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Assessment of methods for covalent binding of nucleic acids to magnetic beads, Dynabeads™, and the characteristics of the bound nucleic acids in hybridization reactions

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ABSTRACT

Dynabeads™ are magnetic monosized beads with high stability, high uniformity, unique paramagnetic properties, low particle-particle interaction, and high dispersibility.Different reactive groups; hydroxyl, carboxyl and amino groups can be attached to the surface. Several methods for covalent attachment of DNA or oligonucleotides to the beads were investigated. Best coupling yields were obtained by carbodiimide-mediated end-attachment of 5'-phosphate and 5'-NH_2 modified nucleic acids to respectively amino and carboxyl beads. The carboxyl beads showed a low degree of non-specific binding, while a better yield of end-attached nucleic acids was obtained using the amino beads. The DNA-beads worked efficiently in hybridization experiments, and the kinetics of hybridization approach those of solution hybridization.

INTRODUCTION

Hybridization of DNA immobilized onto solid supports is a commonly used technique in molecular biology for the isolation, identification and genetic analysis of specific DNA sequences. The commonest method for binding DNA to a support, is the transfer of DNA onto nitrocellulose filters or nylon membranes (Southern blot, colony and plaque blot, dot and slot blot). The disadvantages of this method are that DNA is non-covalently attached which may result in loss of DNA from the support, and that only a small amount of the DNA is available for hybridization (2).

In recent years a number of chemical methods for the attachment of DNA to solid supports through stable covalent linkage, have been developed. These methods can be divided into two groups. Firstly carbodiimide has been used for end-attachment of DNA to cellulose (3), Sephadex (4), or Sephacryl (5), and

secondly DNA can be immobilized via its bases reacting with activated supports such as CNBr-activated agarose (6) and diazotized Sephacryl (5). However, the coupling methods in the last group involves the bases of the DNA which will become incapable of base pairing. Therefore, the best alternative seems to be the carbodiimide methods. However, the effeciency of attachment of long polynucleotides through their 5'-phosphate ends onto cellulose (3) and Sephacryl (7) is very poor. Kremsky et al. (8) found that introducing electrophilic groups at the 5'-end of the DNA and a nucleophilic residue on the support, favours the single-point end attachment rather than the multiple point attachment through the bases. Other workers have modified the 5'-end with nucleophiles (9) to increase the yield of end attached nucleic acids (10).

When filters or membranes are used as the support in mixed phase hybridization, the kinetics of hybridization are very slow, ten to twenty fold slower than the corresponding hybridization reaction in solution. By using some sort of particle support which can be kept in a homogenous solution during the hybridization reaction, the kinetics of hybridization will approach those of solution hybridizations. Wolf et al. (1) have investigated the hybridization kinetics of DNA end-attached to latex particles , while Gingeraas et al. (2) studied the hybridization properties of nucleic acids end-attached to Sephacryl 500 dextran.

We wanted to investigate if magnetic beads, Dynabeads^{TM}, could be used as a support for nucleic acids, and to determine the hybridization properties of these DNA-beads. Magnetic beads are non-porous like latex particles, which ensures that the reagents act on the surface of the support. They also provide a large surface area for DNA attachment. In addition, magnetic beads are very easily and rapidly separated from solutions by using a magnet. The beads are kept in the tube with the magnet while solutions are removed or exchanged. This is time saving since centrifugation steps are avoided both during DNA coupling- and hybridization reactions.

In this paper we present the results obtained for different methods of attachment using magnetic beads carrying different active groups on their surfaces. The method used are DNA attached

to diazotized hydroxyl beads, CNBr-activated hydroxyl beads, and carbodiimide mediated attachment to either hydroxyl, amino, or carboxyl beads. The amounts of non-specifically bound and end-attached DNA to the different beads were also determined. We also demonstrate that the DNA bound to the beads hybridizes very efficiently with complementary sequences.

MATERIALS AND METHODS

Magnetic beads

The Dynabeads^{TM} were provided by Dynal (Oslo,Norway). They are all paramagnetic monodispersed polymeric particles made of polystyrene with a magnetite core and with a diameter of 4.5 um.

A large number of beads with different surfaces and reactive groups were tested for the non-specific binding of nucleic acids. Only those beads with a relatively low non-specific binding were used for covalent attachment of DNA. They are listed in Table 1 and varies in type of surface and active groups (OH, COOH, NH_2) that can be used for chemically coupling of DNA.

In all experiments described the beads were separated from solutions with a magnet. Solutions were removed/exchanged with a pipette while the beads were kept in the tubes with the magnet.

DNA-preparations

Sonicated calf thymus DNA (ctDNA) was used for determination of non-specific binding of single- and double-stranded DNA to different beads and for covalent attachment to beads via diazotization. The ctDNA was fragmented by sonication in ice using a MSE, PG. 621 sonicator, using full amplitude (20 microns, 6 cycles with 20 second on, 40 second off). This treatment resulted in DNA preparations in which the majority of the DNA fragments are approximately 500bp. (Determined by comparison with a molecular size-markers on an agarose gel.) The sonicated DNA was end-labelled by phosphorylation (11). The DNA was first dephosphorylated using calf intestinal phosphatase (Sigma) then end-labelled by T4-polynucleotide kinase (New England BioLabs) and (γ-^{32}P)-ATP (Amersham). The labelled DNA was diluted with cold sonicated ctDNA to a suitable specific activity (cpm/ug).

The DNA preparations were made single-stranded by first heating for 5-10 minutes on a boiling water bath followed by rapid cooling in ice.

Lambda-DNA (48,502 bp, New England BioLabs), digested with restriction enzyme HinfI (New England BioLabs) and then ^{32}P-end-labelled as described for sonicated ctDNA, was used for carbodiimide-mediated attachment to hydroxyl and amino beads. There are about 120 restriction sites for HinfI in lambda-DNA and digestion gives a DNA preparation in which the majority of the fragments are 400-500 bp.

5'-NH_2 modified lambda/HinfI fragments were used for the linkage reactions with CNBr-activated beads and carboxyl beads. The lambda/HinfI fragments were 5'-NH_2 modified by a direct method described by Chu et al. (12). 100 ug of lambda/HinfI DNA were dissolved in 500 ul 0.1 M imidazolebuffer pH 7 (1-methylimidazole, Sigma), 0.1 M EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide, Sigma) and 0.25 M EDA (ethylenediamine, Sigma) and incubated for 3 hours at 50°C. The modified lambda/HinfI DNA was precipitated with ethanol and lyophilized before reaction with the CNBr-activated beads and to carboxyl beads.

Synthetic oligonucleotides oligo A and oligo B (complementary 36 mers) were respectively used as probe on beads and target in hybridization experiments. The oligonucleotides were ^{32}P-end-labelled by T4-polynucleotide kinase and (γ-^{32}P)-ATP (11).

Non-specific binding of DNA to magnetic beads

50 ug of single- or double-stranded ^{32}P-ctDNA in 500 ul 10 mM Tris-HCl pH 7.4, 10 mM NaCl were added to 1 mg of beads in eppendorf tubes. The tubes were incubated at room temperature for 60 minutes with occasional shaking. The DNA solution was then removed and the beads were washed twice with 2x1 ml 5xSSC then twice with 2x1 ml 2xSSC and finally with 1 ml 2xSSC containing 50% formamide. (20xSSC contains 0.3 M citrate buffer pH 7.0, 3 M NaCl). The amounts of DNA on the beads within the tubes, and the amounts of DNA in the different washing solutions were measured by Cerenkow counting.

Covalent attachment of DNA to magnetic beads

Covalent attachment to hydroxyl beads via diazotization. The preparation of 2-aminophenylthioether-derivatives of the M450 hydroxyl beads (APTE-beads), with subsequent diazotization and coupling of denatured ^{32}P-labelled ctDNA, were mainly performed as described by Bünemann et al. (5). Non-specific binding was

determined by using non-activated beads. For this and all the following methods the amounts of DNA on the beads within the tubes, and in the different washing solutions, were measured by Cerenkow counting.

Covalent attachment of 5'-NH_2 modified DNA to CNBr-activated beads. 100 mg of M450 hydroxyl beads were CNBr-activated as described by Clerici et al. (13). The beads were kept in 10 mM $NaHCO_3$ pH 8.4 at 4°C and used for coupling within 2 days after activation.

1 mg of CNBr-activated beads was mixed in 2 ml tubes with 100 ug 5'-NH_2-modified lambda/HinfI fragments dissolved in 600 ul 10 mM $NaHCO_3$ pH 8.4. The tubes were incubated for 20 hours at room temperature with gentle shaking. The amounts of non-covalently attached DNA were determined by adding unmodified lambda/HinfI fragments to the CNBr-activated beads.

The beads were washed successively with 1 ml 5xSSC, 1 ml 0.2 N NaOH for 30 minutes at 65°C to make the DNA single-stranded, 1 ml 5xSSC, and 1 ml 2xSSC.

Carbodiimide (CMC) mediated attachment of 5'-phosphate DNA to hydroxyl beads. The carbodiimide mediated attachment of the 5'-phosphate end of DNA to hydroxyl beads was performed by using modified procedures described by Gilham (3) and Rickwood (4). 100 ug ^{32}P-end-labelled denatured ctDNA and 2 mg beads were mixed in eppendorf tubes in 150 ul 0.04 M MES pH 6 (sodium 2-(N-morpholino)ethane sulfonate) containing 10 mg CMC (1-cyclohexyl-3-(2-morpholino ethyl) carbodiimide). Non-specific binding of DNA to the beads was determined by mixing DNA and beads in 0.04 M MES without CMC. These mixtures were spread on siliconized microslides to cover an area of approx. 1 cm^2. The microslides were incubated at 45°C, and at 2, 5 and 8 hours after start of reaction, they were transfered to a water-saturated atmosphere at room temperature for 1 hour. After 8 or 12 hours the microslides were left in the water-saturated atmosphere for another 12 hours.

The beads were then transfered to eppendorf tubes in 10 mM Tris-HCl, 10 mM NaCl pH 7.4 and washed first with 1 ml 10 mM Tris- HCl pH 7.4, 1 M NaCl, then twice with 1 ml 10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 50% formamide, and lastly with 1 ml 10 mM Tris-HCl pH7.4, 0.15 M NaCl.

Carbodiimide (EDC) mediated attachment of 5'-phosphate DNA or oligonucleotides to amino beads. 50 ug ^{32}P-lambda/HinfI fragments were dissolved in 600 ul 0.1 M imidazolebuffer pH 7, 0.1 M EDC and mixed with 1 mg of amino beads in 2 ml tubes (12). Non-covalent attachment of DNA was determined by carrying out the reaction in the absence of EDC. The tubes were incubated for 3 hours at 50°C with gentle shaking. The beads were washed as described for CNBr-activated beads.

6 ug ^{32}P-labelled oligo A (600 pmole) were reacted with 5 mg L255 or R469 amino beads in a volume of 1 ml.

Carbodiimide (EDC) mediated attachment of 5'-NH_2 DNA to carboxyl beads. 100 ug 5'-NH_2 modified lambda/HinfI DNA were dissolved in 600 ul 0.1 M imidazolebuffer pH 7, 0.1 M EDC and mixed with 1 mg of R452 carboxyl beads in 2 ml tubes. Non-covalent attached DNA was determined by omitting EDC in the reaction mixture. The tube were incubated for 20 hours at room temperature with gentle shaking. The beads were washed as previously described.

The extent of 5'-end attached DNA to both amino beads and carboxyl beads was obtained from the acid hydrolysis of the phosphoramidate bound (0.01 M HCl, pH 2.0, 37°C, 4 hours), followed by washing of beads with 0.4 M NaOH pH 12.0. The loss of radioactivity from the beads provides an estimate of end attachment (10).

Hybridization with probe-beads.

The R469 amino beads were used as the probe. support for the hybridization experiments, and the oligo A (36 mer) was covalently attached to the beads using carbodiimide as previously described.

All the hybridization experiments include prehybridization of one milligram of probe beads in 300 ul 5xSSPE (20xSSPE consists of 0.17 M phosphate buffer pH 7.4, 3 M NaCl, and 0.02 M EDTA), 10xDenhardt's solution (50xDenhardt's consists of 5g Ficoll, 5g polyvinylpyrrolidone and 5g BSA in 500 ml of H_2O), 0.1 % SDS and 300 ug sonicated and denatured ctDNA. Prehybridization was carried out for 30-60 minutes at 37°C with gentle shaking. For hybridization of target nucleic acids, the beads with attached oligonucleotides were incubated in 300 ul of the same

solution without ctDNA, for 1-2 hours at 37°C with gentle shaking. After hybridization, the beads were washed with 3x1 ml 2xSSC, 0.05 % SDS at room temperature.

RESULTS

Non-specific binding of DNA to magnetic beads

Different washing buffers were used to try and remove as much as possible of the non-specifically bound DNA from the beads. Most of the DNA comes off with the 2 first steps in the washing procedure regardless of if SSC- or Tris-NaCl buffers with a low or high molarity of NaCl, were used. Even if the washing buffers contained 50% or 70% formamide, 6 M urea or 6 M guanidine-HCl, no more DNA could be removed from the beads. Since the DNA-beads are supposed to be used in hybridization experiments, the SSC hybridization buffer were chosen as washing solution. The washing procedure was usually ended when no more radioactivity could be detected in the washing solutions by Cerenkow counting. In most cases a 3 steps washing procedure (1 ml 5 x SSC, then two washes with 1 ml 2 x SSC) was sufficient to remove unbound DNA. When the beads were stored in dilute salt solutions at 4°C for 2 weeks only insignificant release of DNA could be detected.

A large number of beads with different surfaces and reactive groups, were tested for non-specific binding of single-stranded DNA. The six which showed the lowest non-specific binding, where chosen for covalent attachment of DNA (Table 1). Beads with amino groups on their surface, bind non-specifically more DNA (approx. 0.8 ug/mg) than beads with hydroxyl or carboxyl groups (approx. 0.2-0.3 ug/mg). One of the amino beads (L255) showed a relative low non-specific binding, most probably because they only carry a small number of amino groups on the surface.

Covalent attachment of nucleic acid to magnetic beads

The beads used for covalent attachment of DNA (Table 1) can be divided into 3 groups; hydroxyl, carboxyl or amino beads, according to which active group they carry on the surface. These active groups have been used for covalent attachment of DNA to the beads.

The M450 hydroxyl beads were activated either by

TABLE 1
Non-specific binding of denatured ctDNA to magnetic beads

Bead no.	Active group	Non-specific binding ug ss DNA/mg
M450	OH	0.2
L255[a)]	OH(NH_2)	0.3
R240	NH_2	0.8
R442	NH_2	0.9
R469	NH_2	0.7
R452	COOH	0.2

50 ug of denatured ^{32}P-ctDNA in 500 ul 10 mM Tris-HCl pH 7.4, 10 mM NaCl were added per mg beads and incubated in eppendorf tubes at room temperature for 60 minutes with occasional shaking.
a) L255 carries only residual amounts of NH_2-groups on their surface.

diazotization or CNBr before used for coupling of DNA. Noyes et al. (14), Bünemann et al. (5) and Langdale et al. (7) have reported to be able to immobilize DNA to different types of support (cellulose, Sepharose, Sephacryl, Sephadex) via diazotization. The procedure described by Bünemann et al. (5) was scaled down (100:1) and used for coupling of denatured ctDNA to M450 hydroxyl beads. We could however only slightly increase the amount of DNA on the beads above the level of non-specifically bound DNA (respectively 0.15 and 0.10 ug/mg).

By using the procedures described by Clerici et al. (13) we were able to attach between 2 and 3 ug of 5'-NH_2 modified DNA to CNBr-activated M450 hydroxyl beads. The CNBr-activated beads should be freshly prepared, since they show decreased chemical activity after storage at 4°C. Activated beads that had been stored for 10 weeks bound only half the amount of DNA when compared to freshly prepared beads.

Carbodiimide (CMC) mediated attachment of 5'-phosphate DNA to beads. This method involves specific activation of the terminal phosphate group of the DNA in the presence of the beads. The activation is brought about by carbodiimide, and the

TABLE 2
Carbodiimide mediated attachment of DNA to magnetic beads

Method of attachment	Bead no.	DNA attached ug ss DNA/mg	Non-covalently attached DNA ug ss DNA/mg	%	%End-attachment
CMC a)	M450	0.9	0.8	93	
	L255	6.4	4.2	66	
EDC b)	R442	1.4	0.3	21	20
	R240	6.0	1.8	30	60
	R469	11.3	0.8	7	65
	L255	2.9	0.2	7	30
EDC c)	R452	2.4	0.02	1	30

Three different carbodiimide methods were used.
a) 100 ug denatured ^{32}P-ctDNA in 150 ul 0.04 M MES pH 6 containing 10 mg CMC were mixed in eppendorf tubes with 2 mg beads. The reaction mixtures were spread on micro slides and dried as described in Material and Methods. Non-covalently attached DNA was determined by omitting CMC in the reaction mixture.
b) 50 ug native lambda/HinfI DNA in 600 ul 0.1 M imidazole buffer pH 7, 0.1 M EDC were added per mg of amino beads. The reaction mixtures were incubated for 3 hours at 50°C with gentle shaking. Non-covalently attached DNA was determined in absence of EDC in the reaction mixture. % end-attachment was determined by acid hydrolysis of the phosphoramidate bond.
c) 100 ug native 5'-NH_2 modified lambda/HinfI DNA in 600 ul of 0.1 M imidazole-buffer pH 7, 0.1 M EDC were added per mg of carboxyl beads. The reaction mixture were incubated for 20 hours at room temperature with gentle shaking. Non-covalently attached DNA and % end-attachment were determined as described in b).

condensation with the beads is achieved by concentrating the reaction mixture onto them. This method has been used by Gilham (3) and Rickwood (4) for covalent attachment of DNA to hydroxyl groups on respectively cellulose and Sephadex.

We applied this method for the attachment of DNA to hydroxyl beads. The results in Table 2 show that the amount of non-specifically bound DNA on the beads when the DNA is dried/concentrated onto them, is higher than when DNA is mixed with the beads in a solution (Table 1). The presence of carbodiimide increases the amount of DNA on the M450 hydroxyl

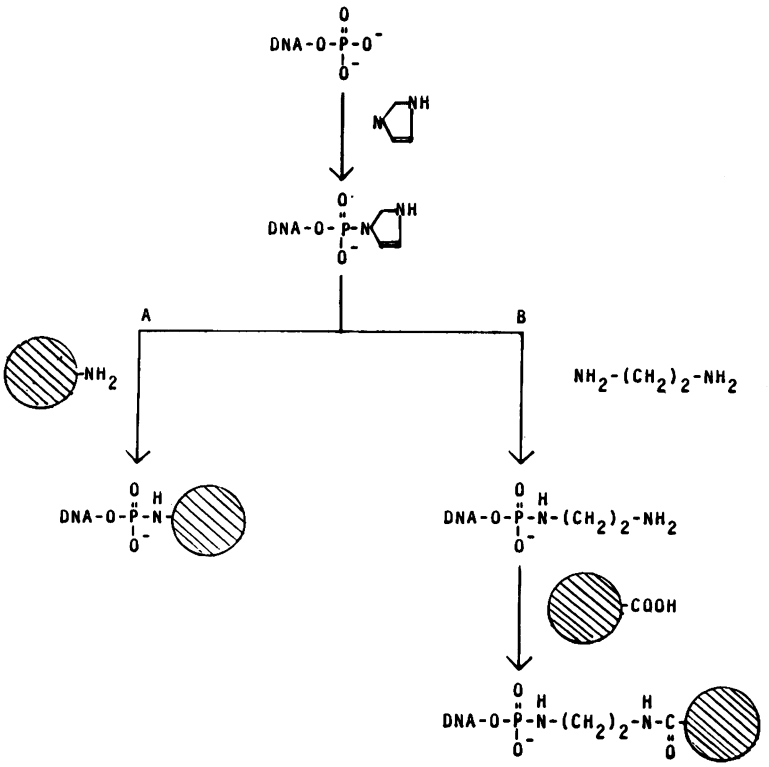

Figure 1. A. Reaction of amino beads with 5'-phosphorimidazolide derivatives of DNA generated in imidazolebuffer pH 7, in presence of EDC. B. Reaction of carboxyl beads with 5'-aminoethylphosphoramidate DNA derivatives in imidazolebuffer pH 6, in presence of EDC.

beads only slightly above the level of non-specific binding. The L255-beads bind nearly twice as much DNA in presence of carbodiimide, and the high non-specific binding is probably due to the residual amino groups on their surface.

Carbodiimide (EDC) mediated attachment of 5'-phosphate-DNA or -oligonucleotides to amino beads. Both lambda/HinfI fragments and synthetic oligonucleotides were attached via a phosphoramidate linkage (Figure 1A) to 3 different amino beads by the method described by Ghosh et al. (10). The results in Table 2 show that the amount of DNA attached to the different beads varies from 1.4-11.3 ug/mg.

The R469 beads which carry an amino group at the termini of an polyethylene glycol linker (8 atoms), bind a larger amount of DNA than R240 beads which carry the amino group on a shorter linker (3 atoms). When the linker is made longer (number of atoms > 20) as in the case of for the R442 beads, a decrease in the amount of DNA bound to the beads is observed. This is probably due to secondary structures of the linkers which results in the terminal amino group becoming unavailable for coupling.

The amount of non-specifically bound DNA varies among the beads (7-30%) probably according to number of amino groups per unit of surface area. The R469 beads that binds the largest amount of DNA covalently(11 ug/mg), show the lowest non-specific binding.

The acid lability of the phosphoramidate bond (9) is used for measuring degree of end-attachment by acid hydrolysis. The amount end-attached DNA varies between the different beads from 20-65%, and again, the R469 bead seems to be the preferable one with 65% of the DNA end-attached.

We were able to attach twice as much DNA to the R469 beads by performing the reaction in imidazole buffer pH 7 for 3 hours at 50°C (12), instead of pH 6, for 24 hours at room temperature (10). An increase in molarity of EDC from 0.1 M to 0.2 M resulted in a 20% decrease in amount of DNA on the R469 beads (data not shown).

The results of attaching oligonucleotides (36 mer) to amino beads are shown in Table 3. As expected the L255 beads which carry only a small number of NH_2 groups on their surface, bind the least amount of 36 mer. The amino beads also show a significant non-specific binding of oligonucleotides.

Carbodiimide (EDC) mediated attachment of 5'-NH_2 DNA to carboxyl beads. The reaction used for attaching DNA to carboxyl beads (10) is illustrated in Figure 1B. Amino groups introduced at the 5'-end of the lambda DNA fragments using a one-step reaction method described by Chu et al. (12), results in a greater nucleophilicity of the terminal primary amino group of the alkyl linker as compared to the amino functionalities of the bases. It was therefore expected that the carboxyl groups on the beads would react preferentially with these primary amino groups.

TABLE 3
Carbodiimide (EDC) mediated attachment of 5'-phosphate oligonucleotides to amino beads

Bead no.	Oligonucleotide attached		Non-covalently attached DNA		%End-attachment
	pmole/mg	(ug/mg)	pmole/mg	%	
L225	18	(0.18)	5.4	30	25
R469	64	(0.64)	25.6	40	60

600 pmole (6 ug) of oligo A (36 mer) were dissolved in 1 ml of 0.1 M imidazole, pH 7, 0.1 M EDC and mixed with 5 mg of amino beads, and incubated for 3 hours at 50°C. % non-covalently attached oligonucleotide was determined by omitting EDC in the reaction mixture. % end-attachment was determined by acid hydrolysis of the phosphoramidate bond.

The result of attachment of DNA to the R452 carboxyl bead is shown in Table 2. As expected, because of the weakly anionic property of the carboxyl beads, the non-specific binding is reduced compared to the amino beads.

Both a one-step and a two-step reaction (12 and 15 respectively) were used for introduction of amino groups at the 5'-end of the DNA-fragments. Several groups have reported to have obtained 70-90% 5'-NH_2-modification of oligonucleotides. Chu *et al*. (9) obtained a 70% conversion of double-stranded DNA fragments (5kb) to 5'-phosphorimidazolides. We were only able to obtain a 30-40% conversion of lambda/*Hinf*I fragments (10-20% for sonicated ctDNA), and how much of the 5'-phosphorimidazolides that are converted to 5'-NH_2-DNA in the second step of the reaction is not known. This means that the DNA-preparations used for attachment to carboxyl beads consist of no more than 30-40% 5'-NH_2-DNA. Only 20-30% of the covalently bound DNA was end-attached to the beads. Ghosh *et al*. (10) also report obtaining a lower degree of end-attachment to carboxyl than to amino support.

The attachment reaction was performed at room temperature for 20 hours. A change in reaction temperature and time to 50°C and 3 hours as used for the amino beads, resulted in a 20 fold decrease in amount of DNA bound to the carboxyl beads.

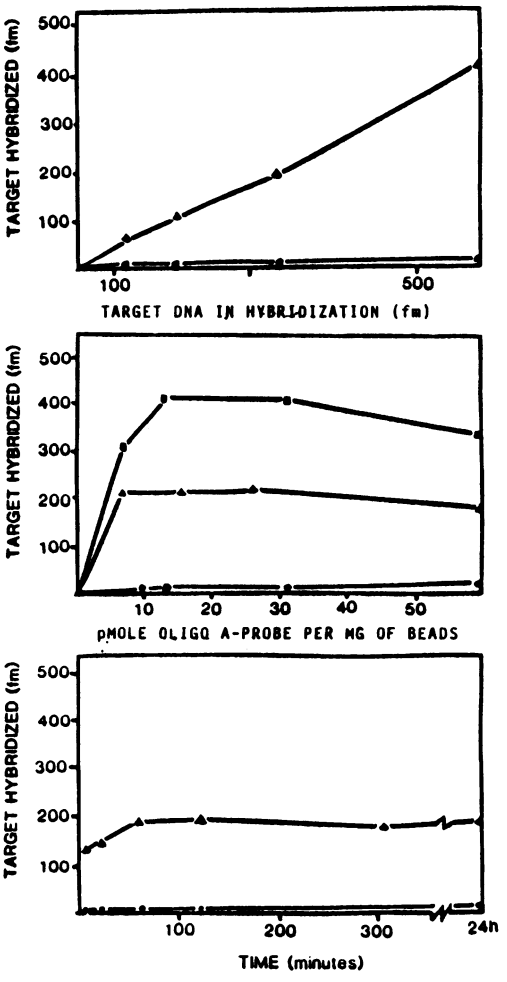

FIGURE 2. Hybridization properties of end-attached oligo nucleotide probe. A. Increasing amount of target in hybridization. 12 pmoles oligo A probe (36 mer) per mg of R469 amino beads in hybridization with increasing amount of complementary oligo B target (▲). B. Increasing amount of probe in hybridization. 6-60 pmoles oligo A probe per mg of R469 amino beads were used in hybridization with a fixed amount of complementary oligo B target (▲ 300 fmoles, ■ 600 fmoles). C. Hybridization efficiencies as function of time of hybridization. 12 pmoles oligo A probe per mg of R469 amino beads and 300 fmoles of complementary oligo B target (▲) were used in each hybridization reaction which was performed for different lengths of time. Non-complementary denatured pBR322/RI fragments (●) were used to measure non-specific adsorption.

Hybridization properties of probe-beads.

The hybridization properties of the probe beads were investigated using the oligo A (36 mer) as probe and the complementary oligo B as target. The R469 amino beads were chosen as support, because large amounts of nucleic acids can be attached within a few hours, and they show a high degree of end-attachment (60-70%). Unfortunately, they show a high level of nonspecific binding (40%). However, non-specifically bound oligo A probe is not able to hybridize with complementary sequences (data not shown). The amount of oligo A probe used in the hybridization experiment is therefore presented as amount of covalently attached probe.

The hybridization properties of the probe beads are illustrated in Figure 2. Figure 2A shows the result of an experiment where a constant amount of oligo A probe (12 pmoles) was used to hybridize increasing amount of oligo B target (75-600 fmoles). The hybridization efficiency seems to be constant with increasing concentrations of target, and approximately 70% of the available target can form a hybrid. The results indicate that the fixed amount of probe was in excess of the amount of target in the 75-600 fmoles range.

To determine whether it is possible to hybridize all of the oligonucleotide target in the solution, the amount of immobilized probe per milligram of beads was increased (6-60 pmoles), while the concentration of target was kept constant (300 or 600 fmoles). Figure 2B shows that between 6 to 60 pmoles of probe a relatively constant percentage of target is hybridized (60-70%), and the hybridization efficiency is consistent with that observed in Figure 2A. However, there seems to be a small decrease in amount target hybridized at higher probe concentrations. This is probably due to the fact that the availability of target becomes the limiting factor. Optimal hybridization efficiency is obtained by using between 10-15 pmoles of probe per milligram of beads.

Figure 2C shows the hybridization efficiencies measured as a function of time of hybridization. Constant amounts of both probe (12 pmoles) and target (300 fmoles) were used in each hybridization reaction, which was performed for different lengths

of time (10 minutes to 24 hours). The hybridization of oligonucleotide target seems to be complete after 60 minutes and it remains hybridized to the probe after 24 hours. The non-specific background of adsorbed non-complementary DNA is low in all three experiments.

DISCUSSION

All the beads show some degree of non-specific binding of DNA (Table 1). The amount of non-specifically bound DNA varies with the type of active group. The beads that bind the least amounts of DNA are those with carboxyl or hydroxyl groups on their surface. A washing procedure that could remove all non-specifically bound DNA, was not found. Since the DNA-beads are to be used in hybridization experiments 5 x SSC and 2 x SSC were chosen as washing solutions.

Several methods have been used in the effort to attach DNA to the beads convalently. One of the methods is based on the reaction between the bases of the nucleic acid and groups activated by diazotization. Noyes et al. (14) and Moss et al. (16) managed to attach 67 and 4 ug of DNA per mg of diazotized cellulose respectively, while Bünemann et al. (5) only managed to attach 0.5-0.8 ug per mg diazotized Sephacryl. However, we were not able to attach any significant amounts of DNA to diazotized M450 hydroxyl beads. A possible explanation might be that scaling down the procedure (100:1) somehow resulted in less favourable conditions.

The principle for the other methods is to immobilize the nucleic acids to the supports via their 5'-terminal end. Clerici et al. (13) was able to increase the degree of end-attached oligonucleotides to CNBr-activated cellulose by introducing reactive amino groups at the 5'-end of the oligonucleotides. The assumption is that this will favour the single-point attachment of the 5'-end rather than the multiple-point attachment of the aromatic groups of the bases. The CNBr-activation method has been applied on a variety of solid supports carrying hydroxyl groups on their surface (agarose, cellulose, polyacrylamide, Sephacryl). However, activated polysaccharides have the highest capacity for binding DNA. We were able to attach 2-3 ug of 5'-NH_2 modified

DNA M450 hydroxyl beads. However, the disadvantage of using a CNBr-activated support is that its reactivity decreases during storage.

Water-soluble carbodiimide (CMC) can be used to activate the 5'-terminal phosphate group of the DNA specifically. When the DNA is activated in the presence of hydroxyl groups on the support, it can be covalently attached through phosphodiester linkages at their 5'-phosphate termini. Gilham (3) and Rickwood (4) used the method of covalent attachment of DNA to cellulose and Sephadex, respectively. We managed to attach DNA to the hydroxyl beads (Table 2), but since the non-specific binding was in the range of 66-93%, and the procedure used resulted in beads sticking together in lumps, no further attempts to improve this method were done.

Instead of drying the DNA onto the beads, we used the water-soluble carbodiimide (EDC) to generate phosphoramidate linkages between primary amines on the beads and 5'-phosphate groups on DNA (Figure 1A). This reaction, in contrast to the CMC-reaction, was performed in solution. Chu et al. (9) have reported the method to give high coupling yields, and Gingeraas et al. (2) have used the method for end-attachment of DNA to a number of commercial amine-derivatized supports.

The amount of DNA that can be attached to the different amino beads varies probably with the molarity of amino groups on the bead surface and with the availability of the groups. Amino groups that are carried at the termini of an extension arm that is to long appear to be unavailable for reaction.

As expected the amino beads show a relatively high non-specific binding, and a high degree of end-attached DNA. Both lambda/HinfI fragments (average MW 500 bp) and a synthetic oligonucleotide (36 mer) were attached to the most promising R469 bead. Since very little was done to optimize the coupling reaction, we do not know how many moles of nucleic acids that can be attached per mg bead. Some results (data not shown) indicate that the R469 beads are saturated with long DNA fragments (11 ug or approx 50-60 pmole per mg) when using the present procedure. The coupling yield obtained for the 36 mer is in the same range (64 pmole per mg), but it can probably be increased since several

groups have reported that the coupling yields decreases with increasing length of the oligonucleotide or DNA fragment.

Water-soluble carbodiimide (EDC) was also used to generate amide linkage between carboxyl groups on the beads and 5'-amino-groups on the DNA (Figure 1B). A number of synthetic routes for introduction of amino groups at the 5'-end of oligonucleotides have been reported (12, 15, 17, 18), and they result in 70-90% 5'-NH_2 modification of the phosphate groups. Applied Biosystems now supplies extra chemicals/equipment to their DNA-synthesizer that make it possible to add amino groups to the 5'-end of the oligonucleotides as the last step of the synthesis.

However, a method for introduction of 5'-NH_2 groups to long DNA-fragments has not been reported, therefore one of the methods described for oligonucleotides (12) was used to introduce amino groups at the 5'-end of lambda/HinfI fragments. Since we in the first step of the reaction (Figure 1B) only obtained 30-40% conversion of the 5'-phosphate to 5'-phosphoramidate, the final DNA preparation will at most consist of 30-40% 5'-NH_2 modified DNA fragments. This might be the reason for obtaining a lower coupling yield to the carboxyl beads (approx. 20 pmoles lambda/HinfI fragments per mg R452 beads) compared to the amino beads (60 pmole per mg R469).

As expected the carboxyl beads show less non-specific binding compared to the amino beads, but unfortunately only 20-30% of the DNA fragments are end-attached. Ghosh et al. (10) also observed a lower degree of end-attachment to carboxyl supports compare to amino supports. Again very little was done to improve the coupling reaction or to determine the maximum amount of DNA that can be attached using the procedure described here. However, we suppose that the yield is dependent on the molarity of the carboxyl groups, the availability of the reactive groups, the degree of 5'-NH_2 modification of the DNA preparation, and the length of the DNA fragment or oligonucleotide. We would therefore expect a reaction between a 100% 5'-NH_2 modified oligonucleotide preparation and the carboxyl beads to result in more efficient coupling reaction.

When the carboxyl and amino beads are used as a support for

the DNA, carbodiimide (EDC) is used both for coupling of DNA and modification of the 5'-phosphate groups. It is known that carbodiimide in slightly alkaline solutions also will modify the bases of nucleic acids. This might effect the hybridization properties of the immobilized DNA. However, this is probably only a problem when denatured DNA or oligonucleotides are being coupled to the beads. When native DNA is used, the bases are most probably protected from carbodiimide modification, and the attachment of DNA via the bases is thereby supressed. Chu et al. (9) have reported that the physical and biological properties of native DNA were not effected when modification was carried out with 0.1 M or 0.2 M EDC for 2 hours at pH 6 and 25°C. When the DNA is being attached to amino beads, it is in contact with carbodiimide for 3 hours, while when carboxyl beads are used in the reaction, the DNA is in contact with carbodiimide for more than 20 hours. The longer reaction-time needed for the carboxyl beads may result in that they bind DNA fragments with a higher degree of base modification. However, the advantage of using carboxyl beads is their low level of non-specific binding.

The hybridization experiments indicate that beads carrying oligonucleotide probes have hybridization kinetics comparable to that obtained for the corresponding reaction in solution.

The R469 amino beads which were used as support showed a considerable non-specific binding of DNA, and therefore a prehybridization step was necessary. The prehybridization step seems to be important in two ways, firstly to saturate the beads with non-specifically bound DNA, and secondly to remove adsorbed probe from the support surface through the displacement properties of macromolecules present in the buffer. Gingeraas et al. (2) suggest that coating the support with macromolecules prevents the end-attached probe from being adsorbed along their length and thereby become unavailable for hybridization.

The hybridization efficiencies obtained for oligonucleotide probes immobilized to the beads, are 70% of total target DNA for oligonucleotide target. These results are similar to those obtained by Gingeraas et al. (2) using end-attached oligonucleotides to Sephacryl 500 dextran support. The limitations on hybridization efficiencies seem to be independent of the amount

of immobilized probe used to hybridize a fixed amount of target, or the concentration of target to be hybridized by a fixed amount of support bound probe. These results suggest that the availability of the target in these hybridizations is limiting.

The advantage of using oligonucleotides in contrast to long DNA fragments as probes, is due to their faster kinetics of hybridization. They can also be obtained in large quantities and in a high degree of purity by using automated nucleic acid synthesizers. However, it is of great interest to also be able to use long DNA probes. Preliminary hybridization experiments showed that increasing amounts of end-attached DNA fragments (approx. 600 bp) in a solution with excess amounts of target, were able to hybridize increasing amounts of target DNA.

In summary, carbodiimide (EDC) mediated end-attachment of oligonucleotides or DNA fragments to amino- or carboxyl-modified beads in solution, seems to be the best coupling method. In hybridization experiments the carboxyl beads are probably the best alternative because of their low non-specific binding. The hybridization kinetics of the DNA-beads approach those of solution hybridization.

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