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A one step derivatization of controlled pore glass for oligonucleotide solid-phase synthesis

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Abstract—A one step anchoring of various diols on bare controlled pore glass (CPG) support via an adsorption mechanism allowed us to synthesize different (oligonucleotide–3′-diol) conjugates with high purity, through standard phosphoramidite chemistry. Diol loading on CPG proved to be efficient and reproducible. This methodology virtually allows the synthesis of any 3′-modified oligonucleotide, using any reporter molecule containing a diol moiety adsorbed on the CPG. Moreover, vicinal diol as glycol or glycerol enabled the release of some part of oligonucleotide-3′-OH.

1. Introduction

In the field of solid-phase DNA synthesis, actual strategies used for CPG support derivatization are performed by multi-step reactions, which are time and reagent consuming. Derivatization protocols generally require first CPG functionalization with an amino spacer and secondly the introduction of a labile linker between the support and the nucleoside, which initiates DNA synthesis, in order to release the resulting oligonucleotide after the synthesis.[1] Universal supports for DNA synthesis have been described by some authors. For instance, acyclic diol universal linker described by Lyttle and coll.[2] enabled the release of free ODN in solution by a phosphate rearrangement of the linker in ammonia. More recently, Azhayev and Antopolsky[3] proposed a 3-amino-1,2-propanediol based linker, which also rearranged in ammonia, leading to an efficient cleavage of oligonucleotides. In both cases, a phosphate diester function being directly bound to the ODN and bearing a vicinal diol as radical, allowed to induce ODN specific cleavage under basic conditions.

We report here on DNA solid-phase synthesis using new diol derivatized CPG supports resulting from a simple one step adsorption reaction of diols on the silica beads. With relation to the diol immobilized on the solid surface, the achievement of ODN synthesis followed by deprotection reaction led to the corresponding ODN–3′-O–PO_2–O–R–OH (R = alkyl, alkyloxy, substituted, etc.). Preliminary amazing results obtained with this strategy demonstrated that a large range of diols can initiate oligonucleotide synthesis.

In this paper, we focused more specifically on the adsorption studies of short polyethyleneglycol (PEG) molecules. In the antisense approach, oligonucleotides protected on their 3′-position by PEG were reported to be of great interest.[4] The 3′ protection increased ODN stability in biological fluids. ODN–PEG conjugates were also described to form stable and stealthy complexes when associated to PEI.[5]

In addition, we also investigated on the possibility to elaborate a universal support by adsorption of vicinal diol as glycol or glycerol on CPG to generate a ODN-3′-OH. Preliminary results on this latter strategy are described herein. This work opens interesting perspectives in the elaboration of new supports for solid-phase synthesis.

Keywords: Diol adsorption; Solid-phase synthesis; Oligonucleotide; CPG.
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2. Experimental

2.1. Diol adsorption on CPG

Hundred milligram of CPG 2000 Å (Fluka) were incubated overnight at 80 °C in 1 mL of pure diol. CPG was filtrated, washed with acetone and dried in a speed vac at 80 °C over 1 h. Then 20 mg of CPG were poured into an SNAP FIT Column (Applied Biosystems) to perform the synthesis of $(GACT)_2$ sequence. ODN syntheses were achieved on an EXPEDITE 8900 DNA synthesizer (Applied Biosystems) using standard phosphoramidite chemistry at 1 μmol scale. The deprotection step was done using 500 μL of a concentrated solution of ammonia either at 80 °C for 2 h or at 55 °C for 16 h without noticing any difference. Ammonia solution was evaporated and the dry residue was quantified twice by UV spectrometry after solubilization in water. Measurements were achieved on a 96-well Spectramax 190 (Molecular Devices) spectrophotometer at 260 nm.

Reverse phase HPLC analyses were performed on a Waters Alliance 2795 system using a XTERRA C18 MS 2.5 μm column 4.6 × 50 mm (Waters), with an acetonitrile gradient from 4.5% to 8.5% (method A, Fig. 1) or from 3% to 7% (method B, Fig. 3) in triethylammonium acetate buffer (50 mM), for 30 min at 60 °C and at 1 mL/min. A part of this crude product was subjected to HPLC purification for mass analysis.

Mass spectra were performed on a MALDI-TOF system from Bruker using 3-hydroxypicolinic acid as matrix.

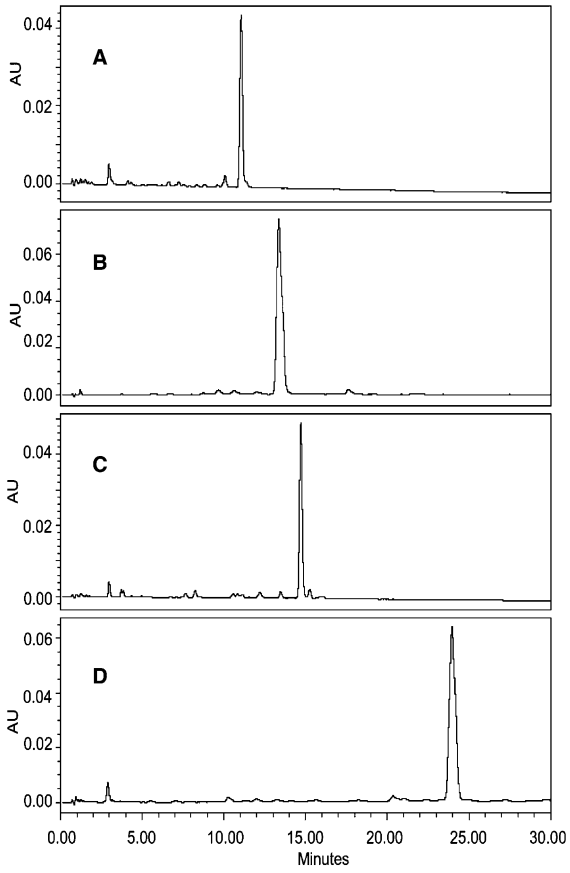
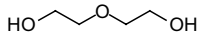
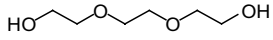
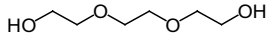
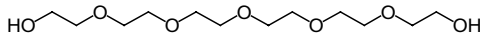
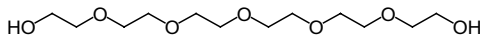
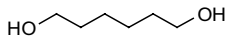
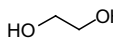
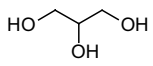


Figure 1. HPLC analyses of $(GACT)_2$ synthesis from different CPG; (A) commercial dT CPG from HTI; (B) diethylene glycol functionalized CPG; (C) triethylene glycol functionalized CPG; (D) hexaethylene glycol functionalized CPG.

Table 1. Oligonucleotide syntheses from different diol derivatized CPG (pore size of 2000 Å)

Entry	Diol	ODN sequence	Nmol/mg[a]	Coupling yield[b] (%)	MS analysis[c] (M−H) g/mol	Expected mass of ODN–diol (M−H) g/mol
1		$(GACT)_2$	6.7	98.2	2574.8	2577.7
2		$(GACT)_2$	4.4	99	2621.1	2621.7
3		$(GACT)_4GA$	3.2	97.8	5733.4	5735.6
4		$(GACT)_2$	4.7	98.6	2754.1	2753.7
5		$(GACT)_4GA$	3.2	97.8	5866.1	5867.6
6		$(GACT)_2$	6.5	97.8	2574.2	2575.7
7		$(GACT)_2$	4.9	98.3	(1) 2532.9 (2) 2408.9	2533.7
8		$(GACT)_2$	5	99	(1) 2562.9 (2) 2562.8 (3) 2409.2	2563.7
9	Control: dT CPG (HTI) 500 Å, functionalized at 35 μmol/g	$(GACT)_2$	34.5	97.9	2409.7	2408.7
10	Control: CPG (Fluka)	$(GACT)_2$	0.3	/	/	

Note: Specific surface of CPG 2000 Å = 9.2 m^2/g and specific surface of CPG 500 Å ~ 45 m^2/g.

[a] Nanomoles of oligonucleotides per milligram of CPG (recovered from ammonia treatment).

[b] Average nucleotide phosphoramidite coupling yield per cycle (%), calculated from integration of HPLC peak areas and with the following equation: $100 \times \left(\sqrt[n]{\frac{(\%\ \text{area of expected ODN})}{100}} \right)$, where n is the number of internucleotide linkages in the sequence.

[c] MALDI-TOF MS analysis in negative mode of main peaks observed on HPLC.

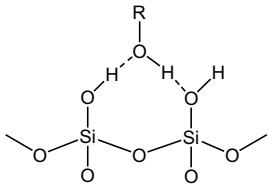


Figure 2. Hypothesis of diol adsorption mechanism through hydrogen bonding on SiO_2 as reported in the literature.[7]

3. Results and discussion

We describe herein a simple method for CPG derivatization that allows automated DNA synthesis to be achieved with efficient yield. A one step adsorption reaction of diol on CPG under gentle stirring overnight at 80 °C led to a stable anchoring of the molecule on the silica surface and the different steps of phosphoramidite DNA synthesis were achieved on the derivatized CPG without any desorption of the diol.

As indicated in Table 1, experiments were carried out with different diols. Molecules as diethylene glycol (entry 1), triethylene glycol (TEG) (entries 2 and 3), hexaethylene glycol (HEG) (entries 4 and 5), 1,6-hexanediol (entry 6) enabled the growth of oligonucleotides.

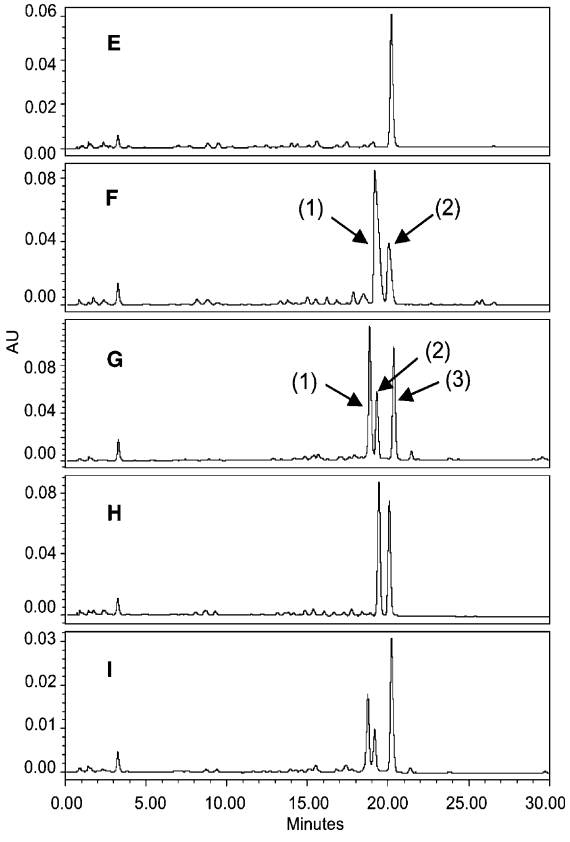


Figure 3. HPLC analyses of $(GACT)_2$ synthesis from different CPG; (E) commercial dT CPG from HTI; (F) glycol functionalized CPG; (G) glycerol functionalized CPG; (H) coinjection of E+F; (I) coinjection of E+G.

The synthesis was initiated from one of the two hydroxyls of the diol, meanwhile the second hydroxyl was engaged in strong hydrogen bounds with silica. HPLC analyses of ODN crude materials revealed that the syntheses were of good quality (Fig. 1). Compared to the A chromatogram, which corresponds to the analysis of $(GACT)_2$ synthesis obtained from a commercial dT-CPG (HTI, Germany), main peak observed on HPLC (chromatograms B–D) corresponded to the oligonucleotide bearing a diol at its 3′ extremity. The elution time increased in relation with the nature of the diol previously adsorbed on the CPG. In regards to peak areas obtained on chromatograms, average coupling yields per cycle of nucleotide synthons were calculated. As shown in Table 1, all values were superior to 97% and confirmed the ODN synthesis efficiency. The same purity was observed on HPLC even after synthesis of the longer sequence $(GACT)_4$ GA from both TEG and HEG functionalized CPG (Table 1, entries 3 and 5). To confirm our results, ODN–diol conjugates were purified by collecting fractions from HPLC and characterized by MALDI-TOF MS (Table 1).

It is important to notice that dimers of oligonucleotides were not detected in crude materials, showing that adsorption mainly occurred at the hydroxyl function and not at the ether function. Furthermore, when adsorption reaction was performed with molecules bearing a single primary alcohol function like di(ethylene)glycol methyl ether or hexa(ethylene)glycol methyl ether, automated DNA synthesis was not allowed. Finally, when diol derivatized CPG was treated 2 h in ammonia at room temperature just before to run oligonucleotide synthesis, no difference was observed regarding the nucleic acid crude material recovered from synthesis, but, on the other hand, when the treatment of 2 h in ammonia was achieved at 80 °C, a drastic decrease of ODN synthesis was noticed, which presumably resulted from diol desorption. This experiment demonstrated that a strong link existed between diol and silica surface.

In the literature, Ogawa and coll. recently described oriented adsorption of diols on SiO_2 in order to perform selective monoprotection of the molecules.[6] They reported that strong interactions occurred between primary alcohols and silanols that allowed reaction to be achieved in organic solvents. Anchoring mechanism was not completely elucidated but probably resulted from hydrogen bond type interactions.[7] This specific reaction was preferentially observed with primary alcohol[6] and induced an oriented anchoring of the diols on the silica surface (Fig. 2). In all cases, the authors

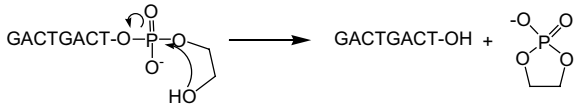


Figure 4. Phosphate rearrangement mechanism in ammonia of oligonucleotide-3′-glycol.

Table 2. Influence of specific surface area on the amount of oligonucleotide synthesized from CPG

CPG	Specific surface area (m^2/g)	Diol	Coupling yield (%)[a]	ODN (nmol/mg)[b]	ODN (nmol/mg/m^2)[c]
CPG 2000 Å (Fluka)	9.2	TEG	97	5.9	6.41
CPG 1000 Å (Fluka)	21.8	TEG	97.3	13.3	6.04
CPG 500 Å (Fluka)	48.6	TEG	97	38.8	7.9

[a] Average nucleotide phosphoramidite coupling yield per cycle (%), calculated from integration of HPLC peak areas.
[b] Nanomoles of oligonucleotide recovered from ammonia treatment per milligram of CPG (related to diol functionalization ratio).
[c] Corrected ODN amount.

described the achievement of only one reaction on the adsorbed molecule. In accomplishing the synthesis of an 18-mer oligonucleotide, we performed in our case a succession of many reactions on CPG, including the different reagents of phosphoramidite DNA synthesis chemistry[1] and numerous washes in acetonitrile.

In order to investigate on the possibility to elaborate a universal CPG support generating a ODN-3′-OH, we synthesized $(GACT)_2$ sequence on glycol functionalized CPG (Table 1, entry 7) and glycerol functionalized CPG (Table 1, entry 8). After ammonia deprotection, ODN population released from glycol CPG showed two important peaks on HPLC (F, Fig. 3). As confirmed by mass analyses (Table 1) and HPLC coinjection with $(GACT)_2$ control (H, Fig. 3), the first peak (1) eluted at 19.2 min corresponded $(GACT)_2$-3′-glycol and the second one (2) eluted at 20 min corresponded to 5′,3′-OH-$(GACT)_2$. This latter product resulted from the well known rearrangement of a glycol phosphate oligonucleotide diester under basic conditions, which liberated 5′,3′-OH-$(GACT)_2$ and more stable cyclic ethylene phosphate diester (Fig. 4).

The same pattern was observed with glycerol (entry 8). Oligonucleotide synthesis from glycerol CPG led to the formation of three different compounds (G, Fig. 3). The ODNs of peaks (1) and (2) on HPLC had the same mass, which corresponded to $(GACT)_2$-3′-glycerol. The two compounds could result from ODN syntheses initiated from primary or secondary hydroxyls of the molecule. The third peak on HPLC eluted at 20.3 min corresponded to 5′,3′-OH-$(GACT)_2$ as indicated by MALDI-TOF MS (2409.2 g/mol) and HPLC coinjection with control (I, Fig. 3). We assume that the same rearrangement reaction occurred during ammonia treatment leading to some part of 5′,3′-OH-$(GACT)_2$ in the mixture. Virtually all vicinal diols and more favourable 'pre-structured' vicinal diol can lead to alkaline cleavage. Additional 2 h treatment in ammonia at 85 °C for mixtures of entries 7 and 8 in Table 1 favoured the appearance of 5′,3′-OH-$(GACT)_2$ in HPLC analyses. But reaction was still incomplete, even after additional hours of ammonia treatment.

Finally, we focused on the amount of ODN synthesized via our strategy, in relation to the specific surface area of CPG used for diol adsorption. Previous results in Table 1 were obtained using CPG from Fluka with pore size of 2000 Å. The specific surface offered by this material was 9.2 m^2/g. After diol derivatization and ODN synthesis, the amount of product recovered from ammonia was quantified by absorbance at 260 nm. A functionalization ratio of ODN per milligram of CPG from 4 to 6 nmol/mg was estimated by this method (Table 1). In order to increase the amount of oligonucleotide produced per milligram of support, CPG with higher specific surface area were used. In Table 2 are reported data obtained with three different triethylene glycol functionalized CPG, with pore size of 2000, 1000 and 500 Å. As expected, the quantity of synthesized ODN increased, in relation with the increase of specific surface area of the support. It is important to notice that the ODN amount obtained by using CPG 500 Å (38.8 nmol/mg) was comparable to the one of commercial dT CPG 500 Å (HTI) (Table 1, 34.5 nmol/mg, entry 9). This suggests that an adsorbed diol approximately occupies the same surface than a covalently linked diol. Moreover in our case, the pre-treatment of CPG under a sulfo-chromic solution, which is classically performed prior to silanization reaction in order to expose silanols on surface was not necessary to reach an efficient diol anchoring rate.

This study revealed that diol adsorption reaction led to an efficient CPG loading. In relation to standard commercial silica supports, comparable amount of oligonucleotides can be obtained by this strategy. Furthermore, when dividing the ODN amounts (nmol/mg) recovered from the different CPG by the respective specific surface area values, resulting corrected ODN amounts (nmol/mg/m^2) were comparable for the three CPG (Table 2). Thus, diol adsorption process appeared to be efficient and reproducible.

4. Conclusion

In summary, we have demonstrated that a large range of diols can be adsorbed on CPG in an oriented way and can initiate oligonucleotide synthesis. This strategy virtually allows one to easily access to any 3′-modified ODN, when using any molecule containing a diol moiety and which can be adsorbed on silica. Moreover, preliminary results obtained with molecules bearing a vicinal diol proved the possibility to synthesize 5′,3′-OH-ODN. Experiments are in progress with other reporter molecules and will be presented in due time.

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