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Covalent attachment of oligonucleotides to solid supports

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ABSTRACT

Coupling efficiencies for the covalent attachment of oligonucleotides (17-29 bases in length) to solid supports derivatized with alkyl-amino and -carboxylic functionalities have been determined. Attachment efficiencies of 60-80% were obtained for coated long-chain alkylamino controlled pore glass (CPG) supports. Similar efficiencies of immobilization were observed for carboxyl-bearing supports, which additionally exhibited lower levels of non-covalent binding. The extent of terminally linked oligonucleotide was determined to be 50-55% of the overall attachment in the carbodiimide-mediated coupling reaction of a 5'-aminohexyl phosphoramidate derivative of a 29-mer to Sephacryl carboxyl support. While lower overall efficiencies of attachment were obtained in the reaction with Sephacryl N-hydroxysuccinimide-activated carboxyl support, greater than 80% of this coupling results in end-attached oligonucleotides.

INTRODUCTION

The use of nucleic acid hybridization of DNA immobilized on solid supports has become a fundamental technique in biochemistry and molecular biology for the detection, isolation, and genetic analysis of specific DNA sequences. Commonly employed procedures of molecular biology such as dot hybridizations (1), Southern blots (2), colony (3) and plaque (4) transfers are based on the non-covalent immobilization of nucleic acids or DNA restriction fragments on insoluble matrices (5) such as nitrocellulose filters and nylon membranes. While nitrocellulose filters remain the preferred choice as solid supports, the low retention efficiency and the relative inaccessibility of the immobilized nucleic acid to the probing sequence, due to only a small part of the target being available for hybridization, limits its potential sensitivity (6).

The simplicity and convenience of the above mixed-phase hybridizations have led, in recent years, to the development of a number of chemical methods for the attachment of DNA to solid supports through stable covalent linkages. In general, these chemical methods fall into two categories: a) carbodiimide-mediated end attachment of DNA to cellulose, Sephadex (7), or Sephacryl (8) through terminal phosphate groups; and b) immobilization of the nucleic acid via one or more of its bases to suitably activated polysaccharide matrices. Examples of the latter approach include the coupling of DNA to cyanogen bromide-activated supports (9) and to celluloses and Sephacryl via diazotized aromatic amines (8,10). The tethering of nucleic acids to solid supports via stable phosphodiester bonds has found a number of applications (11), but low levels of attachment (8), high non-specific binding to the support (12), and a decrease of coupling efficiencies with increasing length of DNA (13) have rendered this process inefficient. In contrast, good coupling yields of DNA to cyanogen bromide and diazotized arylamine-activated supports have been reported (8-10). However, the use of these supports is restricted to the immobilization of long DNA fragments (8,12) since such chemistry results in multiple attachments, which in turn inhibit hybridization at these points. Furthermore, preparative-scale purification of these fragments to generate specific probes for immobilization is a tedious and expensive process. We have therefore focussed our attention on short oligonucleotide probes for immobilization on solid supports.

Our choice of oligonucleotides in the 20-50 base length range has been influenced by a number of factors. By using automated nucleic acid synthesizers, it is now possible to obtain large quantities of oligonucleotides which can then be obtained in a high degree of purity using reverse phase and ion exchange chromatography. The covalent end attachment of such probes, via spacer arms, to a variety of solid supports results in the extension of the oligonucleotide into the hybridizing medium, which thereby approximates the conditions of solution hybridization. Furthermore, the use of oligonucleotide probes is advantageous in contrast to larger fragments due to their shorter kinetics of hybridization, thus allowing samples to be screened quickly for the presence of target sequences.

In this paper, we present our findings on the attachment efficiencies of oligonucleotides to various supports using phosphate-amine and carboxylic acid-amine functional group pairs to generate phosphoramidate and amide linkages, respectively. We have also investigated a number of supports possessing widely different surface and pore properties for the purpose of determining the optimum support system which would give rise to high levels of attachment, predominant end linkage for greater accessibility to the immobilized probe, and low levels of non-specific binding. The accompanying paper (6) examines the hybridization efficiencies of such support-bound oligonucleotides.

MATERIALS AND METHODS

T4 polynucleotide kinase was purchased from Boehringer Mannheim, [γ-^{32}P]-ATP (7,000 Ci/mmole) from ICN, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) from Calbiochem. Glutaric acid monomethyl ester was obtained from Fluka AG, and sheared salmon sperm DNA, yeast tRNA, polyethylene glycol, polyvinylpyrrolidone, chondroitin sulfate A, and cyanogen bromide were obtained from Sigma. Chondroitin sulfate C and 1% cross-linked divinylbenzene-polystyrene chloromethyl resin (1.1 mM Cl/g, 200-400 mesh) were obtained from U.S. Biochemical Corp. All other chemicals were purchased from Aldrich. Long-chain alkylamine and carboxyl controlled pore glass (500 Å pore size, 125-177 μ diameter) were obtained from Pierce. Sephacryl S-500 was obtained from Pharmacia, and 12% cross-linked polystyrene-divinylbenzene resin (200-400 mesh) was purchased from Polysciences.

Coating of Long-Chain Alkylamine Controlled Pore Glass (CPG):

To one gram of long-chain alkylamine CPG support was added 10 ml of 0.1% yeast tRNA in 10 mM Tris and 1 mM EDTA, pH 7.4 (TE). The mixture was agitated using a rotary mixer and, after 16 hours, the support was filtered through a coarse sintered glass funnel, washed with three 40-ml volumes of TE buffer, and dried under high vacuum for 10 hours. The amino groups were titrated by the picric acid method (14), and the level of amino group substitution was found to vary from 10-50 μmoles/g. The process for coating the glass with sheared salmon sperm DNA is identical,

while for polyethylene glycol and polyvinylpyrrolidone, 1% solutions in TE are used.

Preparation of 1% Cross-Linked Polystyrene-Amine Support:

Derivatization of the 1% cross-linked polystyrene resin was carried out according to the procedure of Horiki et al. (15). To 10 grams of 1% cross-linked divinylbenzene-polystyrene chloromethyl resin (U.S. Biochemical Corp.) in 80 ml of N,N-dimethyl formamide were added 2.6 grams of N-t-butyloxycarbonyl-6-aminocaproic acid and 1.28 grams of potassium fluoride, and the mixture was stirred at 80° for 16 hours. The resin was then filtered, washed with methylene chloride, methanol, and acetone, and then subjected to high vacuum (0.01 torr) for 3 hours. Deprotection of the amino functionality with 30% trifluoroacetic acid in methylene chloride for 30 minutes provided the amino-derivatized polystyrene support. Picric acid titration of the amine groups gave a substitution level of 0.17 mmol/g resin.

Preparation of Polystyrene Carboxyl Resin:

An adaptation of the procedure of Bayer et al. (16) was used for the preparation of polystyrene carboxyl resin. To 10 grams of 12% polystyrene-divinylbenzene resin was added 150 ml of 1,2 dichloroethane, and the mixture was brought to a reflux. A filtered mixture of 20 mmoles of aluminium chloride and 10 mmoles of glutaric acid monomethyl ester chloride in 10 ml nitrobenzene was added over a five-minute period, and the reaction mixture was refluxed for an additional four hours. The resin was filtered through a sintered glass funnel and sequentially washed with dioxane:4N HCl (3:1), dioxane:water (3:1), dioxane, ethanol, acetone, and finally methanol, and then dried in a dessicator under vacuum.

To 2 grams of potassium hydroxide in a three-necked flask was added 100 ml of ethylene glycol, and the mixture was heated to 100°C. When the potassium hydroxide was fully dissolved, 5 grams of the derivatized resin were added, along with 3 ml of hydrazine hydrate. The mixture was brought to a reflux and maintained for 3 hours, after which a portion of the liquid was distilled off until the reflux temperature reached 198°C. The flask was then stoppered, and the reflux was continued for 12 hours. The resin was then washed as described for the previous step.

Preparation of Sephacryl-Carboxyl Support:

Cyanogen bromide activation of Sephacryl S-500 was carried out as described by Bünemann (8). To a suspension of 20 grams of freshly prepared cyanogen bromide-activated Sephacryl S-500 in 200 ml of 10 mM potassium phosphate, pH 8.0, was added 16 grams of 6-aminocaproic acid in 4 portions at intervals of 30 minutes, and the mixture was agitated with a blade stirrer for a period of 20 hours. The derivatized Sephacryl was filtered through a sintered glass funnel and washed exhaustively with 10 mM phosphate, pH 8.0, 1 M phosphate, pH 8.0, 1 M KCl, 0.1 M NaOH, and finally water. The wet cake was then suspended in 0.1 M MES, pH 6.0, and stored at 4°C.

Preparation of Chondroitin Sulfate-Coated CPG Supports:

A 2-gram portion of CPG long-chain alkylamine support (Pierce Chem. Co., substitution of 43 μmoles-NH_2/g) was added to 2 grams of chondroitin sulfate (type A or type C) and 250 mg of EDC. The mixture was taken up in 50 ml of 0.1 M HEPES, pH 7.0, and placed on a rotary mixer for 24 hours. The support was collected by centrifugation and washed with 0.1 M HEPES, pH 7.0 (4x, 40 ml each). A ninhydrin test (17) on a portion of the support showed 80-95% coverage of the available amino sites. The remaining sites (presumably sterically inaccessible to chondroitin sulfate) were treated with an excess of glutaric anhydride (0.5 g) in pyridine (40 ml) for 4 hours. The resin was collected by centrifugation and sequentially washed with 95% ethanol (2x, 40 ml), 0.1 M HEPES, pH 7.0 (4x, 40 ml), and 95% ethanol (2x, 40 ml). The support was dried *in vacuo* for 24 hours. A ninhydrin test on a portion of this resin was negative, confirming the absence of any amino sites.

Preparation of N-Hydroxysuccinimide-Activated Carboxyl Supports:

A suspension of carboxy-derivatized Sephacryl S-500 in 0.1 M MES, pH 6.00, was transferred to a sintered glass funnel and was given successive washes with water, 70:30, 50:50, and then 30:70 water:acetone mixture, followed by three washes with acetone, and finally three washes with acetone which had been dried over 4 Å molecular sieves. After filtering, the support was dried under high vacuum (0.01 torr) for ten hours.

To 0.5 grams of dry Sephacryl S-500 carboxyl or CPG-carboxyl

support in 15 ml of dry DMF were added 0.5 grams of dicyclohexylcarbodiimide and 0.3 grams of N-hydroxysuccinimide. The mixture was agitated for 16 hours and then washed with dry DMF (3x, 20 ml) and methylene chloride (2x, 20 ml) to remove excess reagent and dicyclohexylurea. The support was then dried for three hours in vaccuo (0.01 torr).

Synthesis and Purification of Oligonucleotides:

Oligonucleotides were synthesized using the solid-phase phosphoramidite method on an Applied Biosystems 380A automated DNA synthesizer. The purification of tritylated oligonucleotides was carried out using C-18 reverse-phase, semi-preparative chromatography (10 x 250 mm column) using a gradient of 15-35% acetonitrile in 0.1 M triethylammonium acetate, pH 6.6, over 40 minutes. Detritylation using 80% acetic acid in water for one hour, followed by G-50 Sephadex chromatography, provided oligonucleotides which were further characterized by HPLC on an RPC-5 column (18) (4.6 x 250 mm, solvent A, 2 mM Tris, pH 12; solvent B, 2 mM Tris, 200 mM perchlorate, pH 12; gradient 10% B to 50% B over 40 minutes). This last step can also provide additional purification for smaller quantities of oligonucleotides. Enzymatic phosphorylation of the oligonucleotides at their 5′ termini using T4 polynucleotide kinase and cold ATP or [γ-^{32}P]-ATP was performed according to the protocol of Maniatis, et al. (19).

Preparation of 5′-Aminohexyl and 5′-Cystaminyl Phosphoramidate Derivatives of Oligonucleotides:

Reaction of the 5′-phosphorylated oligonucleotides with 1,6-diaminohexane in the presence of 0.1 M EDC in 0.1 M N-methylimidazole, pH 6.0, was carried out according to the direct coupling protocol described by Chu et al. (20) to afford the 5′-aminohexyl phosphoramidate derivatives. The 5′-cystaminyl phosphoramidate derivatives were generated by treatment of the preformed 5′-phosphorimidazolide derivatives with 0.25 M cystamine, pH 7.7, following the two-step method (20,21). The modified oligonucleotides were isolated by ethanol precipitation and were obtained in yields similar to those in the reported procedures.

Determination of Coupling Efficiencies:

Quantitation of covalently coupled DNA was achieved by incorporating a trace of kinase-labeled [^{32}P] oligonucleotide into

each reaction, and after completion of the cycle of washes at each time point, Cerenkov counts were determined in a Beckman LS 7800 liquid scintillation system.

Covalent Attachment of 5' Phosphorylated Oligonucleotides to Amine Supports:

Time Course for the Attachment of Oligonucleotides to Coated, Long-Chain Alkylamine CPG: To a set (6-8) of silanized eppendorf tubes containing 10 mg each of sheared salmon sperm DNA-coated amino CPG (20-30 μmole/g amine substitution) was added 0.11 ml of a freshly prepared solution of 2-10 x 10^{-7} M [^{32}P]-labeled, 5' phosphorylated oligonucleotide in 0.1 M CDI, 0.1 M N-methylimidazole, pH 6.00. As a control to assess non-specific binding, an oligonucleotide solution without the diimide was added to another set of CPG-containing eppendorf tubes. At various times, the support was given quick washes with sodium hydroxide, pH 12, until no further loss of radioactivity was observed. The reactions were generally followed for 50 hours, with occasional agitation of the reactants in the tubes.

Determination of Coupling Yields to Amine Supports: Attachment of oligonucleotides to amine supports was followed by reacting 25 pmoles of the [^{32}P]-labeled 5' phosphorylated oligonucleotides with 50 mg of the support in 0.55 ml of 0.1 M N-methylimidazole, 0.1 M CDI, pH 6.00, for 24 hours, followed by washes with sodium hydroxide, pH 12, to remove unbound oligonucleotide. Non-specific binding was determined by carrying out the reaction in the absence of CDI.

Determination of Coupling Efficiencies of Carboxyl Supports:

Reactions were carried out in duplicate at 23°C. To 50-75 mg of carboxyl support was added a solution of 25-30 pmoles of [^{32}P]-labeled 5'-aminohexyl or a 5'-cystaminyl phosphoramidate-derivatized oligonucleotide in 0.75 ml of 0.15 M CDI, 0.1 M MES, pH 6.0. The mixture was gently agitated in a rotary mixer for one hour or 16-20 hours. Successive washes with 0.1 M potassium pyrophosphate, pH 8.5 (for chondroitin sulfate A- and chondroitin sulfate C-coated long-chain alkylamino CPG supports), or sodium hydroxide, pH 12 (for CPG carboxyl and Sephacryl carboxyl supports), were carried out until there was negligible loss from the support. Duplicate controls were run in the absence of the di-

imide to estimate levels of non-specific binding. Attachment to supports by amino residues other than the primary amine in the HDA linker was measured by reacting 5'-phosphorylated oligonucleotides (without the 1,6-diaminohexane linker) with carboxyl supports in the presence of diimide. Alternatively, the extent of the end attachment was obtained from the acid hydrolysis of the phosphoramidate bond (HCl, pH 2.00, 37°, 4 hours), followed by washes of support with sodium hydroxide, pH 12.00. The loss of radioactivity from the support provides an estimate of end attachment.

The extent of end attachment of the 5'-cystaminyl phosphoramidate-derivatized oligonucleotide was followed by cleavage of the disulfide bond with dithiothreitol (DTT). Thus, 50 mg of oligonucleotide-derivatized support were incubated for one hour at 23°C with 0.2 ml of 0.1 M DTT, 0.1 M HEPES, and 1 mM EDTA, pH 7.7. The support was then washed with NaOH, pH 12.00, to remove dissociated oligonucleotides. The residual radioactivity on the support provides an estimate of terminal linkage.

Attachment of Oligonucleotides to N-Hydroxysuccinimide-Activated Supports:

To a duplicate set of tubes containing 10 mg of N-hydroxysuccinimide-activated carboxyl Sephacryl support were added 20-25 pmoles of 5'-aminohexyl or 5'-cystaminyl phosphoramidate of a 29-base oligonucleotide in 0.2 M HEPES, pH 7.7, and the mixture was agitated for one hour on a rotary mixer. Duplicate controls using 5' phosphorylated oligonucleotide were also run to estimate levels of non-terminal attachment through the bases. Three cycles of washes were then carried out with NaOH, pH 12.00, to remove non-specifically bound DNA, and the efficiency of covalent attachment was determined by Cerenkov counting. The hydrolysis of the phosphoramidate bond under acidic conditions or the DTT reduction of the disulfide linkage provides an estimate of end attachment.

RESULTS

Immobilization on Amine Supports:

The attachment of oligonucleotides via the phosphoramidate linkage to solid supports possessing amine functionalities at the termini of extension arms was the focus of our initial investiga-

tion. This immobilization procedure was dictated by a number of considerations: (1) the high coupling yields for a carbodiimide-mediated reaction of primary amines with 5' phosphate groups of oligonucleotides (20); (2) the availability of a number of amine-derivatized supports from commercial sources which have found extensive application in nucleic acid and peptide syntheses; and (3) the acid lability of the phosphoramidate bond (20) which affords a simple means of measuring the efficiency of end attachment of oligonucleotides. A controlled pore glass support (CPG, Pierce Chemicals) possessing a 20 Å extension arm terminating in an amine functionality and a 1% cross-linked polystyrene divinylbenzene amine resin were used as supports. The polystyrene-amine matrix, possessing a pentylamine extension arm, was generated by modification of a chloromethylated polystyrene resin with t-butyloxycarbonyl-6-aminocaproic acid, followed by deprotection of the amino groups with trifluoroacetic acid (15).

The reaction of amine supports with a [^{32}P]-labeled 5'-phosphorimidazolide derivative (20) of a 17-mer was studied first. The level of non-specific binding was assessed by using the [^{32}P]-5'-phosphorylated oligonucleotide. Our results indicated that the efficiency of attachment to a CPG support leveled off at 35% after 50 hours, a third of which was the result of non-specific binding (data not shown). While coating of the solid support with agents such as sheared salmon sperm DNA, polyethylene glycol, yeast t-RNA and polyvinylpyrrolidone was partially effective in reducing this non-specific adsorption, a limitation of the coupling procedure was the competing hydrolysis reaction of the activated phosphorimidazolide moiety. This undesired reaction was circumvented by generating the phosphorimidazolide *in situ* by reacting the 5'-phosphorylated oligonucleotide with the amine groups in the presence of the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in N-methylimidazole buffer, pH 6.00 (Fig. 1). The time course for the reaction of a 17-mer (Fig. 2) with coated supports showed significantly higher levels of attachment, with 80% being covalently attached after a 24-hour period, while non-specific binding accounted for only 5% of the input DNA.

The efficiency of attachment of longer oligonucleotides to the CPG amine support is somewhat dependent on the size of the

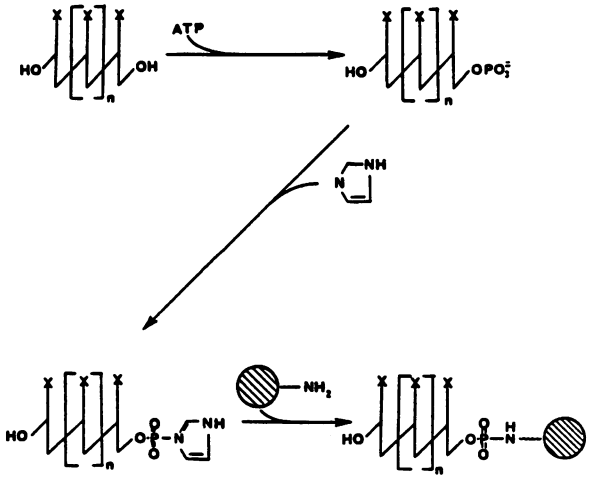

Figure 1: Immobilization of amino controlled pore glass support with 5' phosphorimidazolide derivatives of oligonucleotides generated *in situ* in N-methyl imidazole, pH 6.00, in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

nucleic acid (Table 2). The attachment of a 29-mer to the CPG support leveled off at 67% after 24 hours, with only 6% non-covalent binding. The 1% cross-linked polystyrene-divinylbenzene amine resin was found to be unsatisfactory as a solid support. While coupling efficiencies approaching 40% were observed in its reaction with a 25-mer, the non-specific binding was significantly higher (10-15%).

The phosphoramidate linkage, formed under the conditions described above, can facilely be cleaved by acidic hydrolysis (HCl, pH 2.00, 37°, 4 hours). Such treatment of the CPG amine support resulted in greater than 90% detachment of the oligonucleotide from the surface, indicating that essentially all the nucleic acid was end attached to the support (Table 1). Though reasonable attachment efficiencies were obtained using CPG amine supports, the level of non-specific binding was still quite unsatisfactory. Consequently, we shifted our focus to other functionalized supports which possessed lower non-specific adsorbtion properties.

Coupling to Carboxyl Supports:

Supports bearing carboxyl functionalities were chosen next

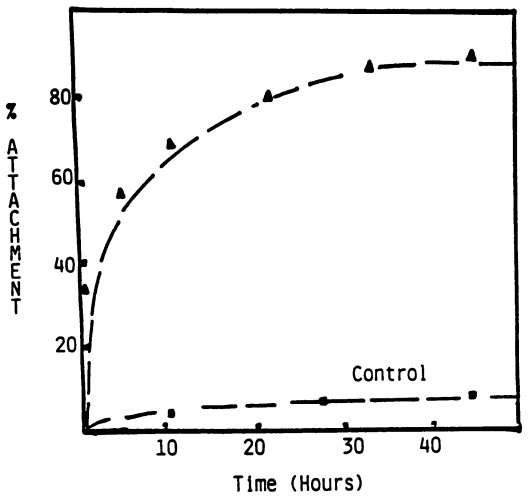

Figure 2: Coupling of a 5′ phosphorylated 17 mer to long-chain amino controlled pore glass support in N-methyl imidazole, pH 6.00, in the presence (▲) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide or absence (■) of diimide. The reaction was followed by incorporation of ^{32}P counts on support.

for study of the coupling reaction to oligonucleotides derivatized at their 5′ end with aminoalkyl functionalities. It was anticipated that the weakly anionic surface property of these supports would be effective in reducing non-specific binding. We also hoped to exploit the greater nucleophilicity of the terminal

TABLE 1

DIIMIDE-MEDIATED ATTACHMENT TO AMINE SUPPORTS

Amine Support[1]	Input DNA (pmole)	Oligo-PO_4 + Diimide[2] % attached (24 hr.)	Oligo-PO_4 (Control)[3] % non-covalently attached (24 hr.)	% end attached (acid hydrolysis)[4]
a) CPG	25	75-80 (17-mer) 67 (29-mer)	5 6	-- 94
b) 1% polystyrene divinyl benzene	25	40 (25-mer)	10-15	--

[1]Coupling efficiencies reported are the average of at least duplicate experiments.

[2]5′ phosphorylated oligonucleotide is mixed with support (50 mg) in the presence of diimide in 0.1 M methyl imidazole, pH 6.00. The sequences of the oligonucleotides were: 17-mer (5′-AATTCACCATGATGTTC-3′); 25-mer (5′-TCCCGTCGAACTAGTGATTATCCTA-3′); and 29-mer (5′-TGCTGCTATGCCTCATCTTCTTGTTGGTT-3′).

[3]5′ phosphorylated oligonucleotide is mixed with support without diimide.

[4]Acid hydrolysis of the phosphoramidate bond with HCl, pH 2.00, 37°C, 4 hours, on immobilized oligonucleotides to determine the extent of end attachment.

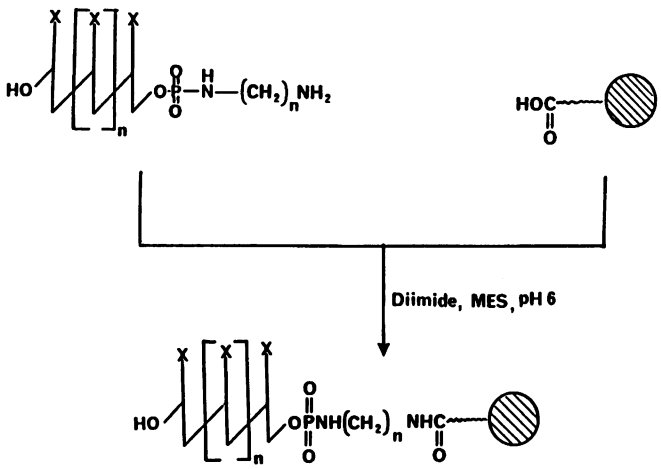

Figure 3: Derivatization of carboxyl supports with 5'-aminohexylphosphoramidate derivatives in MES, pH 6.00, in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

primary amino group of the alkyl linker as compared to the amino functionalities of the bases. It was therefore expected that the carboxyl groups of the support would react preferentially with these primary amino groups to afford predominantly end-attached oligonucleotides.

A number of synthetic routes have reported the introduction of amino groups at the 5' end of oligonucleotides (20,22-24). We focussed on 5'-aminohexyl phosphoramidate derivatives of oligonucleotides (20) since they conveniently allow the introduction of a [^{32}P] label at the 5' end. A number of solid supports possessing widely different physical and surface characteristics were investigated in the immobilization reactions. Sephacryl S-500, chosen for its macroporous and hydrophilic properties (25), was activated with cyanogen bromide following the procedure of Bünemann (8) and then treated with aminocaproic acid to furnish a carboxyl support having a six-carbon extension arm. A 12% cross-linked divinylbenzene-polystyrene resin was included in our studies due to its small exclusion volume and strongly hydrophobic nature. Derivatization of this resin was carried out by Friedel-Crafts acylation of the phenyl groups with glutaric acid monomethylester chloride. Wolff-Kischner reduction, with concomitant hydrolysis, then provided a support possessing pentanoic acid

functional groups (16). A CPG carboxyl support (10 Å extension arm) was obtained commercially. Two other CPG carboxyl supports were generated by covalently coating long-chain alkylamino CPG (Pierce) with the polymers chondroitin sulfate A or C, respectively. This modification was accomplished by diimide coupling of the amino groups of the support with the carboxyl residues of the polymer. The residual amino functionalities were then treated with glutaric anhydride to provide CPG supports possessing both carboxyl and sulfonate functionalities on the surface.

Figure 3 illustrates the reaction used for the attachment of oligonucleotides to the surfaces of these supports. Carboxyl supports were coupled to [^{32}P]-labeled 5'-aminohexyl phosphoramidate oligonucleotide derivatives (20) in the presence of EDC in MES, pH 6.0, and attachment efficiencies were measured after 16 hours. Controls were simultaneously run in the absence of the diimide to determine levels of non-specific binding. Table 2 summarizes the results of the attachment of a 29-mer derivative to the various carboxyl supports. As expected, the levels of non-specific binding were lower than those measured for amine supports. Further reduction of non-specific adsorbtion was achieved with chondroitin sulfate A- and C-coated CPG supports. It is interesting to observe that while the highly anionic sulfonate residues on the surface of these CPG supports appear to block much of the non-specific adsorbtion, respectable attachment efficiencies are still obtained in the coupling reaction. The 12% cross-linked polystyrene-divinylbenzene carboxyl support, which had been chosen for its hydrophobic and highly rigid properties, proved to be disappointing, however, due to the higher levels of non-covalent attachment.

While no significant differences were observed in the coupling efficiencies of the CPG supports and the macroporous, hydrophilic Sephacryl carboxyl support at the end of 16 hours, the initial rate of the reaction appears to be slower with glass supports, as compared to Sephacryl. The reaction is quite rapid with Sephacryl supports and is essentially over after a period of one hour (Table 2). We have also found that the attachment efficiencies of oligonucleotides to the Sephacryl support are independent of size in the 15-29 base-length range (data not shown). Our subsequent work focussed on the Sephacryl carboxyl support,

TABLE 2

DIIMIDE-MEDIATED ATTACHMENT TO CARBOXYL SUPPORTS

Carboxyl Support[1]	Input DNA (pmole)	29-mer-HDA + Diimide[2]		29-mer-HDA (Control)[3]		29-mer-PO_4 + Diimide Control[4]	% end attached after 1 hr. (acid hydrolysis)[5]
		% attached (16 hr.)	% attached (1 hr.)	% non-covalently attached (16 hr.)	% non-covalently attached (1 hr.)	% end attached (1 hr.)	
a) Sephacryl S-500	25	67-75	60-71	1.9	0.5	55-65	50-55
b) 12% polystyrene divinyl benzene	25	50		9			
c) CPG	25	68	18	3	1.1		40-45
d) CPG, condroitin sulfate A coating[5]	25	78		0.5			
e) CPG, condroitin sulfate C coating	25	34		0.4			

[1]50 mg of support was used for determining attachment efficiencies.

[2]Hexylenediamine (HDA) attached to 29-base oligonucleotide (see Table 1 legend for sequence) is reacted with carboxyl support using diimide in 0.1 M MES, pH 6.00.

[3]29-mer-HDA adduct is mixed with 50 mg support without diimide.

[4]5' phosphorylated 29-mer oligonucleotide without HDA is reacted with support in the presence of diimide. The relationship of % end attached =

$$\frac{\%\ \text{attached (29-mer-HDA)} - \%\ \text{attached (29-mer-PO}_4\text{)}}{\%\ \text{attached 29-mer-HDA}}$$

and was used to obtain an approximate extent of end attachment.

[5]Acid hydrolysis of phosphoramidate bond as described in Table 1.

based on the good coupling yields obtained with short reaction times and the support's low, non-specific, background characteristics.

To determine whether the carbodiimide-mediated attachment to Sephacryl carboxyl supports proceeded through the primary amine functionality of the 1,6-diaminohexane adduct or through the potentially available amines in the cytosine residues of the oligonucleotide, diimide coupling was performed for one hour with phosphorylated oligonucleotides having no 1,6-diaminohexane linker. Table 2 shows that approximately 35-45% of the 1,6-diaminohexane adduct is bound to Sephacryl through the bases. Acidic hydrolysis of the phosphoramidate bond between the 1,6-diaminohexane linker and the 5'-terminal phosphate of the immobilized oligonucleotide resulted in the release of 50-55% of the nucleic acid from the Sephacryl support, offering independent confirmation of the extent of end attachment (Table 2). Under acid hydrolysis conditions, the levels of end attachment for CPG carboxyl support were determined to be 40-45% of the quantities originally measured to be attached to this surface.

Because the carbodiimide-mediated attachment of the 5'-aminohexyl phosphoramidate derivatives of oligonucleotides results in significant coupling through the bases (potentially compromising the effectiveness of the immobilized nucleic acid in hybridization reactions), alternate strategies for obtaining end attachment of oligonucleotides have been explored. The selective functionalization of the primary amine group of the linker functionality of 5'-aminohexyl phosphoramidate oligonucleotide derivatives with N-hydroxysuccinimido-biotin (26,27) prompted the investigation of activated carboxyl supports for effecting end attachment of oligonucleotides. Treatment of vacuum-dried Sephacryl carboxyl and CPG carboxyl with N-hydroxysuccinimide and N,N'-dicyclohexylcarbodiimide in DMF under strictly anhydrous conditions provided N-hydroxysuccinimide (NHS)-activated supports. Coupling of the 5'-aminohexyl phosphoramidate derivative of a 29-base-length oligonucleotide to these supports resulted in 52% and 14.5% attachment efficiencies after 1 hour (Table 3). Additionally, greatly decreased attachment through the bases was observed as measured by using phosphorylated oligonucleotides as

TABLE 3

ATTACHMENT TO N-HYDROXYSUCCINIMIDE-ACTIVATED CARBOXYL SUPPORTS

Support[1]	Input DNA (pmole)	29-mer-HDA[2] % attached (1 hr.)	29-mer-PO_4[3] (control) % end attached (1 hr.)	% end attached[4]
CPG	20	14.5	88	80
Sephacryl S-500	20	52	90	83

[1]10 mg of support is used for the attachment experiment.

[2]29-mer-HDA adduct (see Table 1 legend for sequence) is linked to support in 0.2 M HEPES, pH 7.7

[3]5' phosphorylated 29-mer without HDA is reacted with derivatized support.

[4]Acid hydrolysis as described in Tables 1 and 2 is done to determine the extent of end attachment.

controls. Cleavage of the phosphoramidate bonds immobilized on Sephacryl and CPG results in greater than 80% release from these supports, thus suggesting that this chemistry is a viable method for end attachment. However, a variability of the efficiencies of attachment to these NHS-activated supports has been observed. This problem has been attributed to the susceptibility of the succinimido-ester moiety to hydrolysis. Rigorous exclusion of moisture during the preparation of the supports and storage of the vacuum-dried form under nitrogen at 4°C was found to be necessary to insure the stability of the activated carboxyl functionality on the surface.

The attachment of a 5'-cystaminyl phosphoramidate derivative (21) of a 29 base-length oligonucleotide to Sephacryl carboxyl and NHS-activated Sephacryl supports has also been studied (Table 4). The thiol-sensitive disulfide bond of the cystamine linker allows a mild reductive method for evaluating the efficiency of end attachment when such oligonucleotide derivatives are coupled to solid supports. The results from Table 4 indicate close agreement of the end-attachment efficiency obtained from the DTT-mediated reduction of the disulfide bond and acidic hydrolysis of the phosphoramidate linkage.

TABLE 4

ATTACHMENT OF CYSTAMINE-29-MER ADDUCT TO SEPHACRYL SUPPORTS

Support	Input DNA (pmole)	29-mer-cys[1] % attached (1 hr.)	29-mer-PO_4[2] (control) % end attached (1 hr.)	% end attached[3] (acid hydrolysis)	% end attached[4] (DTT reduction)
Sephacryl S-500 carboxyl (diimide-mediated coupling)	25	72	55	58	66
Sephacryl S-500 (NHS-activated)	25	15	83	87	86

[1]Cystamine linker (cys) attached to 29-mer (see Table 1 legend for sequence) is reacted with 50 mg of Sephacryl carboxyl support using diimide in 0.1 M MES, pH 6.00, and to 10 mg of NHS-activated Sephacryl in 0.2 M HEPES, pH 7.7.

[2]5' phosphorylated oligonucleotide without cystamine reacted with the supports, as described in (1).

[3]End attachment was determined by acid hydrolysis of the phosphoramidate bond, as described in Table 1.

[4]End attachment was determined by dithiothreitol (DTT) reduction of the disulfide bond with 0.1 M DTT, 0.1 M HEPES, 1 mM EDTA, pH 7.7, 23°, 1 hr.

CONCLUSION

The chemistries of covalent coupling of oligonucleotides to solid supports are dependent upon the reactive functionalities available at the surface of the support. The factors which must be considered in choosing the chemistry for coupling are: (1) the coupling yields; (2) the ease of manipulation of the support; (3) the magnitude of the non-specific background binding; (4) the ease of modulation of the chemical reactivity of the functionalities of the support; and (5) the extent of end attachment of the coupled oligonucleotide. In this study, we have investigated the attachment characteristics of oligonucleotides to a variety of solid supports possessing amine and carboxyl functionalities. The solid supports possess these reactive groups at the termini of alkyl extension arms. Covalent 5'-end attachment of oligonucleotides via these functional moieties results in tethered nucleic acids which extend out into the solution and can thereby function as probes to target DNA sequences or affinity ligands to DNA binding proteins.

Reaction of 5'-phosphorylated oligonucleotides with coated CPG amine supports in the presence of a water-soluble carbodiimide generated end-attached oligonucleotides in good yields, linked via phosphoramidate bonds (Table 1). Although coating the

CPG-amine support with various reagents was found to be partially effective in reducing non-specific binding (presumably by masking the interaction of the amino groups with the charged phosphate backbone of the nucleic acids), the non-adsorbtion properties of these coated supports were still quite significant.

High attachment efficiencies were also obtained in the diimide-mediated coupling of a 5'-aminohexyl phosphoramidate derivative of a 29 base-length oligonucleotide with carboxyl supports (Table 2). In addition, lower levels of non-specific binding were observed as compared to CPG-amine supports, due to the weakly anionic surface properties of these supports. Further reductions of non-covalent attachment resulted from using chondroitin sulfate A- and chondroitin sulfate C-coated CPG carboxyl supports, due to their negatively charged sulfonate groups on the surface. The rapid attachment of oligonucleotides to the Sephacryl carboxyl support (1 hour, 60-71%), coupled with its low non-specific adsorbtion properties, made this support the preferred system of choice for hybridization studies (6).

An estimate of the extent of end attachment to the Sephacryl carboxyl support was obtained from the acid-catalyzed hydrolysis of the phosphoramidate bond between the diamine linker and the 5'-phosphate group of the oligonucleotide. A significant amount of the reaction with the support proceeds through the amines of the bases, and only 50-55% of the oligonucleotide is end attached via the primary amine functionality of the linker arm. Independent confirmation of the degree of attachment was obtained in the diimide-mediated coupling to Sephacryl carboxyl supports of the 5'-phosphorylated oligonucleotide having no diaminohexyl linker (Table 2). Additionally, a cystaminyl phosphoramidate oligonucleotide derivative was utilized in the coupling reaction to exploit the thiol-sensitive disulfide moiety of the linker arm. The end-attachment efficiencies obtained from the dithiothreitol-mediated cleavage of the disulfide linkage were found to be in close agreement with the values provided by the acid hydrolysis of the phosphoramidate bond (Table 4).

Conversion of the carboxyl functionalities to their N-hydroxy succinimide-activated esters afforded a method for differentiating their reactivity towards the primary amine of the linker arm and the amine of the bases of the 5'-aminohexyl or 5'-cysta-

minyl phosphoramidate oligonucleotide derivatives (Tables 3 and 4). The rapid reaction results in predominantly end-attached oligonucleotides. However, the overall attachment efficiencies were lower than those obtained from diimide-mediated coupling reactions, due to the competing hydrolysis of the activated esters.

Our current research effort is therefore directed towards modifications of the present strategy, to generate more stable activated supports which, upon reaction with oligonucleotide derivatives, afford exclusively end-attached nucleic acids with optimum attachment efficiencies.

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