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A Novel Phosphoramidite Method for Automated Synthesis of Oligonucleotides on Glass Supports for Biosensor Development

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Abstract

Two protocols for functionalization of glass supports with hexaethylene glycol (HEG)–linked oligonucleotides were developed. The first method (standard amidite protocol) made use of the 2-cyanoethyl-phosphoramidite derivative of 4,4'-dimethoxytrityl-protected HEG. This was first coupled to the support by standard solid-phase phosphoramidite chemistry followed by extension with a thymidylic acid icosanucleotide. Stepwise addition of the linker phosphoramidite graduated at 1% (relative to the total sites available) per step at 50°C resulted in an optimal yield of immobilized oligonucleotides at a density of 2.24×10^{10} strands/mm^2. This observed loading maximum lies well below the theoretical maximum loading owing to nonspecific adsorption of HEG on the glass and subsequent blocking of reactive sites. Surface loadings as high as 3.73×10^{10}/mm^2 and of excellent sequence quality were achieved with a reverse amidite protocol. The support was first modified into a 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite analog followed by coupling with 4,4'-dimethoxytrityl-protected HEG. This protocol is conveniently available when using a conventional DNA synthesizer. The reverse amidite protocol allowed for control of the surface loading at values suitable for subsequent analytical applications that make use of immobilized oligonucleotides as probes for selective hybridization of sample nucleic acids of unknown sequence and concentration.

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Index Entries: Covalent immobilization; glass supports; solid-phase synthesis; phosphoramidite chemistry; linker; hexaethylene glycol; hybridization; biosensor; oligonucleotides; nucleic acids.

Introduction

The selective hybridization of DNA or RNA of unknown sequence with immobilized oligonucleotides has shown great capacity for the elucidation of sequence information and the detection of mutations and polymorphisms, hence providing the possibility of distinguishing between sequences with single nucleotide changes *(1)*. This principle is currently applied to biochip arrays that are capable of multiplexing the partially selective binding reactions. This approach has potential for DNA sequencing by hybridization, and development of nucleic acid oligomer libraries for screening ligand binding *(2)*. Another recent development in analytical methodology couples oligonucleotide probes to a transducer to provide a biosensor with fast response times on the order of minutes, as well as high sensitivity and selectivity *(3,4)*. Such a sensor could find widespread use for detection of pathogenic microorganisms in areas such as medicine and the food industries *(5)*. The ability to control the coverage density of oligonucleotides on the sensor is crucial for efficient hybridization with complementary DNA. Maximal surface loading might not be desired because steric hindrance may prevent interaction of complementary strands needed for the formation of DNA duplexes, whereas low loadings provide for little signal during analysis.

Most biochip arrays and biosensors rely on the use of silicon dioxide supports such as glass, fused silica, or quartz for immobilizing oligonucleotide probes because these substrates are well suited for fluorescence detection and possess a relatively homogeneous chemical surface that is amenable to modification *(6)*. The use of a spacer arm or linker to attach the oligonucleotide probe to the solid support significantly increases the steric availability of the nucleobases, thus improving hybridization kinetics and efficiencies. To provide hybridization efficiencies that approach those of solution phase reactions, the linker must be at least 28 Å long *(7)*, and other properties such as charge, hydrophobicity, and solvation have additional effects *(8)*. Because most RNA and DNA hybridization studies are carried out in an aqueous environment, the linker is required to have hydrophilic characteristics in order to prevent aggregation of adjacent linker molecules. The use of hexaethylene glycol (HEG) appears to be a very promising approach because of its length, chemical stability, and low hydrophobicity, and has previously been used to attach oligonucleotides to solid supports *(9–11)*. However, the sequence distribution and quantity of the resulting linker-oligonucleotide conjugates have not been thoroughly studied. Furthermore, some of the existing methods did not incorporate any steps to cap unfunctionalized sites subsequent to the linker deposition, and it is possible that a considerable fraction of the oligonucleotides were immobilized directly on the surface of the solid support. This would have formed oli-

gonucleotide probes that are not readily available for hybridization with complementary sequences owing to steric hindrance between the surface and the analyte *(8)*.

In this article, we describe and compare the immobilization of HEG on controlled pore glass using two different approaches that are conveniently available using an automated DNA synthesizer. The first method utilizes a 4,4'-dimethoxytrityl-protected 2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite derivative of HEG (CEP-HEG-DMT) that is attached to the support employing the standard protocol for automated DNA synthesis. The second strategy includes activation of the surface rather than the use of an activated HEG. In this latter approach, entitled reverse amidite protocol *(12)*, the support is first converted into its phosphoramidite analog and subsequently functionalized with dimethoxytritylated HEG.

The linker is easily attached to the glass surface, allows control of the coverage density, uses readily available chemicals, and provides an oligonucleotide linker that is not labile to mild base deprotection reagents *(13)*. Employing the new reverse amidite protocol, subsequent oligonucleotide synthesis on the linker leads to the formation of immobilized full-length strands in high yield with purities that compare well with those obtained by standard oligonucleotide synthesis. Because the linker can be cleaved off the support by extended treatment with concentrated ammonia without affecting any internucleotide bonds, quality control to determine loading and sequence length of oligonucleotides is feasible using high-performance ion-exchange liquid chromatography (HPIEC), polyacrylamide gel electrophoresis (PAGE), and electrospray ionization mass spectrometry (ESI-MS).

Materials and Methods

Chemicals and Instrumentation

Acetic anhydride, aqueous ammonia (30%), calcium hydride, dichloroacetic acid, 1,2-dichloroethane, dichloromethane, diethyl ether, ethanol, HCl, hydrogen peroxide (30%), iodine, 2,6-lutidine, methanol, phosphorus pentoxide, pyridine, tetrahydrofuran, and trichloroacetic acid were purchased from BDH (Toronto, Ontario, Canada). Ammonium acetate, *N*,*N*-diisopropylethylamine, HEG, 1-methylimidazole, sodium bicarbonate, sodium sulfate, and trimethylchlorosilane were obtained from Aldrich (Milwaukee, WI). All phosphoramidite reagents, DNA synthesis grade acetonitrile and tetrazole, 5-ethylthio-tetrazole, 4,4'-dimethoxytritylchloride and controlled pore glass (CPG) (pore size of 1000 Å, particle size of 100–200 µm, surface area of 33.2 m^2/g) functionalized with 5'-*O*-dimethoxytrityl-2'-deoxythymidine in prepacked columns were obtained from Dalton (Toronto, Ontario, Canada). Underivatized CPG (pore size of 500 Å, particle size of 80–120 µm, surface area of 37.5 m^2/g) was purchased from CPG (Lincoln Park, NJ). Dry argon from Canox (Bramalea, Ontario, Canada) was used to provide an inert atmosphere for the preparation of reagents for DNA synthesis, automated oligonucleotide synthesis, and for the synthesis of the HEG-DMT and CEP-HEG-DMT. Water was obtained from a Milli-Q

five-stage cartridge purification system (Millipore, Mississauga, Ontario, Canada) and had a specific resistance of not less than 18 MΩ-cm. Sterile water was prepared from glass double-distilled water by treatment with diethylpyrocarbonate (0.1% [v/v]; Aldrich) and autoclaving (121°C for 20 min, 1 atm overpressure). Organic solvents were dried and distilled as needed and all other chemicals were of reagent grade or better.

Linker deposition as well as oligonucleotide synthesis was done on a 391EP DNA synthesizer (Applied Biosystems, Foster City, CA). Distillation of solvents and preparation of reagents for oligonucleotide synthesis followed the method of Hudson and Damha *(14)* with two exceptions: a 0.25 *M* solution of 5-ethylthio-tetrazole in acetonitrile was used to activate nucleotide phosphoramidites or CEP-HEG-DMT, and detritylation was achieved with a 2% (v/v) solution of dichloroacetic acid in 1,2-dichloroethane. Typically, oligonucleotide synthesis was done on the 1-µmol scale 2-cyanoethyl-*N*,*N*-diisopropyl-phosphoramidite cycle in the trityl off mode as supplied by Applied Biosystems with the exception of extended nucleoside coupling times (10 min). The dimethoxytrityl effluents were collected and acidified with 5% (v/v) trichloroacetic acid in 1,2-dichloroethane, and absorbance at 504 nm was measured in a 1-cm quartz cuvet on an HP 8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, CA).

Linker Synthesis

Synthesis of 18-*O*-(4,4'-dimethoxytrityl)-3,6,9,12,15,18-hexaoxaoctadecan-1-ol (HEG-DMT)

HEG 1.55 g, 5.5 mmol was dissolved in 5 mL of anhydrous pyridine, and 1.69 g (5 mmol) of 4,4'-dimethoxytritylchloride in 10 mL of pyridine was added dropwise at room temperature with stirring over 1 h. The reaction mixture was then stirred overnight. Thin-layer chromatography analysis (dichloromethane:diethyl ether [1:1], staining with HCl fumes) indicated completion of the reaction. The solution was shaken with water (400 mL)/triethylamine (1 mL) to hydrolyze any remaining dimethoxytritylchloride. The crude product was extracted three times with 100 mL of dichloromethane, and the organic phase was washed with water/triethylamine to remove any residual HEG and pyridine. The solvent was removed under reduced pressure, and the residue was purified by column chromatography with silica gel using a solvent system of dichloromethane:diethyl ether (1:1) with 0.5% triethylamine to yield 2.2 g (76%) of HEG-DMT. R_f in dichloromethane:diethyl ether (1:1) = 0.10. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained on a Gemini 200 spectrometer (Varian, Palo Alto, CA). ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 7.47–7.19 (m, 9H), 6.81 (d, 4H, J = 8.8 Hz), 3.78 (s, 6H), 3.74–3.51 (m, 22H), 3.22 (t, 2H, J = 5.8 Hz), purity (HEG-DMT) = 93%.

Synthesis of 18-*O*-(4,4'-dimethoxytrityl)-3,6,9,12,15,18-hexaoxaoctadec-1-yl-*O*'-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (CEP-HEG-DMT)

HEG-DMT (1.2 g, 2 mmol) was dried overnight *in vacuo* and dissolved in 10 mL of anhydrous tetrahydrofuran (THF) under argon. After addition

of 0.75 g (6 mmol) of anhydrous *N*,*N*-diisopropylethylamine, 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite (0.52 g, 2.2 mmol) was added dropwise at room temperature over 1 h with stirring. The solution was stirred overnight and the reaction was monitored by TLC (dichloromethane:diethyl ether [1:1]) the next day. The reaction mixture was then transferred to a separatory funnel and combined with 250 mL of ethyl acetate. The solution was washed twice with 5% aqueous sodium bicarbonate and five times with saturated brine. After drying over sodium sulfate, the organic phase was removed under reduced pressure and the crude product was purified by flash chromatography using a solvent system of 50% dichloromethane in diethyl ether/0.5% triethylamine to give 1.25 g (80%) of CEP-HEG-DMT. R_f in dichloromethane:diethyl ether (1:1) = 0.25. ^{1}H-NMR ($CDCl_3$, 200 MHz) δ: 7.47–7.19 (m, 9H), 6.81 (d, 4H, *J* = 8.8 Hz), 3.78 (s, 6H), 3.74–3.51 (m, 26H), 3.22 (t, 2H, *J* = 5.8 Hz), 2.75 (t, 1H, *J* = 6.6 Hz), 2.64 (t, 1H, *J* = 5.9 Hz), 1.44–1.15 (m, 12H), purity (CEP-HEG-DMT) = 95%.

Linker Addition by Standard Amidite Protocol

Synthesis on Prefunctionalized CPG

A 0.15 *M* solution of CEP-HEG-DMT in 50% acetonitrile/THF (v/v) and a prepacked 0.2-µmol scale column containing 10 mg of 5'-*O*-dimethoxytrityl-2'-deoxythymidine-long-chain alkyl amine (lcaa) functionalized CPG were introduced to the DNA synthesizer. The CEP-HEG-DMT solution was prepared fresh prior to synthesis and used immediately. After deprotection of the 5'-hydroxyl group from the dT functionalized lcaa support, one cycle of a standard 0.2-µmol scale synthesis cycle with the CEP-HEG-DMT reagent was done with the coupling time extended to 16 h. This linker addition cycle was followed by 19 cycles (10 min coupling time) of 5'-*O*-dimethoxytrityl-2'-deoxythymidine-3'-*N*,*N*-diisopropyl(2-cyanoethyl)-phosphoramidite addition to give **1** (Fig. 1), with DMT yield shown in Fig. 2. To analyze the product for yield and purity, a portion of the CPG was removed and treated with concentrated ammonia (16 h, 55°C) to liberate the oligonucleotides from the CPG so that further analysis by HPIEC, PAGE, and ESI-MS could be done.

In a comparable synthesis, four cycles of 5'-*O*-dimethoxytrityl-2'-deoxythymidine-3'-*N*,*N*-diisopropyl(2-cyanoethyl)-phosphoramidite addition on prepacked 5'-*O*-dimethoxytrityl-2'-deoxythymidine-lcaa CPG were done prior to the addition of CEP-HEG-DMT. After the linker immobilization, 15 more cycles of dT addition were carried out to obtain **2** (Fig. 1).

Synthesis on Pure CPG

Prior to synthesis, the substrate was cleaned using a combined base/acid treatment. First, the CPG was suspended in a 1:1:5 (v/v/v) mixture of 25% ammonia, 30% hydrogen peroxide, and water. After stirring the suspension for 5 min at 80°C, the substrate was rinsed with hydrogen peroxide and then treated for 5 min with a 1:1:5 (v/v/v) mixture of 15 *M* HCl, 30% hydrogen peroxide, and water at 80°C. After rinsing thoroughly with

hydrogen peroxide, methanol, dichloromethane, and diethyl ether, the cleaned substrate was dried and stored *in vacuo* and over P_2O_5. To use the DNA synthesizer to functionalize the surface, the CPG was transferred to custom-made Teflon synthesis columns with an id of 6 mm and a length of 7 mm. Prior to synthesis, the columns were heated to 150°C in a vacuum oven for 16 h to remove any residual solvents.

A Teflon column containing 50 mg of dried CPG was attached to the DNA synthesizer and heating tape was used to maintain the column temperature at 25 or 50°C during the linker addition cycle. The CPG was washed with anhydrous acetonitrile, and solutions of 0.025 *M* (1% scale) CEP-HEG-DMT/acetonitrile and 0.25 *M* 5-ethylthio-tetrazole/acetonitrile were delivered simultaneously to the column at a ratio of 1:3 (v/v). For synthesis on the 10% scale, a 0.25 *M* solution of CEP-HEG-DMT in acetonitrile was used. After a coupling time of 90 min and subsequent acetonitrile washing, 14 more CEP-HEG-DMT additions were done, each followed by an acetonitrile washing. Oxidation of the phosphite moieties was then done according to the standard protocol for phosphoramidite chemistry. The column was removed from the synthesizer; washed thoroughly with water, methanol, and diethyl ether; and dried under vacuum overnight. To cap any nonfunctionalized sites on the surface, the column was treated overnight with a 10% (v/v) solution of trimethylchlorosilane in anhydrous pyridine. Subsequently, the substrate was washed with pyridine, dichloromethane, methanol, and diethyl ether. Ten milligrams of the CPG was transferred to another Teflon column and dried overnight *in vacuo*. Using the 1-µmol scale cycle on the DNA synthesizer, dT_{20} was assembled onto the HEG to form **3** (Fig. 1), which could be recovered after cleavage from the surface with concentrated ammonia, for quality control analysis.

Linker Addition by Reverse Amidite Protocol

Figure 3 outlines the reaction scheme of the reverse amidite protocol.

General Procedure

A 50-mg portion of cleaned and dried CPG in a Teflon column was washed with anhydrous acetonitrile and equal volumes of 0.5 *M* 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite and 0.6 *M* *N*,*N*-diisopropylethylamine, both dissolved in anhydrous acetonitrile, and were delivered to the column *(12)*. Ten phosphitylation cycles with a reaction time of 90 min each and intermediate acetonitrile washing steps were done. An alternative approach involved the use of a mixture of equal volumes of 0.5 *M* 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropyl-phosphoramidite and 0.25 *M* tetrazole in acetonitrile, and this solution was delivered to the column in 10 cycles.

Linker addition was then done by treatment of the 2-cyanoethyl-*N*,*N*-diisopropyl-phosphite functionalized CPG with equal volumes of 0.2 *M* HEG-DMT/acetonitrile and 0.25 *M* tetrazole/acetonitrile (90 min at room temperature). This procedure was repeated 10 times with intermediate washings with anhydrous acetonitrile. After the final washing step,

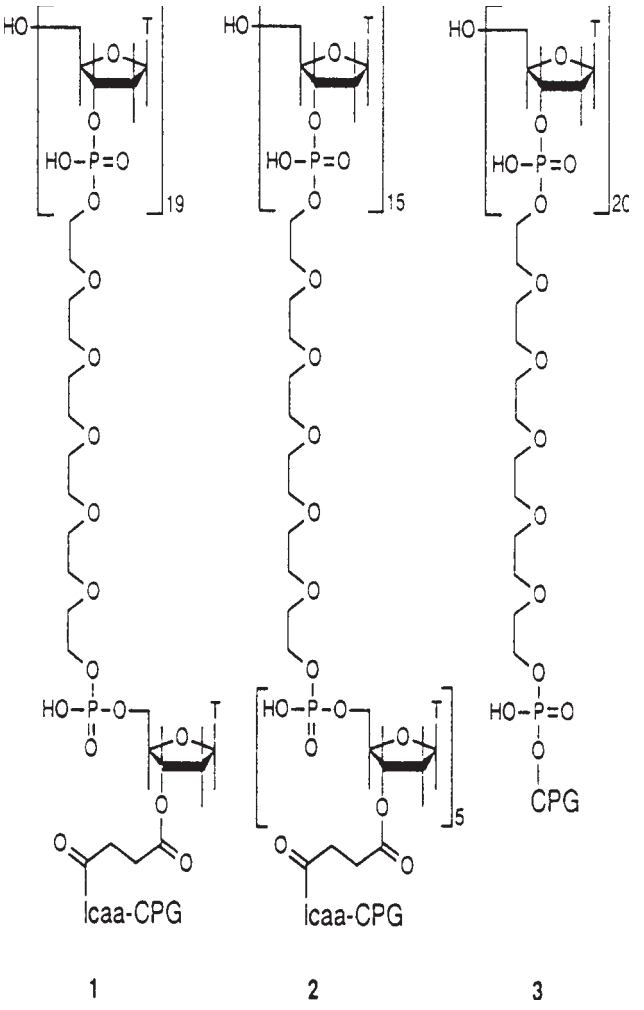

Fig. 1. Chemical structures of immobilized linker-oligonucleotide conjugates obtained with the standard amidite protocol on lcaa-dT functionalized (**1**, **2**) and pure (**3**) CPG.

oxidation, washing, and capping were done as described for the standard amidite protocol. Ten milligrams of functionalized CPG was dried overnight *in vacuo* and functionalized with dT_{20} via automated synthesis to yield **3**.

Determination of Linker Addition Kinetics on Phosphitylated Support

Four columns containing 10 mg of CPG each were attached to the DNA synthesizer in tandem. The support was functionalized with phosphoramidite moieties as already described using 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite/*N*,*N*-diisopropylethylamine. After the first addition of HEG-DMT, the last column in line was removed while purging the remaining three columns with argon to keep their contents

anhydrous. Following 2, 4, and 10 cycles of HEG-DMT treatment, respectively, the remaining columns were removed. After oxidizing, washing, and capping of all the substrates, assembly of dT_{20} was then done on all the CPG samples. The CPG was treated with concentrated ammonia and surface loading was analyzed by HPIEC.

Results and Discussion

In our initial attempts to immobilize HEG on nonfunctionalized CPG to provide a linker for the subsequent synthesis of dT_{20}, we employed a one-step approach using CEP-HEG-DMT as the reactive compound (unpublished results). CEP-HEG-DMT was expected to react with the hydroxyl groups of the support in the same way as the first nucleotide phosphoramidite is coupled during standard DNA synthesis. This endeavor failed for two reasons. First, the bulky HEG-DMT-moiety was sterically hindered, thereby limiting collisions of the CEP groups with surface Si-OH sites in terms of achieving coupling. Second, the hydrophilic nature of HEG facilitated the formation of hydrogen bonds between polyglycol oxygen atoms and hydrogens from hydroxyl groups of the support (unpublished results). It is suspected that most of the CEP-HEG-DMT was adsorbed onto the CPG rather than covalently bound by the terminus, making surface hydroxyl groups unavailable for bond formation. Successive washing steps using hydrophilic solvents eliminated the adsorbed linker molecules from the surface, and subsequent capping with trimethylchlorosilane inactivated the nucleophilic hydroxyl sites on the support.

Linker Addition on Prefunctionalized Glass by Standard Amidite Protocol

The feasibility of CEP-HEG-DMT immobilization under very favorable conditions was examined first. This was done by using commercially available prepacked columns containing CPG functionalized by an lcaa linker onto which one dT is immobilized by means of an intermediate succinate group. The lcaa should reduce steric hindrance and therefore significantly increase coupling efficiency. CEP-HEG-DMT addition on the lcaa-bound dT and subsequent synthesis of dT_{19} should therefore give **1** in good yield. The coupling efficiencies of all steps were monitored by the release of DMT cations, and the results are shown in Fig. 2 (circles). The first data point displays the amount of DMT originating from lcaa-dT-DMT. When CEP-HEG-DMT was added in the second cycle, the number of immobilized DMT groups decreased significantly from 240 to 28 nmol, giving a coupling efficiency of only 12%. The first dT addition, however, led to an unexpected increase in the assay, which indicates that some thymidine was grown directly onto lcaa-dT in addition to immobilization onto the linker. This unwanted side reaction can be explained by nonquantitative capping of surface hydroxyl groups after the linker deposition cycle. During the subsequent immobilization of 18 additional dTs (cycles 4–21), a total

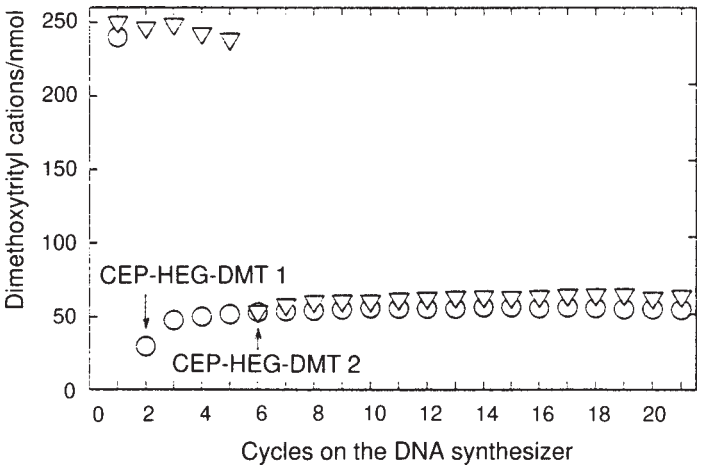

Fig. 2. Dimethoxytrityl analysis during synthesis of **1** (circles) and **2** (triangles). Arrows indicate at which cycles CEP-HEG-DMT instead of CEP-dT-DMT was added to the solid support.

amount of about 50 nmol of oligonucleotides was grown onto the linker and onto the lcaa-dT.

If the assumption that the steric hindrance owing to the bulkiness of CEP-HEG-DMT causes low coupling efficiency is correct, yields should increase when synthesis is carried out further from the surface. An easy way to provide a reaction site distant from the solid support would be to extend the length of the substrate linker by the addition of several nucleobase phosphoramidites before attempting to immobilize CEP-HEG-DMT. Therefore, lcaa-dT_5-OH was synthesized first and then CEP-HEG-DMT was added. Subsequently, 15 additional dTs were grown on the linker to give **2**, having the same molecular mass as **1**. The trityl assay in Fig. 2 (triangles) shows that coupling efficiency was good for the first four dT additions but then decreased to 22% as CEP-HEG-DMT immobilization was attempted. However, this number is about two times higher than the coupling efficiency of 12% during synthesis of **1**. Because the availability of hydroxyl groups for linker coupling was the only difference between the two approaches, it can be assumed that steric hindrance caused by CEP-HEG-DMT represented a limiting factor during the linker deposition step. The following 15 dT additions resulted in nearly constant amounts of DMT cations, which indicated that almost no additional oligonucleotides were immobilized on lcaa-dT_5-OH. Apparently, capping was more efficient than during the synthesis of **1**.

Linker Addition on Pure Glass by Standard Amidite Protocol

Although CEP-HEG-DMT could be immobilized on a linker-activated surface, it remained to be shown how efficiently bare glass substrates could be functionalized. As previously mentioned, treatment of pure CPG with

an excess of CEP-HEG-DMT did not result in covalent immobilization of HEG-DMT. Besides the issue of steric hindrance, adsorption of CEP-HEG-DMT onto the surface was the other major factor that inhibited linker coupling. A strategy to overcome this problem employs a stepwise addition of CEP-HEG-DMT. This gives the CEP-moieties the time to react with Si-OH groups to form the most thermodynamically stable product.

To establish a ratio between surface area and the amount of reagent delivered to this surface, we assumed that all silicon atoms on the surface of the support were available for functionalization. With a Si-O bond length of 150 pm and a bond angle of 109.5°, the area occupied by one Si-atom on a glass surface was estimated to be 5.2×10^{-20} m^2. Additional cations present in glass can be neglected in the calculation because the density of Si-atoms on the surface is always sufficiently high to provide enough immobilization sites. This makes the size of CEP-HEG-DMT the spatially limiting factor regardless of the support being used (glass, fused silica, quartz). The unfunctionalized CPG used in this work had a surface area of 37.5 m^2/g and 50-mg portions were typically used. Hence, several 3.6×10^{19} or 6×10^{-5} mol of Si-OH moieties were available. To provide enough linker molecules to activate all hydroxyl groups, 6×10^{-5} mol of CEP-HEG-DMT had to be introduced into the synthesizer column containing the CPG; for a 0.2-mL vol column the concentration of the solution was 0.3 *M*. The standard 2-cyanoethyl-phosphoramidite protocol delivers only about 25% base amidite to the column whereas the rest of the delivered solution consists of 5-ethylthio-tetrazole. Thus, the initial concentration of the CEP-HEG-DMT solution on the synthesizer port is required to be at least 1.2 *M* to provide enough reagent for all hydroxyl groups. In our unsuccessful one-step attempts, we had used a 0.15 *M* solution to functionalize 10 mg of CPG, which would have been sufficient to activate 62% of available surface sites in only one cycle of CEP-HEG-DMT addition.

To prevent adsorption phenomena, a step-by-step addition was done using a 0.012 *M* solution of CEP-HEG-DMT that should functionalize only 1% of all Si-OH sites in each cycle. An extended coupling time of 90 min was chosen to allow sufficient time for reaction. The coupling reaction was not only conducted at room temperature but also at 50°C to determine whether additional kinetic energy would improve the reaction efficiency. Excess reagent was washed out after the first CEP-HEG-DMT addition and 14 more cycles were run. The synthesis of dT_{20} on the linker followed to give **3**. Data given in rows 4 and 5 of Table 1 indicate that surface loading increased by 50% as the linker was immobilized at 50 instead of 25°C. The purity ratio of $n/n-1$ was almost identical for both temperatures. However, a linker addition at 25°C resulted in the formation of considerable amounts of sequences that were directly immobilized on the surface. An increase in the number of linker addition cycles from 15 to 50 did not further improve the surface loading, but instead led to inferior purity of immobilized oligonucleotides (ratio of $n/n-1$ decreasing to 1.45 instead of 3.30 for 15 cycles; unpublished data). Therefore, we opted to increase the concen-

Table 1
Comparison of Various Linker Immobilization Strategies[a]

Type of support	Immobilization protocol	Surface loading in chains/mm^2	Area occupied per chain/Å^2	Diameter occupied per chain/Å	Purity as ratio $n/(n-1)$
1. CPG-lcaa-dT	None; data for prefunctionalized support	4.35×10^{11}	230	17.1	—
2. CPG-lcaa-dT	Standard amidite: synthesis of 1	5.18×10^{10}	1752	47.2	4.82
3. CPG-lcaa-dT	Standard amidite: synthesis of 2	8.20×10^{10}	1105	37.5	20.18
4. Pure CPG	Standard amidite: 1% CEP-HEG-DMT (25°C)	1.49×10^{10}	6049	87.8	3.45
5. Pure CPG	Standard amidite: 1% CEP-HEG-DMT (50°C)	2.24×10^{10}	4048	71.8	3.30
6. Pure CPG	Standard amidite: 10% CEP-HEG-DMT (50°C)	1.83×10^{10}	4963	79.4	1.82
7. Pure CPG	Reverse amidite/chloroamidite: 1 cycle	1.25×10^{10}	7225	95.9	3.63
8. Pure CPG	Reverse amidite/chloroamidite: 2 cycles	1.73×10^{10}	5242	81.7	5.78
9. Pure CPG	Reverse amidite/chloroamidite: 4 cycles	2.58×10^{10}	3515	66.9	7.09
10. Pure CPG	Reverse amidite/chloroamidite: 10 cycles	3.73×10^{10}	2431	55.6	9.17
11. Pure CPG	Reverse amidite/tetraisopropylamidite: 10 cycles	3.55×10^{10}	2551	56.9	15.23

[a]Data were acquired using quantitative HPIEC. All immobilization protocols were evaluated after synthesis of dT_{20} except for rows 2 and 3, which describe results for synthesis of **1** and **2**. The diameter occupied per chain was calculated assuming that the oligonucleotides are immobilized as densely packed cylinders. Sequence purity is described as ratio $n/n-1$ in which n gives the amount of the desired product and $n-1$ accounts for sequences that contain the linker but lack one thymidine. The purity for standard amidite (1% CEP-HEG-DMT, 25°C) (row 4) is worse than expressed by the index because a significant amount of structures that do not contain the linker could be detected.

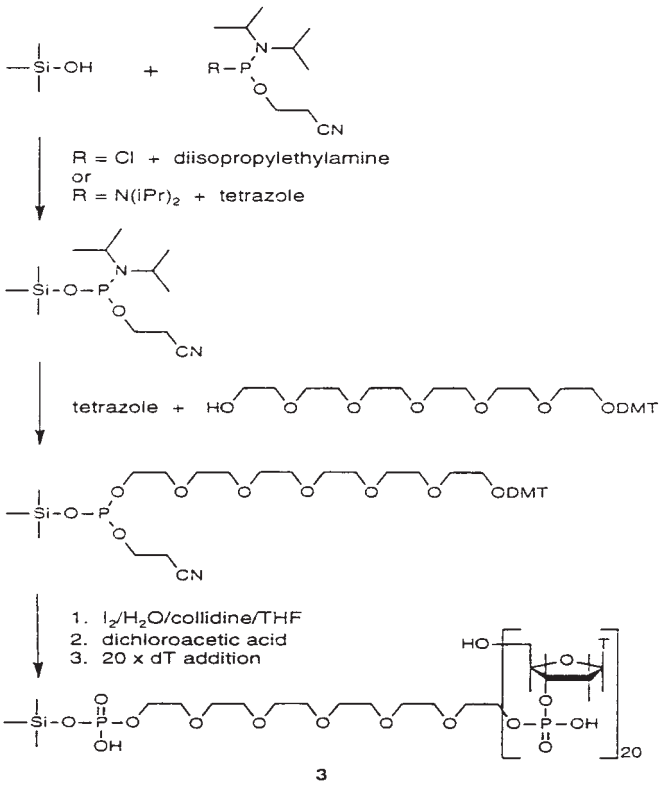

Fig. 3. Derivatization of glass substrates via the reverse amidite protocol.

tration of the CEP-HEG-DMT solution 10-fold to allow activation of 10% of all hydroxyl groups at each of the 15 steps. Surprisingly, row 6 of Table 1 shows that this modification resulted in lower surface loading and drastically reduced purity. Apparently, higher concentrations of CEP-HEG-DMT led to adsorption of linker rather than to formation of covalent bonds. Note that we checked that a poor oligonucleotide synthesis was not caused by an insufficient amount of base phosphoramidite being delivered to the column. The 1-µmol synthesis scale that was used provided enough material to functionalize all available sites even at the highest possible linker densities. However, the standard amidite protocol was shown to be incapable of providing the desired high surface loadings, and therefore a second method was developed.

Linker Addition on Pure Glass by Reverse Amidite Protocol

The alternative approach as outlined in Fig. 3 incorporated the phosphoramidite on the support rather than using the phosphoramidite derivative of HEG. The substrate was first phosphitylated with a mixture of 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite and *N*,*N*-diisopropylethylamine. Both reagents were delivered simultaneously to the

reaction column on the automated DNA synthesizer in concentrations far in excess of the hydroxyl groups on the support. After washing with acetonitrile, the phosphitylation was repeated nine more times. This many treatments may not be required to generate a fully activated surface, but because the success of the reaction could only be determined after completion of the oligonucleotide synthesis, we decided to ensure maximal surface coverage with phosphite groups. Coupling of the linker was then done by repeatedly delivering HEG-DMT and tetrazole simultaneously to the activated support. The addition of dT_{20} produced the same product **3** as was obtained with the standard amidite protocol. Row 10 in Table 1 (reverse amidite/chloroamidite: 10 cycles) shows that yield and purity of the product are significantly better than in any of the previous syntheses on pure glass.

Only a small amount of trityl cations was recovered when the immobilized linker was DMT deprotected during the first cycle of oligonucleotide synthesis (data not shown). At the immobilization of the first nucleobase, the quantity of released trityl cations increased significantly and remained constant throughout the further synthesis. The same observation was made when the standard amidite protocol was carried out, and we have no explanation for this finding. Degradation of DMT during the washing and capping procedure could be ruled out because a sample of CPG was tested before these steps and showed only small quantities of immobilized dimethoxytrityl groups.

During standard oligonucleotide synthesis, water leads to inactivation of the dissolved phosphoramidite species by competing with the hydroxyl groups on the support. Therefore, the concentration of all phosphoramidite reagents is chosen to be far in excess of the support-bound moieties. Any trace amounts of water have only a negligible effect on the synthetic yield, and the availability and reactivity of the support are not greatly reduced. The same is true when CEP-HEG-DMT is used as the phosphoramidite derivative, especially at the 10% scale when 15 steps deliver an excess of phosphoramidite in comparison to surface hydroxyl groups. By contrast, the reverse protocol employs a support-bound phosphoramidite species that will be irreversibly inactivated when contacted with water or any nucleophilic compound other than HEG-DMT. Absolute anhydrous conditions are essential because the phosphitylation as well as the linker addition steps are repeated. Every new cycle bears the risk of introducing water and inactivating the solid support. After completion of the linker addition and oxidation of the trivalent phosphorus, the immobilized HEG-DMT is sufficiently stable against nucleophilic attack. In fact, washing with water and methanol at this point is essential to remove excess HEG-DMT and to inactivate all phosphoramidite sites not functionalized with linker molecules.

The stepwise addition of dilute solutions of CEP-HEG-DMT to functionalize only a certain percentage of the surface was shown to be useful for immobilization of rather pure oligonucleotides on CPG when the surface coverage of the linker was kept low. This approach was possible

because information about the area per weight ratio of the CPG was provided by the manufacturer. Typically, linker deposition was done on 50 mg of CPG, which has a surface area of about 1.9 m^2. Although CPG can be used for hybridization studies in a number of applications *(9)*, biochip arrays and biosensors rely on the use of wafers and fibers that have considerably smaller surface areas. For example, when 50 optical fiber sensors with a diameter of 400 µm and a length of 45 mm are to be functionalized, their combined surfaces would only account for 0.15% of the surface of 50 mg CPG, which would necessitate the use of very dilute reagents on the synthesizer. Another problem arises with the observation that the surface of cleaned fused silica fibers or quartz wafers is not completely flat but shows elevations that add considerably to the surface (unpublished data). This finding provides an explanation for previously reported surface densities as high as 10^{12} oligonucleotide chains/mm^2 giving a diameter for each chain of only 11.3 Å *(15)*. Thus, an exact reagent-to-surface ratio can hardly be established owing to the unknown surface area of most silicon dioxide–based substrates.

The reverse amidite protocol made use of very concentrated reagents throughout the entire linker deposition. During each of the 10 phosphitylation steps with 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite/*N*,*N*-diisopropylethylamine, sufficient reagent to activate all hydroxyl groups on the surface was delivered to the column. The same was true for the subsequent linker addition in which the HEG-DMT concentration was sufficient to allow coupling to all theoretically available sites in one cycle. The excellent yield of the subsequent DNA synthesis and the quality of the immobilized oligonucleotides demonstrate that the use of excess reagent did not result in adsorption of linker molecules on the surface, as was observed with the standard amidite protocol. This result can be explained by a change in surface polarity as the Si-OH groups are phosphitylated. The polar hydroxyl groups are replaced by a trivalent phosphorus and associated relatively hydrophobic moieties. Formation of hydrogen bonds between polyglycol oxygen atoms and hydrogens from the hydroxyl groups is thus prevented, and interactions between surface and linker molecule are limited to formation of covalent bonds. Furthermore, a previously phosphitylated surface generated using the reverse amidite protocol significantly reduces interactions of covalently immobilized linker molecules with any remaining surface groups, and leaves a maximum number of binding sites available for coupling.

Kinetics of Linker Addition on Pure Glass by Reverse Amidite Protocol

Coverage density of oligonucleotide probes on the surface affects the hybridization with complementary strands. It is therefore desirable to achieve control of the number of linker-oligonucleotide conjugates immobilized on the support.

The diameter of a single-stranded DNA is about 20 Å and an additional 20 Å is necessary to provide enough space during hybridization. Hence, the maximum surface coverage should correspond to a pattern in which each oligonucleotide probe is immobilized in the middle of a circle with a diameter of about 40 Å. Studies by Guo et al. *(6)* indicated that hybridization of an immobilized 15mer with a 157 nucleotide polymerase chain reaction (PCR) product is optimal when each oligonucleotide probe occupies an area between 400 and 1300 $Å^2$. Assuming that strands are arranged in a close-packed configuration, this area corresponds to a diameter of 21–41 Å available to each chain for 15mers. A simple model would involve the presumption that the oligomers rotate as rigid rods to spatially fill cones that have the apex located at the surface that is used for immobilization. Consideration of the geometry associated with extension of 15mers to 20mers as used in this work suggests an increase in average area of up to 2400 $Å^2$ (presuming the same angle of rotation for a cone). Note that when hybridization of longer PCR products was studied by Guo et al. *(6)*, a 30% lower surface density was shown to be favorable. These results and considerations suggest that the synthetic goal should be to reproducibly and reliably control 20mer immobilization to achieve average molecular areas on the order of 2000 $Å^2$ or greater for optimization of detection of PCR products in the size range of about 150 nucleotides or larger.

To control deposition of oligonucleotides, three different steps in the reverse amidite protocol can be modified through changes in concentrations and reaction times: phosphitylation, linker coupling, and the first cycle of oligonucleotide addition. Phosphitylation is supposedly a relatively fast reaction owing to the high reactivity of the reagents. Furthermore, because a completely phosphitylated surface reduces adsorption phenomena we decided not to modify this step. The first cycle of oligonucleotide synthesis was left unmodified for two reasons. First, coupling of base phosphoramidites is a fast process because it takes place away from the surface. Reaction times are short to an extent where variations would become irreproducible. Second, incomplete functionalization of linker molecules during the first base phosphoramidite addition would lead to synthesis of shorter sequences. This is supported by our observation that acetic anhydride capping after the first cycle is not quantitative (unpublished results).

The stepwise addition of HEG-DMT, however, is slow owing to steric hindrance. We therefore decided to vary the number of cycles during which HEG-DMT is delivered to the column. Samples were subject to 1, 2, 4, and 10 coupling cycles and dT_{20} was then synthesized on the terminal hydroxyl of the HEG. The data of the coupling reaction shown in Fig. 4 reveal that 1.25×10^{10} chains/mm^2 were immobilized during only one cycle. Ten cycles gave a coverage density of 3.73×10^{10} chains/mm^2, corresponding to a chain diameter of 55.6 Å. It was observed that the sequence purity of the oligonucleotides (i.e., the ratio between the n and the $n-1$ sequence) improved significantly with increasing number of immobilized linker-oligonucle-

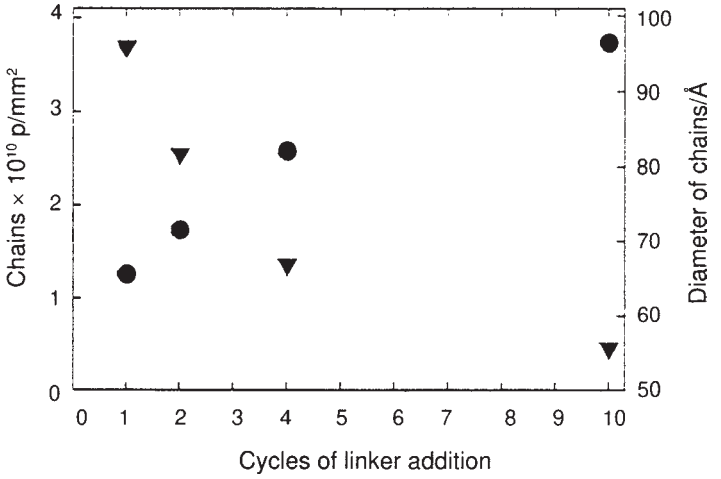

Fig. 4. HEG-DMT deposition on CPG that was previously activated via the reverse amidite protocol using 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite/*N*,*N*-diisopropylethylamine. After immobilizing the linker in varying densities, dT_{20} was grown and the product was quantified with HPIEC. Surface loading is given as the number of immobilized chains $\times 10^{10}/mm^2$ (circles, left axis) and as diameter in angstroms occupied by each chain (triangles, right axis).

otide conjugates (rows 7–10 in Table 1). This is probably caused by better availability of the 5' end of the growing chains as they are forced to arrange perpendicularly to the surface with increasing packing density.

Linker Addition on Pure Glass by Alternative Reverse Amidite Protocol

Despite the successful immobilization of oligonucleotides with the 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite/*N*,*N*-diisopropylethylamine approach, HPIEC analysis still indicated the presence of some failed sequences. Another problem that arose with the use of 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite is of technical nature. We often observed clogging of the delivery lines on the DNA synthesizer owing to precipitation of the reagent. Although this problem may be solved by reducing the concentration of the reagents, we decided to employ a modified chemistry for the phosphitylation of the support.

In the alternative reverse amidite protocol, the surface was phosphitylated with a mixture of 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphoramidite and tetrazole (R = N[iPr]$_2$ in Fig. 3). The subsequent linker addition followed the same protocol as the 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite method, and 10 cycles of HEG-DMT addition were done. The yield of the subsequent synthesis of dT_{20} compared well with that of the 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite method (cf. rows 10 and 11 in Table 1). The product of the alternative approach

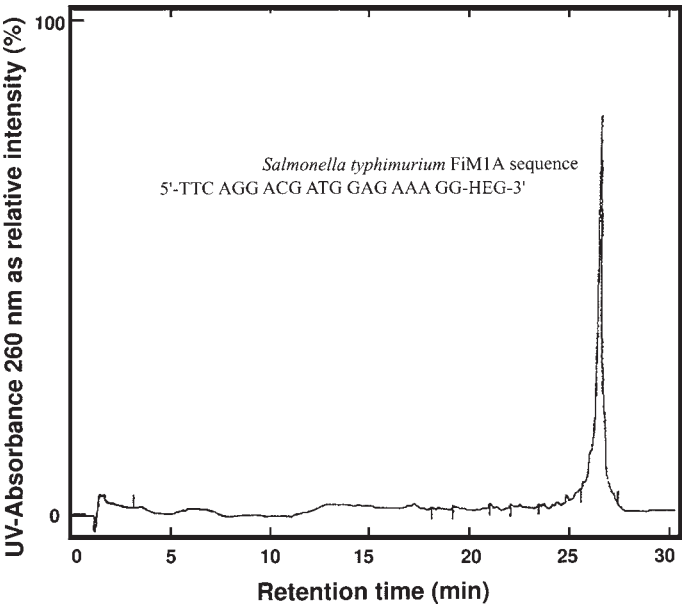

Fig. 5. HPIEC chromatogram showing the purity achieved for synthesis of a 20mer of mixed-base sequence. Immobilization was done using the alternative reverse amidite protocol.

showed an improved purity that approached that of standard oligonucleotide synthesis on lcaa-succinate-activated supports.

The present study focused on the immobilization of oligomers prepared from dT for development of methodology. Applications of immobilized oligomers make use of oligomers of mixed-base sequence. Figure 5 shows an HPIEC chromatogram for a 20mer sequence prepared by the alternative reverse amidite protocol on CPG. This chromatogram confirms the purity of the synthesized oligomer, and recovery yields indicate that oligomers of mixed-base sequence can be immobilized with a density similar to that determined for dT_{20}.

Conclusion

Two different methods of activating glass substrates with HEG-linked oligonucleotides were developed. Their principal difference was that either the linker (standard amidite protocol) or the solid support (reverse amidite protocol) was phosphitylated to provide a reactive compound. The standard amidite protocol employing CEP-HEG-DMT at 50°C gave surfaces with moderate packing densities of immobilized linker-oligonucleotide conjugates and satisfactory sequence integrity when 1% of all surface sites was activated in one step. Our efforts to further increase surface loading failed owing to electrostatic and steric interactions between HEG and the surface. Yet, control over the packing density is one of the prerequisites for

applications of linked oligonucleotide probes in the fields of biochip arrays and biosensors.

The reverse amidite method for deposition of HEG proved to be the method of choice for several reasons. First, the protocol always used an excess of reagents to ensure that yield and base purity of the immobilized oligonucleotides were independent of the substrate surface area. Thus, the large surface area of CPG served to optimize the protocol, and the optimized reaction conditions were successfully transferred to a synthesis on fused silica optical fibers or wafers with smaller surface areas. This was confirmed by batch derivatization of 20 to 30 optical fibers, followed by cleavage of the oligomers from the surfaces and then HPIEC analysis to determine synthetic yields quantitatively.

Second, the reverse amidite protocol allowed for high surface densities with the option to reduce the amount of immobilized oligonucleotides to any desired value by varying the number of HEG-DMT addition steps. Furthermore, the possibility of increasing or decreasing the coupling time for each step offered additional control over the surface loading. Coverage densities obtained in our study already appear quite promising for hybridization experiments.

Third, base purity of the immobilized strands was on the order of standard automated DNA synthesis. Low abundance of failed sequences is especially important because purification of oligonucleotides that are permanently bound to the surface is not feasible. In addition, possible applications of oligonucleotide probes (e.g., detection of single-base mismatches) require the use of probes with a high degree of sequence purity.

Finally, the reverse amidite protocol could be done almost completely on an automated DNA synthesizer, thereby minimizing labor. The capping and washing steps necessitated that the reaction column be taken off the present instrument. However, the recent advent of multiport models allows the automation of these steps.

The stability to hydrolysis of the covalent bond to the surface that is formed in the reverse amidite protocol is relatively poor in comparison to some other methods of coupling such as that using 3-glycidoxypropyltrimethylsilane (GOPS). The stability is sufficient for development of one-shot biosensors. Future work will employ the general strategy for surface activation that is represented by the reverse amidite protocol and will search for coupling reactions with improved resistance to hydrolysis.

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