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Immobilization of DNA via oligonucleotides containing an aldehyde or carboxylic acid group at the 5′ terminus

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ABSTRACT

A general method for the immobilization of DNA through its 5′-end has been developed. A synthetic oligonucleotide, modified at its 5′-end with an aldehyde or carboxylic acid, was attached to latex microspheres containing hydrazide residues. Using T4 polynucleotide ligase and an oligonucleotide splint, a single stranded 98mer was efficiently joined to the immobilized synthetic fragment. After impregnation of the latex microspheres with the fluorescent dye, Nile Red, and attachment of an aldehyde 16mer, $5x10^5$ bead-DNA conjugates could be detected with a conventional fluorimeter.

INTRODUCTION

Many of the commonly used techniques of molecular biology require the immobilization of DNA onto some solid support for use in the fractionation and identification of specific sequences (1). Such methods as binding of polynucleotides onto nitrocellulose (2) or nylon membranes are used extensively; however, because the bonds between these supports and DNA are presumably non-covalent, a certain level of release from the support could be expected to occur (3). Other fractionating supports such as diazotized cellulose (3,4) or cyanogen bromide activated agarose (5, 6) suffer from additional shortcomings; because the DNA is immobilized covalently to these supports through the exocyclic amines of the bases, there is a low, but real, level of mismatch hybrids that can form (7). In addition, this form of immoblized DNA has hybridization rates that are, at best, ten fold slower than the corresponding reaction in solution (8).

It seemed that the concept of a single point, covalent attachment, as first described by Gilham (9), would solve some of these problems. Although this method for preparing support-bound homopolymers is quite satisfactory (10, 11), the attachment of long polynucleotides through their 5′-phosphate onto cellulose (12) or Sephacryl (13) is cumbersome to execute and suffers from low yields. We reasoned that by introducing an electrophilic group at

the 5'-end of DNA and a nucleophilic residue on a support, there would be little chance for the exocyclic amine groups on the bases to react and thus become incapable of base pairing. This modification could be readily achieved by ligating a modified oligonucleotide to the DNA to be immobilized. This approach of chemical and enzymatic reactions should also permit an immobilization scheme in which the synthetic oligonucleotide is attached first, followed by the ligation of the polynucleotide of interest. Other workers have reported the specific modification of the 5'-end of polynucleotides with nucleophiles (14-18), as well as the immobilization of 5'-amine polythymidylic acid to cyanogen bromide-activated cellulose (19).

In an attempt to find a support that would have minimal influence on the behavior of attached DNA, non-porous polystyrene latex spheres were examined. These particles are amenable to chemical functionalization and the spheres provide a great deal of surface area for macromolecular attachment. Their non-porous nature ensures that reagents will act at the surface of the support and not be required to diffuse into a polymer. The small particles, with diameters less than 1 micrometer, generally exist as colloidal suspensions and would be expected to interfere only minimally with the hybridization kinetics of tethered DNA.

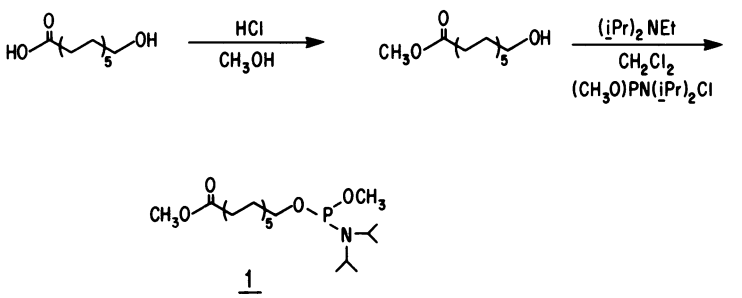
We also noted an additional advantage of the beads, specifically, their ability to absorb fluorescent dyes (20). This property of the non-crosslinked polystyrene beads can allow them to act as non-radioactive labelling moieties for the detection of DNA. Because the beads may be impregnated with a great deal of dye, it is potentially a far more sensitive system than linking individual dye molecules (16, 21, 22).

We describe here one system of chemical and enzymatic reactions that leads to DNA of any length that is attached covalently to a sub-micron, hydrophilic-surface latex sphere through a unique site at the 5'-end. We also outline how these latex spheres, once impregnated with a fluorescent dye, can serve as a nonradioactive label for DNA. An accompanying paper outlines a second method for immobilization and discusses the hybridization behavior of attached DNA (23).

RESULTS AND DISCUSSION

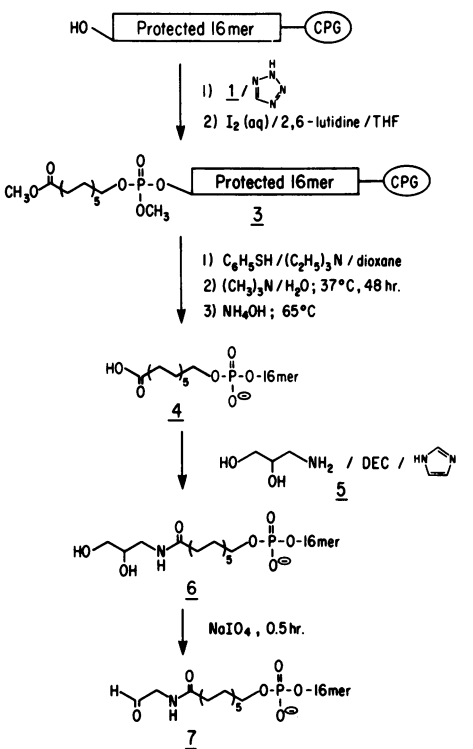
Preparation of Modified Oligonucleotides

Automated phosphoramidite-based DNA synthesis (24) provides a simple, high-yield method to introduce unique reactive groups into the 5'-end of DNA by adding suitably functionalized phosphoramidites at the final cycle of a synthesis. To this end, we have prepared several phosphoramidites containing



Scheme I. Synthesis of carboxylic acid linker arm phosphoramidite 1.

various ω-functionalized aliphatic linker arms (25) which is intended to both tether the DNA to a solid support as well as to permit the immobilized polynucleotide to be held away from the support surface. The preparation of a carboxylic acid linker arm phosphoramidite 1 is outlined in Scheme I.



Scheme II. Preparation of 5'-modified oligonucleotides.

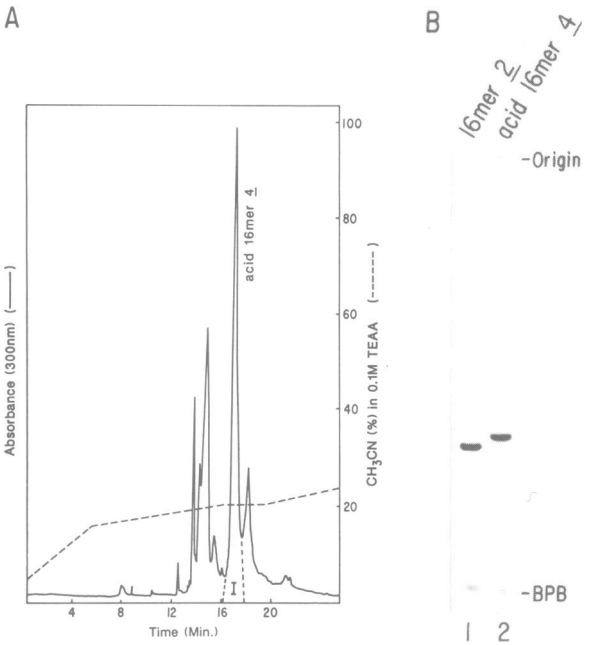


Figure 1. Panel A. HPLC profile for preparative purification of acid 16mer 4. The column effluent corresponding to Peak I contained the desired 5'-modified oligonucleotide. Panel B. 20%/7M urea polyacrylamide gel analysis of unmodified 16mer 2 (lane 1) and HPLC purified acid 16mer 4 (lane 2). Bands were visualized by UV-shadowing.

These studies have been carried out with the 16mer sequence, d(C-G-A-A-G-C-T-T-G-G-A-T-C-C-G-C) 2, chosen to contain recognition sites for HindIII and BamHI restriction endonucleases. Scheme II outlines the chemistry involved in the final cycle of the synthesis of 5'-modified carboxylic acid 16mer 4. The product on the support was deprotected under standard conditions but with an additional step of a treatment with aqueous trimethylamine to effect the hydrolysis of the methyl ester 3 to the carboxylic acid 4. This avoided the formation of the undesired amide that occurs when the molecule is cleaved from the support with aqueous ammonia. It should be noted that the attached aliphatic linker arm of acid 16mer 4 provided a lipophilic purification handle for easy HPLC separation from failed sequences (26) (Figure 1A). It also had a significant effect on the electrophoretic mobility of the 16mer on a polyacrylamide gel (Figure 1B).

An aldehydic linker arm 16mer was also prepared in order to examine coupling via reductive amination to various nitrogen nucleophiles. Reaction

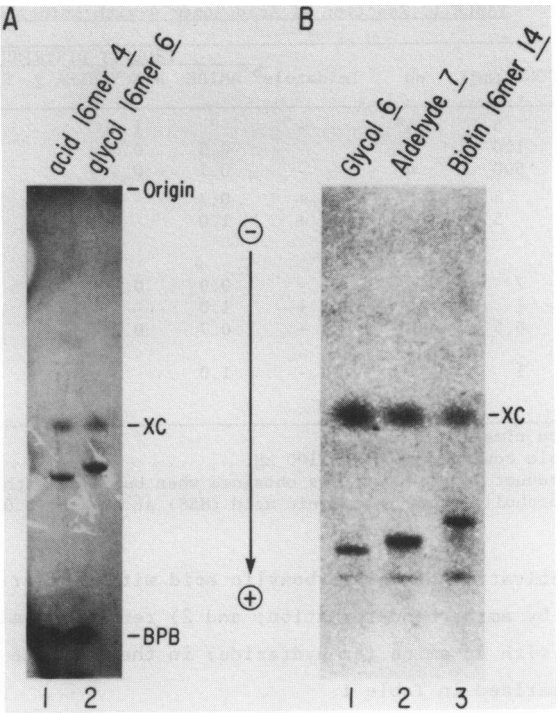


Figure 2. 20%/7M urea polyacrylamide gel analyses. Panel A shows the conversion of acid 16mer 4 (lane 1) to the glycol 16mer 6 (lane 2). Panel B shows the electrophoretic mobility of glycol 6 (lane 1), its $NaIO_4$ oxidation product, aldehyde 7 (lane 2) and its reductive amination product with biotin hydrazide: alkylbiotinylated 16mer 14 (lane 3). The faster moving band in each lane of panel B corresponds to a minor contaminant of acid 16mer 4. Bands were visualized by UV-shadowing.

of the acid 16mer 4 with 3-amino-1,2-propanediol 5 (Scheme II) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) afforded a convenient route to the glycol 16mer 6 (Figure 2A), a stable precursor of the reactive aldehyde 16mer 7. The glycol was cleaved oxidatively with $NaIO_4$ to give aldehyde 7 (Figure 2B). It is preferable to execute this oxidation step just prior to a coupling reaction in order to limit potential side-reactions of the aldehyde function with the DNA bases (27).

Solution Reactions of Modified Oligonucleotides

The reactions in solution of the linker arm oligonucleotides with various nucleophiles were examined as models of the eventual solid-support attachments. Two principal strategies have been used in these coupling

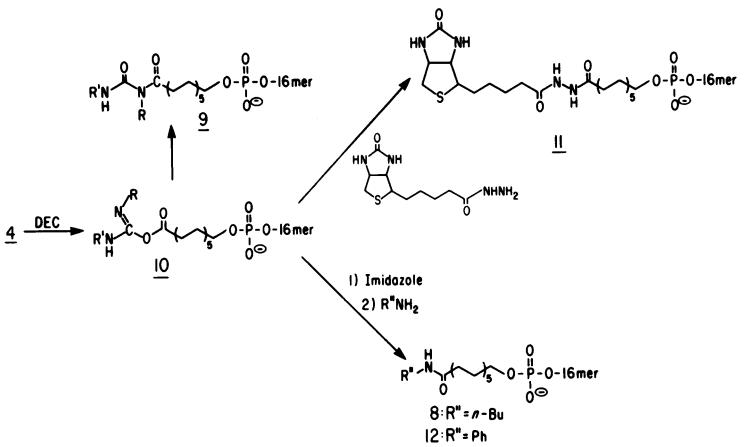
TABLE 1. Reaction of Acid 16mer 4 with Amines[a]

AMINE	CONC. (mM)	pH	Imidazole[b]	PRODUCT DISTRIBUTION AMIDE	N-ACYLUREA 9	STARTING ACID
n-$BuNH_2$	5	4[c]	-	-	1	-
"	100	4	-	0.5	0.5	-
"	500	4	-	0.7	0.3	-
"	5	4	+	0.7	-	0.3
"	5	6	+	1.0	-	-
Biotin hydrazide	7	4	-	0.9	0.1	-
"	1	4	+	1.0	-	-
"	0.5	4	+	0.7	0.2	0.1
Aniline	1	6	-	1.0	-	-

[a]Reaction time was 3 hr.
[b]Imidazole concentration was 100 mM.
[c]Same product distribution was obtained when buffered with 2-(N-morpholino) ethanesulfonic acid (MES) at pH 5.5, 6.0, or 6.7.

reactions: 1) activation of the carboxylic acid with a water-soluble carbodiimide followed by amide bond formation; and 2) reductive amination of the aldehyde linker with an amine (or hydrazide) in the presence of $NaBH_3CN$. The results are summarized in Table 1.

Typical nucleophile-derivatized solid supports contain rather low loadings of functional groups. Aminohexyl agarose, for example, has only ~5μmol amines/ml gel ("5mM"). Many colloidal supports have even lower loads (See Experimental Section). The kinetically effective concentrations of these nucleophiles may be expected to be even lower than these simple calculations would indicate, due to steric hindrance, inaccessability of some sites to hydrodynamically large DNA, etc. Initially, therefore, n-butylamine at 5mM was reacted with acid 16mer 4 at pH 4 in an attempt to form amide 8 (Scheme III). The reaction afforded, however, a product identified as the N-acylurea 9. This type of byproduct is common (28) in carbodiimide mediated couplings carried out in water at low nucleophile concentration and results from the rearrangement of the initially formed O-acylisourea 16mer 10. Attempts to avoid this by changing the pH to 5.5, 6.0 or 6.7 or increasing the concentration of n-$BuNH_2$ to 100 or 500 mM were unsuccessful, presumably due to the low concentration of free amine at these pHs. In addition, carrying out the reaction at higher pH is undesirable for several reasons: hydrolysis of the carbodiimide; the tendency of the activated ester to undergo rearrangement to the N-acylurea (29); and, addition of DEC to the bases (30).

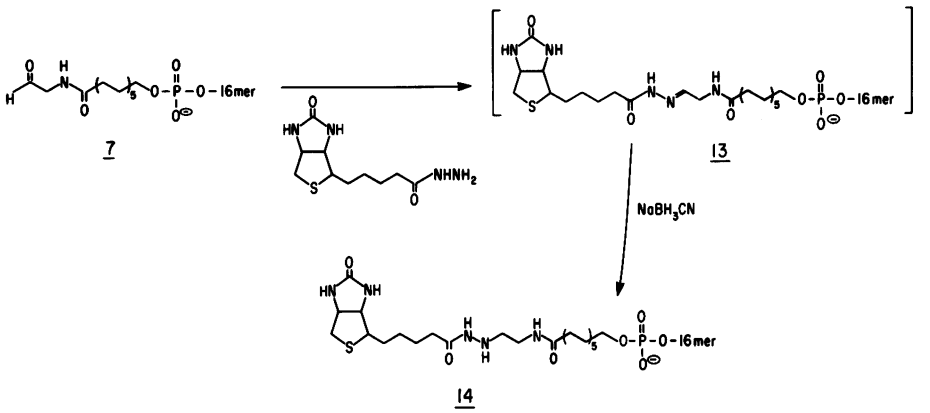


Scheme III. Reaction of various nucleophiles with carboxylic acid 16mer 4.

The formation of the N-acylurea 9 can be easily suppressed by the addition of imidazole. In the presence of imidazole the initially formed O-acylisourea 10 is converted to the corresponding imidazolide before it can rearrange. This intermediate reacts either with the amine or water; in the latter case the acid 16mer 4 is formed and can be reactivated with excess DEC. In this way we were able to get complete conversion of the acid 16mer to amide 8 with the n-$BuNH_2$ concentration as low as 5mM.

While these results with n-$BuNH_2$ were encouraging, many interesting solid supports were expected to present effective concentrations of nucleophile lower than 5mM. We hoped to achieve greater reactivity by choosing a nucleophile with a lower pK_a. In reactions of a series of nitrogen nucleophiles with an activated ester, Jencks and Carrioulo found that the loss in reactivity with lower basicity of the nucleophile was often more than compensated by the higher fraction present as free base (31). We therefore examined the reactivity of aniline (pK_a=5) and an α-effect nucleophile, biotin hydrazide (pK_a~2.5). Thus, as can be seen in Table 1, the reaction of the acid 16mer 4 with biotin hydrazide at pH 4 in the absence of imidazole occurred efficiently at 7mM to afford the acylbiotinylated 16mer 11 (Scheme III). As expected, addition of imidazole suppressed the formation of N-acylurea 9 and gave quantitative conversion to 16mer 11 at biotin hydrazide concentrations as low as 1mM. Similarly, aniline at 1mM gave only the expected amide 12 when the coupling was executed at pH 6.

The other route that was explored involved the reductive amination of



Scheme IV. Preparation of alkylbiotinylated 16mer 14.

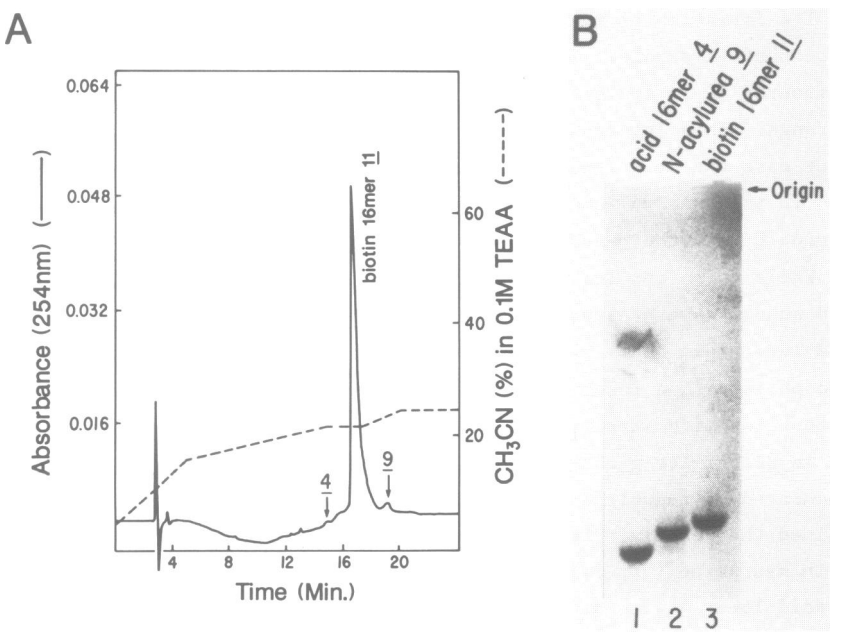


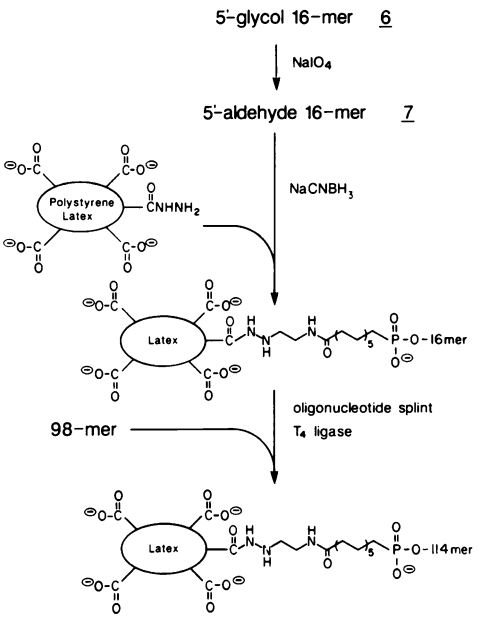
Figure 3. HPLC (panel A) and 20%/7M urea polyacrylamide gel analysis (panel B, lane 3) of crude product from reaction of acid 16mer 4 with biotin hydrazide. Lanes 1 and 2 of panel B show mobility of acid 16mer 4 and N-acylurea 16mer 9, respectively.

the aldehydic oligonucleotide 7 with a hydrazide nucleophile. Condensation of aldehyde 7 with biotin hydrazide (Scheme IV), followed by reduction of the intermediate imine 13 with $NaBH_3CN$, afforded the alkyl biotinylated oligonucleotide 14 (Figure 2B). This system has the advantage of using an exceedingly mild reagent with virtually no competing side reactions. The alkylbiotinylated 16mer 14 was produced in 80% yield in three hours with a biotin hydrazide concentration of 250μM (data not shown).

Because the acylbiotinylated 16mer 11 was easily prepared in high yield (Figure 3) by a more direct route than that of 16mer 14, the utility of this type of biotin labeling was explored. First, this oligonucleotide was shown to bind selectively to streptavidin/agarose (see Experimental Section). Second, the 5'-end modification has been found not to interfere with certain enzymatic reactions of DNA. For example, oligonucleotide 11 as well as others have been used as primers for DNA polymerase as a method for the preparation of 5'-biotinylated single-stranded DNA. Likewise, we have ligated these synthetic DNAs to both cloned single- and double-stranded DNA by means of an oligonucleotide splint and T4 polynucleotide ligase and achieved selective binding to streptavidin supports (32). Lastly, as has been shown by others (33, 34), acylbiotinylated 16mer 11 would be expected to serve as hybridization probe after labeling with an enzyme/streptavidin conjugate.

Preparation of functionalized latex microspheres as solid supports and fluorescent labels

From the solution reaction data, all of the tested amines, after immobilization, could be expected to be sufficiently reactive to permit bond formation with the 5'-modified oligonucleotides 4 and 7. Having already noted the potential advantage of polystyrene latex spheres as a solid support (see Introduction), we chose first to examine particles functionalized with hydrazide groups. These surface-functionalized beads could be readily prepared from the carboxylate analogues by a limited derivatization with hydrazine. It was important to keep residual acid groups on the bead surface for two reasons: 1) at pH >4 they would be expected to keep the particles in monodisperse form under hybridization conditions and thus afford better kinetic properties for the immobilized DNA (23) and 2) the electrostatic repulsion between these carboxylates and DNA should minimize non specific binding to the micropheres. Although aniline or alkylamine containing microspheres were commercially available, we anticipated that the alkylamine functionalized bead at pH >4 would act as an ion exchange resin, making it



Scheme V. The two step method for attachment of DNA to hydrazide latex spheres.

quite difficult to wash away any unattached DNA. Also, because aromatic amines have been reported to react with water-soluble carbodiimides (35), aniline supports could also be converted to ion exchange resins.

Accordingly, a hydrazide derivatization procedure was developed that consisted of diluting an aqueous suspension of latex (2.5-5.0% by weight) with an equal volume of formamide. Water was removed in vacuo, leaving a suspension of monodisperse beads in the organic solvent. Carbonyldiimidazole (CDI) was added to activate the carboxylate groups followed by treatment with hydrazine. After reacting for several hours, excess hydrazine was removed by dialysis to give the hydrazide latex beads. The amount of surface functionalization of the bead was determined by a trinitrobenzenesulfonic acid (TNBS) difference assay (36).

In addition to functioning as a solid support, polystrene latex microspheres can serve as a nonradioactive label due to their ability to absorb and hold small, hydrophobic molecules such as aromatic dyes (20). We chose to impregnate the latex with the fluorescent dye, Nile Red (9-diethyl-amino-5H-benzo[a]phenoxazine-5-one), for two reasons: 1) its emission maximum was expected to be around 600nm, a region where few other molecules

would be expected to fluoresce and thus have less background interference and 2) it is extremely hydrophobic and thus would not be expected to leach out of the particle (37). Upon impregnating a 0.06μm hydrazide bead as described in the Experimental Section, a detection limit of $5x10^5$ beads/mL ($8x10^{-16}$M in beads) was determined. A similar detection limit was obtained after attachment of aldehyde 16mer 7 (see below).

Two step method for attachment of DNA to solid support

The immobilization method is outlined in Scheme V.

Step one. Chemical attachments of 16mers 4 and 7 to latex hydrazide beads. Both the acid 16mer 4 and aldehyde 7 were reacted with several different hydrazide latex beads ranging in size from 0.06 to 0.19μm diameter as well as one containing Nile Red. Results are summarized in Table 2. In order to distinguish specific 5'-end attachments to the latex microsphere as opposed to nonspecific adsorption of the DNA, carbodiimide mediated couplings of acid 16mer 4 were also carried out in the absence of DEC. Likewise aldehyde 16-mer 7 attachments were executed in the presence and absence of $NaBH_3CN$. To help quantitate the specificity of these two attachment methods a selectivity number S can be defined by the ratio of attachment with and

TABLE 2. Comparison of Attachment to Hydrazide Latex Beads with Carboxylic Acid/DEC or Aldehyde/$NaBH_3CN$

Reaction	Support diam (um)	Hydrazides DNA	Density[a]	Yield(%)[b]	S[c]
Acid 4 +DEC[d]	.19	10	500	11	3.8
+DEC+imidazole[d]			1000	22	7.6
-DEC+imidazole			174	3	
Aldehyde 7 +$NaBH_3CN$	.07	36	375	67	61
-$NaBH_3CN$			6	1.1	
+$NaBH_3CN$	.06	48	314	69	49
-$NaBH_3CN$			6	1.4	
+$NaBH_3CN$	.07	6	80	3.4	
-$NaBH_3CN$			1	.04	85
+$NaBH_3CN$	.06[e]	35	358	63	40
-$NaBH_3CN$			9	1.6	

[a]Pmol attached DNA/mg Latex
[b]Based on amount of DNA added.
[c]"S"=the ratio of attachment in the presence of DEC (or $NaBH_3CN$) to the attachment in their absence.
[d][DEC]=25mM; [Imidazole]=100mM; pH4.
[e]Impregnated with Nile Red.

without DEC or $NaBH_3CN$. Thus,

$S_{acid} = \frac{\% \text{ attached with DEC}}{\% \text{ attached without DEC}}$ and $S_{aldehyde} = \frac{\% \text{ attached with } NaBH_3CN}{\% \text{ attached without } NaBH_3CN}$

As can be readily seen from Table 2, the S value for attachment with aldehyde 7 is nearly ten times better than that of acid 16mer 4. Although higher loads of 16mer per bead were obtained with the carbodiimide/acid method, these beads upon extensive washing in both the attachment and control reactions continued to release DNA. A similar shortcoming was not seen for the reductive amination method. In addition, the aldehyde attachment method gave yields in the 60-70% range, quite adequate for working with valuable oligonucleotides, and worked equally well with Nile Red impregnated beads. Also, 16mer-bead conjugates made by this method were shown to be monodisperse when analyzed by photon correlation spectroscopy. These results suggest that 5'-modified DNA is best immobilized via an aldehyde group and reductive amination.

Step two. Ligation of DNA to the 16mer-bead.

To illustrate the second step of the attachment method, we chose to join a synthetic 98mer to the 16mer-bead by means of T4 polynucleotide ligase and an oligonucleotide splint. As described in the Experimental Section the ligation was carried out in usual fashion with the exception that spermidine was omitted from the reaction mixture to prevent aggregation of the latex beads. Although the reaction was allowed to proceed for 24 hours, kinetic analysis of similar experiments showed the reaction to be complete within one hour or less (data not shown). The desired DNA-bead conjugate (39% yield), obtained as the retentate of the diafiltration step, was analyzed by polyacrylamide gel electrophoresis (Figure 4) and shown to be almost exclusively (97%) the product of ligation of the 98mer to the 16mer-bead. Two important control reactions were carried out. First, repeating the above procedure with a latex bead containing no attached 16mer, gave particles that contained only 0.06% of the starting 98mer. Second, in the absence of T4 ligase, aliquots withdrawn at various times during the ligation reaction, showed no 114mer-bead conjugate at the origin of a polyacrylamide gel, indicating that the 98mer was not held to the bead through hybridization with the oligonucleotide splint (data not shown). We expect that double stranded DNA should ligate equally well to the 16mer-beads when they are converted to double stranded form with a synthetic oligonucleotide.

CONCLUSION

We have described a two step DNA immobilization procedure that is applicable to any sequence and is unique in that it leaves all bases

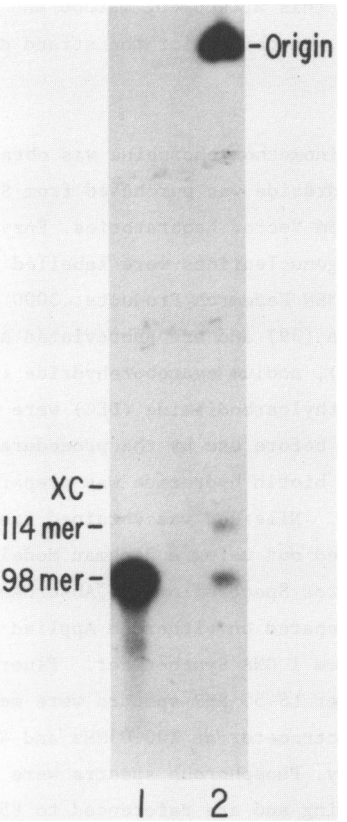


Figure 4. Autoradiogram of a 5% polyacrylamide gel analysis of the 114mer-bead conjugate (lane 2). The [5'-^{32}P]98mer (lane 1) was included as a marker.

available for hybridization. Beginning with a readily prepared 5'-aldehyde or carboxylic acid containing oligonucleotide, a covalent bond is formed between the 5'-electrophilic group and a nucleophile on the solid support. This is followed by the joining of either single or double stranded DNA to the immobilized oligonucleotide by T4 polynucleotide ligase. Alternatively, the ligation can be carried out in solution, leading to uniquely functionalized DNA that can be directly attached (23). Through the use of polystyrene latex particles impregnated with fluorescent dyes, this attachment method can serve as a highly sensitive nonradioactive DNA labeling procedure.

The accompanying paper (23) describes the hybridization behavior of this attached DNA and its use in hybridization assays. Future reports will des-

cribe the application of this attachment method and signalling system to various nucleic acid assays, in particular the strand displacement format (38).

EXPERIMENTAL SECTION

Chlorodiisopropylaminomethoxyphosphine was obtained from American Bionetics. (d)-Biotin hydrazide was purchased from Sigma; streptavidin-agarose was purchased from Vector Laboratories. Enzymes were obtained from commercial sources. Oligonucleotides were labelled with [α-^{32}P]cordycepin-5'-triphosphate (DuPont NEN Research Products; 3000 Ci/mmol) according to the procedure of Tu and Cohen (39) and are abbreviated as [3'-^{32}P]Xmer. Carbonyldiimidazole (CDI), sodium cyanoborohydride ($NaBH_3CN$) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC) were purchased from Aldrich. The $NaBH_3CN$ was purified before use by the procedure of Borch, et al. (40). A stock solution of 15mM biotin hydrazide was prepared by heating the reagent in H_2O at 37°C for 15min. Nile Red was obtained from Eastman Kodak. HPLC purifications were carried out using a Beckman Model 332 Gradient Liquid Chromatograph with a Kratos Spectroflow 757 Absorbance Detector. Synthetic oligonucleotides were prepared on either an Applied Biosystems Model 380A or Beckman Instruments System 1 DNA Synthesizer. Fluorescence data were acquired on a Perkin-Elmer LS-5. NMR spectra were measured on an IBM Instruments WP-SY100 spectrometer at 100.03MHz and 40.53 MHz for proton and phosphorous, respectively. Phosphorous spectra were obtained with two level broadband proton decoupling and are referenced to 85% H_3PO_4 in an internal capillary. Proton spectra are referenced to tetramethylsilane.

Preparation of hydrazide latex beads:

Carboxylate-modified latex particles were purchased from Polysciences, Inc. or Seragen, Inc. The beads were assayed by photon correlation spectroscopy at each stage to ensure that they remained as monodisperse spheres using an Autosizer IIc from Malvern Instruments. The following is a representative procedure for introduction of hydrazides: A 2.5% suspension of 0.06 μm carboxylate particles (32 mL, 800 mg latex, ~120 μmol carboxylates/g) was diluted with an equal volume of spectral grade formamide and evaporated overnight at room temperature in a Savant Speed Vac. The anhydrous beads were treated with CDI (400mg, 2.47 mmol) dissolved in formamide (4mL) for 16 hr followed by hydrazine (1.28mL, 40mmol) for 5 hr. The mixture was diluted with several volumes of water and dialyzed against 0.01% sodium n-lauroylsarcosinate (6x10L) or until the dialysate was free of hydrazine as determined by reaction with trinitrobenzenesulfonic acid (TNBS).

The hydrazide titer was measured by the TNBS difference assay (36) and found to be 38μmol/g. The ranges of hydrazide densities for different diameter of beads used were: 0.038μm, 15-25μmol/g; 0.06μm, 30-45μmol/g; 0.07μm, 14-23 mol/g; 0.19μm, 14-30μmol/g.

Preparation of fluorescent hydrazide latex

A 2.5% aqueous suspension of hydrazide beads (0.06μm, 5mL) were treated with 8 X 25μL of a solution of 6 mg Nile Red in 400μL toluene and 150μL $CHCl_3$ with vigorous vortexing. After each addition, the beads were allowed to sit several hours at room temperature to allow evaporation of the organic solvents. The dyed beads were filtered through a silanized glass wool plug and centrifuged (12,000xg, ~20min.) until the remaining suspension was monodisperse as determined by photon correlation spectroscopy. At λ_{ex}=533nm and λ_{em}=622nm, the detection level is $8x10^{-16}$M ($5X10^5$/mL) with a signal-to-noise ratio (41) of 2.

Methyl ester phosphoramidite 1: 12-Hydroxydodecanoic acid (10.82g, 50mmol) was esterified with 3% HCl in MeOH (210mL) at 23°C for 3.5hr. Base extraction was followed by drying and distillation to afford in 90% yield methyl 12-hydroxydodecanoate (10.30g), mp 33.5-35°C. [(Literature: 34.5°C (42)]. ^{1}HNMR ($CDCl_3$): δ3.66 (s, 3H), 3.62(t,2H), 2.88(t,2H), 1.28(br s, 18H). Condensation of the hydroxy ester (1.73g, 75 mmol) with $Cl(CH_3O)PN(i\text{-}Pr)_2$ (2g, 9.75mmol) in the presence of $(i\text{-}Pr)_2NEt$ afforded the distillable phosphoramidite 1 in 60% yield. ^{1}HNMR ($CDCl_3$): δ3.65(s,3H), 3.39(d,3H), 2.32(t,2H), 1.22(br s,18H), 1.21(d,12H) ^{31}P-NMR(CH_3CN/C_6D_6); δ148.9, with a minor peak at 10.7. Phosphoramidite 1 (200mg, 0.53mmol) was oxidized with 15% t-BuOOH in 3.3mL MeOH and chromatographed to yield 190mg of the corresponding phosphoramidate (95%): ^{1}HNMR($CDCl_3$): δ3.87(m,1H), 3.65(s,3H), 3.64(d,3H), 2.27(t,2H), 1.28(br s, 18H), 1.21(d,12H). ^{31}P-NMR($CDCl_3$): δ10.23. Analysis for $C_{20}H_{42}O_5PN$: Calculated: C, 58.94; H. 10.39; P, 7.60; N.3.44. Found: C, 58.78; H, 10.26; P, 7.82; N, 3.42.

General method for synthesis of spacer arm oligonucleotides: Using the 16mer d(CGA AGC TTG GAT CCG C) 2 as an example, the fully protected modified oligonucleotide was prepared on a 7.5μmol scale on the Beckman System 1 DNA Synthesizer. A 0.2M solution of the linker arm phosphoramidite 1 in anhydrous CH_2Cl_2 was loaded into a spare reservoir and the program modified for the addition of this reagent after the detritylation of the last base. These modifications ensured that the long-chain phosphoramidite did not come into contact with CH_3CN which caused it to precipitate. Removal from the support and deprotection was carried out under standard conditions, except that prior

to treatment with aqueous ammonia, the methyl ester was treated with $(CH_3)_3N$ in water (3:1 v/v; 37°C, 48hr). Preparative HPLC purification on an Ultrasphere ODS™ column (250mmx10mm) (Figure 1) gave pure 4, (130 A_{260}units, 812 nmol; 9.6% from starting 3'-nucleoside).

Preparation of glycol 16mer 6

Acid 16mer 4 (300nmol), imidazole·HCl (5μmol, pH 6), 3-amino-1,2-propanediol·HCl 5 (100μmol, pH 6) and DEC·HCl (1.5μmol, pH 6) in H_2O (50μL) were reacted at room temperature for 24 hr. Additional DEC·HCl (1.5 μmol, pH 6) was added seven times over a period of six days. After chromatography over Sephadex G-50, half of the sample (~150 nmol) was purified by reverse phase HPLC to afford glycol 16mer 6 (48 nmol) in approximately 30% yield.

Preparation of aldehyde 16mer 7

To a solution of glycol 16mer 6 (6nmol) in H_2O (20μL) was added 4mL 120 mM $NaIO_4$ dissolved in 20 mM sodium phosphate (pH 7). After the reaction was allowed to proceed for 1.5 hr at room temperature, the aldehyde 7 (4.4nmol) was isolated by a Sephadex G-25 spin column (37) and showed a single band on a 20%/7M urea polyacrylamide gel (Figure 2B).

To aldehyde 16mer 7 (1.4 nmol) was added 15 mM biotin hydrazide (10μL), 500 mM NaOAc (5μL, pH 5) and 100 mM $NaBH_3CN$ in acetate buffer (5μL). After 21 hr at room temperature, samples were analyzed on a 20%/7M urea polyacrylamide gel (Figure 2B) and showed a single band corresponding to the alkylbiotinylated 16mer 14. (Subsequent experiments have shown that the oxidation procedure was complete in 15 minutes and that the coupling was complete in 90 minutes.)

Preparation of acylbiotinylated 16mer 11

Acid 16mer 4 (18nmol), biotin hydrazide (500nmol) and DEC (900nmol) were combined in H_2O (50μL) and reacted at 37°C for 1 hr. The reaction mixture was chromatographed on a Sephadex G-50 column to afford the acylbiotinylated 16mer 11 (17.4nmol). HPLC analysis (Figure 3A) of the product showed that it was ~90% pure and contained <10% of the N-acylurea 16mer 9. Complete snake venom phosphodiesterase and bacterial alkaline phosphatase digestions of 16mer 11 gave only the four nucleosides in the expected ratio, thus verifying that biotin hydrazide had not added to a base, i.e., cytosine. Nucleoside analysis: Theoretical: dA, 3.0; dG, 5.0; dC, 5.0; dT, 3.0. Found: dA, 2.7; dG, 5.0; dC, 4.8; dT, 2.7. The [3'-^{32}P] acylbiotinylated 11 was applied to a streptavidin/agarose column and washed with 1M NaCl/5X Denhardt's solution/20 mM Tris.HCl (pH8); 95% of the labeled acylbiotinylated 16mer 11, as compared with 1.5% of acid 16-mer 4 bound to the column.

Reaction of acid 16mer 4 with n-butylamine, biotin hydrazide or aniline

The following is a typical protocol. Acid 16mer 4 (6nmol), imidazole·HCl (5μmol, pH4), n-butylamine·HCl (250nmol, pH4) and DEC·HCl (375nmol, pH4) in H_2O (50μL) were allowed to react at room temperature for 3 hr. After chromatography on a Sephadex G-50 column, 20%/7M urea polyacrylamide gel analysis showed two bands corresponding to the n-butyl amide 16mer 8 and acid 16mer 4 in an approximate ratio of 7:3. No band corresponding to the N-acylurea 16mer 9 was detected.

Chemical attachments to hydrazide latex spheres

Method A. Attachment of acid 16mer 4 via DEC. A typical 50μL reaction contained [3'-^{32}P] acid 16mer 4 (6nmol, 4.8 x 10^5 cpm) imidazole·HCl (5μmol, pH4) DEC·HCl (1.2μmol, pH4) and 0.19μm hydrazide latex beads (2mg, 60nmol hydrazide). After 2hr. at room temperature, the latex was treated with the following 0.45μm filtered wash solution: 100 mM NaCl, 10 mM sodium phosphate (pH 6.8), 1 mM EDTA, and 0.05% SDS. The latex beads were collected by centrifugation (12,000xg, 10 min). Yields were between 10-25%. See Table 2.

Method B. Attachment of aldehyde 7 via $NaBH_3CN$. A solution of [3'-^{32}P] glycol 16mer 6 (4nmol, 1.2X10^6 cpm) in H_2O (20μL) was added to 100 mM sodium phosphate (pH 7) and treated with 120 mM $NaIO_4$ (5μL). The solution was vortexed and the reaction allowed to proceed for 20 min at room temperature, at which time the excess periodate was destroyed in situ with 550 mM pinacol (5μL). After 30 min., one-half of this solution (27.5μL) was added to a 1.2% suspension of 0.07μm latex hydrazide beads (300μL, 72nmol hydrazides). This was immediately treated with 500 mM NaOAc (50μL, pH5) and 350 mM $NaBH_3CN$ in H_2O (23μL). After 18 hr the reaction was diluted with H_2O (1mL) and formamide (0.5mL). The other half of the oxidized mixture was treated identically, except that no $NaBH_3CN$ was added. From this point forward, the two reactions were treated separately but identically.

The noncovalently bound 16mer was removed by loading the diluted reaction mixture onto a 20% native polyacrylamide gel (3mm thick) and carrying out electrophoresis in a Tris/borate/EDTA buffer (43) containing 0.05% SDS for 15 min. The 16mer-beads were removed and the wells rinsed. The combined washes were diluted with the bead wash solution (see above) and placed in an Amicon diafiltration apparatus equipped with a YM100 membrane. The entire chamber was incubated at 65°C for 2 hr and then the contents subjected to diafiltration; this was repeated once. The 16mer-beads were dialyzed against H_2O (3x7L) over two days at 37°C. Cerenkov counting of the 16mer-bead suspension obtained with $NaBH_3CN$ reduction and that without showed

attachment yields of 67% and 1.1%, respectively (see Table 2).

Enzymatic attachment to 16mer bead conjugate

A mixture of [5'-^{32}P]d(AAT TCC CCG GAT CCG TCC TGA TAA GCC TGG TTG ACG GAA GTG GCA ATC CGG TCA GCG TGG AGG TTC AGT CCG TCA CCG ACG GCG TGA AGG TAA AAG TG) (12pmol, 1.2 x 10^6cpm), and the 33mer splint d(GAC GGA TCC GGG GAA TTG CGG ATC CAA GCT TCG) (20pmol) were heated at 95°C for 2 min and then cooled on ice for 15 min. The 0.07μm 16mer-beads (20pmol 16mer, 2.9x10^{11} beads) were added and the total reaction was made 5mM in $MgCl_2$, 50mM in Tris-HCl (pH 7.6) 10mM in dithiothreitol and 1mM in ATP in final volume of 180μL. T4 polynucleotide ligase (1,200 units) was added and the reaction incubated at room temperature for 24 hr. The unreacted 98mer was removed by diafiltration in a 10mL Amicon Cell with a YM100 membrane at 37°C with a first wash solution of 100 mM NaCl, 10 mM sodium phosphate (pH 6.8), 1 mM EDTA and 0.1% sodium n-lauroylsarcosinate (5 x 1mL) and a second wash solution of 10 mM sodium phosphate (pH 6.8) and 0.1% sodium n-lauroylsarcosinate. Cerenkov counting of the retentate (114mer-bead suspension) showed that 39% (4.2pmol) of the 98mer had ligated to the solid support-16mer conjugate. Analysis of this bead suspension by gel electrophoresis (Figure 4) followed by excision and Cerenkov counting of the radioactive bands showed a band at the origin (97%) which was identified as the 114mer-bead, a band which comigrated with the 98mer (2%) and a slower moving band (1%) which was identified as the ligation product of the 16mer and 98mer. A control reaction with a 0.07μm bead with no 16mer attached showed only 0.06% of the starting 98mer in the retentate.

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