN-3′-TEG recovered after ODN synthesis.**

to HEG occurred during the second adsorption step in the presence of HEG. The reversibility of diol adsorption at high temperature is confirmed by this experiment. It is another argument that supports the hypothesis of an anchoring mechanism involving hydrogen bonding.

3.1.4. Influence of Adsorption Time. Different incubation times were investigated to optimize diol anchoring onto CPG. Experiments were performed at 3.7 M of TEG in diglyme and at a constant temperature of 80 °C. Adsorption was stopped at different incubation times, and the GACTGACT sequence was synthesized on the resulting diol-functionalized CPG. After ammonia deprotection, the oligonucleotide material was quantified and analyzed by HPLC. The amount of ODN in relation to reaction time is shown on the graph in Figure 8. For each experiment, the HPLC analysis of crude material confirmed the high purity of the ODN synthesis. The average nucleotide coupling yield per cycle calculated from HPLC profiles[2] appeared to be higher than 98.2%.

We can observe a plateau of adsorption that is reached at 8 h of reaction. Nevertheless, a reaction time of 16 h was preferred to optimize the anchoring process.

In the experiments performed as part of this study at the constant temperature of 80 °C, the solution viscosity did not interfere with diol diffusion onto CPG. So, to conclude from these results, we have evidence that real kinetics of diol adsorption exist. The kinetics obviously depend on temperature, and we could presumably speed up the adsorption process by working at a higher temperature to reach the adsorption plateau more quickly.

3.2. Oligonucleotide Desorption Studies. Aiming to investigate the parameters controlling the oligonucleotide release from CPG, such as the effects of ammonia or the influence of temperature and reaction time, different desorption conditions were experimented. The main results are shown in Table 1. All experiments were performed using the same diol-loaded CPG support elaborated from standard adsorption reaction conditions (i.e., 3.7 M TEG in diglyme, 16 h at 80 °C).

After oligonucleotide synthesis, the three classic ammonia treatments described in the literature for ODN release from a standard succinyl linker CPG (i.e., 2 h at 80 °C, 4 h at 60 °C, and 18 h at 25 °C) [1] allowed recovery in solution of almost the same amount of ODN/mg of diol-functionalized CPG (entries 1, 2, and 3 respectively). The ODN release from the support appeared to be possible at room temperature when the incubation time was increased to 18 h (entry 3). The possibility of using these weaker basic conditions is interesting when applying our strategy to more base-sensitive diols or oligonucleotides, such as oligonucleotides conjugated with base-sensitive fluorophore groups such as Cy5. In entries 4 and 5 of Table 1, we attempted to examine the possibility of releasing the ODN-3′-diol in neutral conditions, with a water/methanol mixture. After treatment, the deprotection of ODN material recovered from CPG was achieved by 2-h reaction in ammonia at 80°C prior to synthesis analysis with RP-HPLC. These experiments were performed in the light of previous results obtained in this study that revealed that protic solvents such as methanol or water disturb the diol-anchoring process (Figure 2). In these neutral conditions, around 30% of the nucleic acid population was recovered in solution after 2 h at 80 °C (entry 4), and around 80% was released after 2.5-h treatment (entry 5). These results confirm the efficient desorption of ODN-3′-diol in neutral conditions, such as the water/methanol mixture, when heating the medium at 80 °C. Exposing the nucleic acid material for longer time in H_2O/methanol conditions led to deterioration of the ODN (entry 6, Table 1), probably due to the high sensitivity of the cyanoethyl phophotriester internucleosidic linkage to hydrolysis in this medium. Nevertheless, these results open up a very interesting route to the synthesis of base-sensitive oligonucleotides, like phosphotriesters[1] or prooligonucleotides,[6] where cleavage from the support in basic medium must be avoided, for non thermo-sensitive oligonucleotides.

Table 1. **Oligonucleotide desorption study in either basic or neutral aqueous conditions**

entry	desorption	ODN[a]	nucleotide average coupling yield (%)
1	NH_4OH (30%/H_2O) 2 h, 80 °C	3.5	98.5
2	NH_4OH (30%/H_2O) 4 h, 60 °C	3.5	98.5
3	NH_4OH (30%/H_2O) 18 h, 25 °C	4	98.5
4	H_2O/CH_3OH (30/70) 2 h, 80 °C	∼1.5[b]	97.8[b]
5	H_2O/CH_3OH (30/70) 2h30, 80 °C	∼3[b]	97[b]
6	H_2O/CH_3OH (30/70) 16 h, 80 °C	∼3.1[b]	ODN degradation
7	H_2O/CH_3OH (80/20) 16 h, 80 °C	∼3.1[b]	ODN degradation

[a] nmol of ODN recovered from desorption per mg of CPG. [b] analysis performed after ammonia treatment (2 h at 80 °C) of the nucleic acid material released from the CPG.

4. Conclusion

In conclusion, these different studies show that the main parameters influencing TEG adsorption are diol concentration, temperature and the type of solvent used for dilution. The best results were obtained at a diol concentration of 3.7 M in diglyme, an aprotic polar solvent, at a temperature of 80 °C for at least 8 h. Nevertheless, DMF or methanol could be considered in the case of particularly insoluble diols.

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Furthermore, after ODN synthesis, weak basic conditions or even neutral aqueous conditions at 80 °C could be applied to totally recover ODN material from the CPG in a non-thermo-sensitive oligonucleotide sequence. We have demonstrated the versatility of the diol adsorption strategy to synthesize a large range of 3′-oligoethylene glycol-modified oligonucleotides. Experiments on 3′-reactive group oligonucleotide syntheses will be reported in due time.

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