Design and synthesis of polyacrylamide-based oligonucleotide supports for use in nucleic acid diagnostics

Eoin Fahy, Geneva R. Davis, Luke J. DiMichele and Soumitra S. Ghosh*
Life Sciences Research Laboratory, Baxter Diagnostics Inc., 4245 Sorrento Valley Boulevard, San Diego, CA 92121, USA

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ABSTRACT
Polyacrylamide supports, in a range of pore sizes, were investigated as nucleic acid affinity matrices for the detection of target DNA or RNA sequences using a sandwich hybridization format. Bromoacetyl and thiol oligonucleotide derivatives were covalently linked to sulfhydryl- and bromoacetyl-polyacrylamide supports with greater than 95% end-attachment efficiencies. These polyacrylamide-oligonucleotide supports were further derivatized with anionic residues to provide multi-functional supports which show low non-specific binding for non-complementary nucleic acids. While all the polyacrylamide-oligonucleotide supports capture complementary oligonucleotides with high affinity, the pore size was found to be a critical parameter in sandwich hybridization reactions. The superior hybridization characteristics of the Trisacryl support was ascribed to a combination of its macroporous nature, hydrophilicity and the terminal attachment of its capture oligonucleotides.

INTRODUCTION
In recent years, solid support-based sandwich hybridization formats have become very attractive methods in the repertoire of nucleic acid diagnostics for detecting target DNA or RNA sequences (1–5). The efficacy of the assay is derived from the combined specificities of the bead-bound oligonucleotide and detection oligonucleotide probe for the target molecule. The kinetics of the hybridization reaction are rapid when short capture oligonucleotides (~15–30 bases in length) are employed (6) and when these sequences are attached to the solid support through single-point linkages (7). Typical solid supports which have found application for detecting nucleic acids include avidin-coated polystyrene beads (5), nitrocellulose or nylon membranes (3, 8, 9), activated dextran, (10) diazoitised cellulose supports (11, 12), Sephacryl (6, 11, 13), polystyrene matrices (7, 14) and glass (15).

A number of the above hybridization systems have drawbacks which can compromise the sensitivity of detection. In membrane-based approaches, where the target is non-covalently bound to the matrix, the release of the analyte from the supports during hybridization has been observed (16, 17). In the converse case employing DNA probes immobilized on nylon membranes, sandwich hybridization detection of target nucleic acids is compromised by very low capture efficiencies (3). The release of oligonucleotide probes from the solid support is also a potential problem in methods which exploit the biotin-avidin interaction (4, 5) and CNBr- or N-hydroxy-succinimide activated polysaccharide affinity supports are prone to leakage of ligands (18, 19). The reaction of CNBr activated polysaccharide supports with amines also leads to the formation of charged N-substituted isoamides, which confer undesirable ion-exchange properties to the support resulting in increased non-specific binding (18). Furthermore, the capture sequences in a number of these methods are often immobilized through multiple linkages involving the exocyclic amines of the bases (6, 11, 14, 20), thus decreasing the efficiency of the hybridization. Finally, low capture efficiencies and slow rates of hybridization have been noted for glass supports (15).

In order to address the limitations discussed above, we have investigated the suitability of polyacrylamide resins as nucleic acid affinity matrices. The attractive features of these cross-linked supports were their hydrophilicity, availability in a large range of pore sizes, wide limits of chemical and thermal stability, low non-specific adsorption of biological macromolecules and ease of derivatization of the matrices with reactive functional groups (21). In this report, we describe chemical methods for the covalent end-attachment of oligonucleotides to these supports using thioether linkages and have evaluated the hybridization characteristics of these polyacrylamide-based nucleic acid affinity supports in direct capture and sandwich hybridization reactions.

MATERIALS AND METHODS
T4 polynucleotide kinase (EC 2.7.1.78) was obtained from New England Biolabs and bovine serum albumin fraction V (BSA), sodium dodecyl sulfate (SDS), and polyvinylpyrrolidone (PVP, MW 40,000 daltons) were from Sigma. Bio-Gels P-2, P-10, P-60, P-200, P-300 and acrylamide were obtained from Bio-Rad, and Trisacryl GF-2000 was obtained from IBF Corp. N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid (HEPES), 3-(N-
morpholino)-propanesulfonic acid (MOPS), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. HCl (EDC) were purchased from Calbiochem, and all other chemicals were purchased from Aldrich.

The oligonucleotides used in this study were synthesized on an Applied Biosystems 380A automated DNA synthesizer and were purified according to the reverse-phase chromatographic conditions described by Ghosh et al (6). Oligonucleotides were checked for purity by electrophoresis on a 20% polyacrylamide gel. The sequences of the oligonucleotides used in these experiments are as follows: 5'-GCACACAAGTGA-CCTGGAATTAGCAAGCA-3'; 86-32: 5'-TGTCGCTAGTGGCTGTCG-3'; 88-179: 5'-CTATAGATCTCCAGGCAGCCGC-3'; 88-180: 5'-CCGGGCCTCGAGATCTAGA-3'; 88-77: 5'-AATTATAATAGCACTCAGTAT-AGGGGACCTAGGCTAATATGTGGTCTTAAAGG-3'; 86-29: 5'-ACACCATGTTGATTTCTCGAGGGAAGCCT-AGTA-3'. T4 polynucleotide kinase was used for the enzymatic phosphorylation of the oligonucleotides at their 5'-termini (22).

Plasmid pARV7A/2 was constructed by inserting a DNA copy of the HIV genome into the EcoRI site of pUC19 (2, 23). The plasmids pHIVX1 and pHIVX2 are M13-based plasmids containing an approximately 1300-base pair fragment from the vif region of HIV-1 in opposite orientations. The approximately 8600-base single-stranded forms of these plasmids were used for sandwich hybridization. DNA targets were synthesized using a transcription-based amplification system (TAS) described by Kwohl et al (2). Total RNA was extracted from HIV-1-infected CEM cells, and a region of the vif gene was amplified by the TAS procedure using primers 88-77 and 86-29. The 214 base gene was amplified by the transcription-based amplification system (TAS) described by Ghosh et al (24). The level of cystamine attachment and the extent of modification was determined by autoradiography following electrophoresis on a 15% polyacrylamide/8M urea gel.

Preparation of bromoacetyl derivatives of oligonucleotides

The 5'-cystaminyl oligonucleotide derivatives were prepared as described by Ghosh et al (24). The level of cystamine attachment was determined by treating 50 pmol of crude 32P-labeled cystaminyl-derivatized oligonucleotide with 1 µl of calf intestine alkaline phosphatase (Boehringer Mannheim, EIA grade) in 100 µl of 0.1 M Tris HCl, 0.1 M NaCl, 0.01 M MgCl2, pH 9.5, at 23°C. After 2 hours, the reaction mixture was applied to a 5-ml Sephadex G-50 column and eluted with TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0). The first and second radioactive peaks were collected and corresponded with cystaminyl-derivatized oligonucleotides and free inorganic phosphate (cleaved from unreacted 5'-phosphorylated oligonucleotide), respectively. The radioactivity associated with each peak was used to estimate the yield of cystamine attachment.

Preparation of bromoacetyl derivatives of oligonucleotides

Preparation of bromoacetyl derivatives of oligonucleotides

Typically, 20 ml of 6 M hydrazide was added to 1 gram of dry resin, and the suspension was stirred mechanically at 50°C. After 40 minutes, the excess reagent was removed by washing the beads in a sintered glass funnel (porosity C) with 0.2 M NaCl until the filtrate gave a negative test with 2,4,6-trinitrobenzene sulfonic acid (TNBS) (21). The derivatized beads were filtered, weighed, and suspended in TE or 0.1 M K2HPO4, pH 8.0.

Acylation of Biogel hydrazide beads with N-succinimidyl bromoacetate

One gram (wet weight) of Biogel hydrazide beads (50 mmole/dry gram) was washed with two 50-ml volumes of 0.1 M K2HPO4, pH 7.0, and resuspended in 5 ml of the same buffer. The suspension was cooled to 0°C in an ice bath, and N-succinimidyl bromoacetate (10-mole equivalents relative to hydrazide groups) in 250 µl of DMF was added drop-wise with stirring. The reaction mixture was allowed to come to room temperature over 30 minutes. After cooling to 0°C again, another aliquot (10-mole equivalents) of N-succinimidyl bromoacetate in DMF was added and the reaction mixture stirred for 30 minutes at room temperature. The beads were filtered through a sintered glass funnel (porosity C), washed with 50 ml of 0.1 M K2HPO4, pH 7.0, and the acylated beads were stored in 0.1 M K2HPO4 at 4°C. The level of substitution was determined by treatment with dithiothreitol (DTT), followed by titration with 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB, (26)].

Glutarylolysis of bromoacetyl Biogel beads

One gram (wet weight) of bromoacetyl Biogel beads (50 mmole hydrazide/dry gram) was washed with 0.1 M NaCl and then suspended in 20 ml of the same solution. A 100-mg portion of glutaric anhydride was added to this suspension and the pH of the suspension was maintained near 4.0 by the addition of 3 M NaOH, while the reaction mixture was magnetically stirred for 15 minutes. A second 100-mg portion of glutaric anhydride was added, and the reaction mixture was stirred for an additional 15 minutes. The beads were then subjected to three consecutive washes with 40 ml each of 0.1 M K2HPO4, pH 7.0.

Preparation of sulfhydryl derivatives of Biogel supports

One gram (wet weight) of Biogel hydrazide beads (500 mmole-NNHNH2/dry gram) was equilibrated with 0.5 M NaHCO3, pH 9.7. To a suspension in 5 ml were added 30-mole equivalents (relative to NNNHNH2 groups) of N-acetylimidocyanine thiolactone, and the mixture was agitated using a wrist action shaker for 16 hours at room temperature. The beads were washed with 300 ml of 0.1 M NaCl and stored in TE or 0.1 M K2HPO4, pH 8.0.

Glutarylolysis of sulphydryl-Biogel beads

A one-gram sample of sulphydryl Biogel was suspended in 20 ml of 0.1 M NaCl solution and two 100-mg aliquots of glutaric anhydride were added at 15-minute intervals. The pH was maintained near 4.0 by the addition of 3 M NaOH. After a total reaction time of 30 minutes, the beads were washed with 200 ml of 0.1 M NaCl. Hydrolysis of the thioester linkages was then carried out with 10 ml of 0.1 M Tris HCl, pH 8.5, for 1 hour at room temperature.
Preparation of the amine derivative of Trisacryl
A 20-ml suspension of Trisacryl GF-2000 was pipetted into a sintered glass funnel, washed with 200 ml of H2O, and subjected to vacuum suction for 10 minutes. The dried sample (~11 grams) was added slowly to 20 ml of ethylenediamine at 90°C. After one hour, the reaction mixture was cooled by the addition of 30 ml of crushed ice. Excess ethylenediamine was removed from the resin with 400-ml washes with 0.2 M NaCl and 1 mM HCl, followed by an additional 500-ml wash with 0.1 M NaCl. Washing with 0.1 M NaCl was continued until the filtrate gave a negative test with TNBS reagent.

Preparation of the sulfhydryl derivative of Trisacryl
Trissacryl-amine support (5 gm) was equilibrated with 0.5 M NaHCO3, pH 9.7, and the volume was adjusted to 30 ml. One gram of solid N-acetylhomocysteine thiolactone was added, and the reaction vessel was mechanically shaken at room temperature for 2 hours. Then, a second gram of reagent was added, and the mixture was agitated overnight. The beads were washed with 500 ml of 0.1 M NaCl, and the sulfhydryl group concentration was estimated by titrating with DTNB reagent.

Succinylation of sulfhydryl-Trisacryl
A 2-gram sample of sulfhydryl-Trisacryl support was equilibrated in 20 ml of 0.1 M NaOAc, pH 6.0, and treated with 100 mg of solid succinic anhydride. After agitation for 30 minutes, another 100 mg of anhydride was added to the suspension, and the mixture was agitated for a further 30 minutes. The beads were then washed and equilibrated in 40 ml of 0.1 M Tris HCl, pH 8.5. After 1 hour, the support was washed with TE and stored at 4°C.

DTNB test for determination of sulfhydryl and bromoacetyl substitution levels of solid supports
Derivatized support (1–20 mg wet weight) was reduced with 500 μl of 20 mM DTT in 50 mM K2HPO4, 1 mM EDTA, pH 8.0, for 30 minutes. After removal of the supernatant, the beads were washed with 3 ml volumes of 50 mM K2HPO4, pH 8.0. The beads were then treated with 1 ml of 1 mM DTNB in 50 mM K2HPO4, pH 8.0. The absorbance of the supernatant was monitored at 412 nm after 15 minutes (ε = 13,600 for released thiophenolate).

Covalent attachment of 5'-mercaptoethylphosphoramidate oligonucleotide derivatives to bromoacetyl-Biogel beads
The 32P-labeled cystaminyl-containing oligonucleotide (200 pmoles) was reduced with 300 μl of 0.1 M DTT, 0.2 M HEPES, 1 mM EDTA, pH 7.7, for 1 hour at room temperature. The product was precipitated with EtOH/LiCl, the pellet was dissolved in 300 μl of 0.2 M HEPES, 1 mM EDTA, pH 7.7, and precipitated again with EtOH/LiCl. This process was repeated once more to remove the last traces of DTT. Typically, 50 mg of beads were treated with 25 pmoles of freshly prepared thiolated oligonucleotide in 150 μl of 0.1 M K2HPO4, pH 9.0, and agitated under argon atmosphere on a rotary mixer overnight. As a control to determine non-specific binding, a 32P-labeled 5'-phosphorylated oligonucleotide was added to a sample of beads under the same conditions. The beads were washed three times with 1 ml each of 0.1 M Na2PO4, pH 7.5, and three times with 1 ml each of 15 mM NaOH, pH 12. The 32P-labeled oligonucleotide control was also used to estimate end-attachment efficiencies.

Covalent attachment of 5'-bromoacetyl-derivatized oligonucleotides to sulfhydryl-Biogel beads
Sulfhydryl-Biogel support (1 gram wet weight) was reduced with 5 ml of 20 mM DTT, 50 mM K2HPO4, pH 8.0, for 1 hour. Then, the support was washed twice with 40 ml of 50 mM K2HPO4, pH 8.0, followed by two 40-ml washes with 0.1 M triethylenammonium phosphate (TEAP), 1 mM EDTA, pH 9.0. Bromoacetyl-derivatized oligonucleotide (500 pmoles) was dissolved in 1 ml of TEAP, 1 mM EDTA, pH 9.0, and added to the resin. After purging the reaction tube with argon and sealing with parafilm, the reaction mixture was agitated on a rotary mixer overnight. Subsequently, the beads were washed with three 10-ml volumes of 0.1 M Na2PO4, pH 7.5, followed by two 10 ml volumes of TE, pH 8.0. Unreacted sulfhydryl groups were capped by reducing the support with 3 ml of 20 mM DTT, 50 mM K2HPO4, pH 8.0, for 30 minutes. After removal of excess DTT and equilibration in 0.1 M TEAP, 1 mM EDTA, pH 9.0, 3 ml of 5 mM iodoacetic acid in the same buffer were added and allowed to react for 1 hour. Following filtration of unreacted reagent through a sintered glass funnel (porosity C), the bead samples were stored in TE at 4°C.

Covalent attachment of 5'-bromoacetyl-derivatized oligonucleotides to sulfhydryl-Trisacryl
One gram of the solid support was reduced with DTT following the procedure used for Biogel beads and equilibrated in 0.1 M TEAP, 1 mM EDTA, pH 9.0. Five hundred pmoles of bromoacetyl-derivatized oligonucleotide was dissolved in 1 ml of 0.1 M TEAP, 1 mM EDTA, pH 9.0, and the solution was added to the support. The reaction tube was purged with argon, sealed and agitated overnight on a rotary mixer. Then, 19 mg of solid iodoacetic acid were added, and the mixture was left at room temperature for 1 hour. The beads were washed three times with 20 ml each of 0.1 M Tris HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS, four times with 20 ml each of 0.1 M Na2PO4, pH 7.5, followed by two washes with 20 ml each of TE.

Direct capture of 32P-labeled targets on oligonucleotide-derivatized supports
Bead suspensions (50 mg wet weight) were aliquoted into 1.5-ml Eppendorf tubes, and the supernatant was removed after centrifugation. The beads were then prehybridized in 250 μl of hybridization buffer [5 × SSPE (22), 10% dextran sulfate, 0.1% SDS] at 37°C for 30 minutes, after which the supernatant was drawn off. Complementary or non-complementary 32P-labeled oligonucleotides, approximately 30 bases in length, were preincubated in 100 μl of hybridization buffer at 65°C for 5 minutes and then added to the beads containing end-attached oligonucleotides. The beads were incubated at 37°C for 1 hour with occasional shaking. After washing five times with 1 ml each of 2 × SSC at 37°C, the amount of label bound to the supports was determined by Cerenkov counting.

Sandwich hybridization of transcription-amplification systems-generated RNA transcripts on solid supports
Samples of Biogel or Trisacryl (50 mg) were prehybridized as described above. The target RNA was denatured at 65°C minutes
immediately prior to hybridization. Solution hybridization of the target RNA (0.5 fmol) with a complementary 32P-labeled detection oligonucleotide (5 fmol) was performed in a total volume of 20 μl of hybridization buffer for 2 hours at 42°C. The sample was then diluted to 100 μl with hybridization buffer and added to the capture supports. Sandwich hybridization was performed at 37°C for 1 hour with occasional mixing. Finally, the beads were washed five times with 1 ml of 2 × SSC at 37°C. Non-complementary RNA target was used as a control to determine the level of non-specific binding in the assay. More extensive washes of the immobilized sandwich hybridization complexes do not afford any further improvement in signal-to-noise ratio of the assay.

RESULTS
Attachment of thiol-oligonucleotides to bromoacetyl-derivatized polyacrylamide supports

The chemistry of attachment of oligonucleotides to Biogel polyacrylamide supports is illustrated in Figure 1. The Inman procedure (21) was followed to modify the primary amide groups of the matrices with hydrazine. The requisite substitution levels of the derivatized supports (Table 1) were obtained using the Inman relationship: D (mmoles/g) = h.C.t where C is the molar concentration of hydrazine, t is the reaction time in hours and h is a temperature-dependent coefficient (at 50°C, h = 0.123). Treatment of these derivatized supports with a 20-molar excess of bromoacetic acid N-hydroxysuccinimide ester provided essentially quantitative formation of the thiolated oligonucleotides. Reduction of the disulfide linkage with DTT resulted in quantitative formation of the thiolated oligonucleotides.

The overall attachment efficiencies (Table I) of freshly prepared 32P-labeled thiolated oligonucleotides to the bromoacetyl-polyacrylamide supports were in the 5—68% range, showed variability (entries 3—5), and were lower than the 70% attachment efficiencies observed in our previous study using the amide linkage for Sephacryl support (6). However, in contrast with the 55—65% end-attachment observed with the Sephacryl support (6), the thiol-bromoacetyl group coupling strategy provided essentially quantitative end-attachment of the oligonucleotides.

Table 1. Substitution levels and attachment efficiencies for bromoacetyl supports

<table>
<thead>
<tr>
<th>Support</th>
<th>μmoles NH2/gram</th>
<th>μmoles CH2Br/μl</th>
<th>% 86-31-SH attached</th>
<th>% 86-31-PO4 attached</th>
<th>% end attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. P-2-CH2Br</td>
<td>50</td>
<td>8.0</td>
<td>5.0</td>
<td>0.08</td>
<td>98</td>
</tr>
<tr>
<td>2. P-10-CH2Br</td>
<td>50</td>
<td>27.9</td>
<td>13.6</td>
<td>0.44</td>
<td>97</td>
</tr>
<tr>
<td>3. P-60-CH2Br</td>
<td>50</td>
<td>21.6</td>
<td>22.6</td>
<td>0.28</td>
<td>99</td>
</tr>
<tr>
<td>4. P-60-CH2Br</td>
<td>50</td>
<td>21.6</td>
<td>52.4</td>
<td>0.84</td>
<td>98</td>
</tr>
<tr>
<td>5. P-60-CH2Br</td>
<td>50</td>
<td>21.6</td>
<td>68.0</td>
<td>0.15</td>
<td>100</td>
</tr>
<tr>
<td>6. P-200-CH2Br</td>
<td>50</td>
<td>20.3</td>
<td>24.0</td>
<td>0.35</td>
<td>99</td>
</tr>
</tbody>
</table>

Biogel supports were reacted with hydrazine to provide hydrazide matrices, and subsequently acylated to the bromoacetyl form (see Materials and Methods). The functional group densities are presented in μmoles per dry gram. The efficiency of attachment of the thiolated oligonucleotide 86-31 to these supports was determined by measuring the percentage of 32P-labeled thiolated oligonucleotide bound to the beads after the coupling and washing steps. The level of non-specific attachment was estimated using the same 32P-labeled oligonucleotide, but lacking the cystamine group at its 5' end. The end-attachment efficiencies were calculated by using the relationship: (% 86-31-SH bound — % 86-31-PO4 bound) + % 86-31-SH bound).
Hybridization characteristics of the P-60 affinity support

The P-60 support (exclusion volume of 60,000 daltons) was chosen for hybridization experiments since this matrix displayed superior attachment efficiencies in coupling reactions with thiolated oligonucleotides (see Table 1). For comparison, a Sephacryl-based oligonucleotide support (6) was also included in the hybridization studies. Table 2 shows that the two types of P-60 oligonucleotide supports were more efficient than Sephacryl in the direct capture of complementary oligonucleotide target. In sandwich hybridizations with a 8.6 kb single-stranded DNA plasmid, however, the polyacrylamide-based support showed a poorer ability for detection of long single-stranded DNA than Sephacryl. These results suggested that a substantial proportion of immobilized oligonucleotides was in the interior of the P-60 support and was therefore not accessible to the long target for hybridization due to the support's relatively low exclusion limit. Since the coupling reactions for thiol-oligonucleotides with bromoacetyl-derivatized supports gave variable yields (see Table 1), the converse coupling reaction of bromoacetyl-oligonucleotide derivatives with thiol-polyacrylamide supports was next investigated as were polyacrylamide supports with higher exclusion limits (200,000 daltons or greater).

Attachment of bromoacetyl oligonucleotide derivatives to sulphydryl-polyacrylamide supports

Figure 2 shows the synthetic scheme for linking bromoacetyl-oligonucleotides to sulphydryl-polyacrylamid supports. The P-2, P-200 and P-300 Biogel supports were chosen for thiol modification because of their vastly different exclusion volumes. The macroporous Trisacryl GF 2000 resin was also included in the study to provide a support similar to Sephacryl S-500 in having an exclusion limit in the range of $2 \times 10^7$ daltons. Thiol groups were introduced on the Biogel matrices by reacting hydrazide-polyacrylamide supports with N-acetylhomocysteine thiolactone. The Trisacryl support was modified via transamidation with ethylene diamine at 90°C followed by treatment with the thiolactone. The functional group density on the supports was determined by titrating the thiol groups with DTNB reagent (Table 3). Further conversion of the remaining hydrazide groups (Table 3, entries 4—5) with glutaric anhydride

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Table 2. Hybridization characteristics of bromoacetyl-derivatized polyacrylamide supports

<table>
<thead>
<tr>
<th>Immobilized support</th>
<th>Direct capture (%)</th>
<th>Sandwich hybridization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-60—CH$_2$Br—NHNH$_2$</td>
<td>89</td>
<td>0.2</td>
</tr>
<tr>
<td>P-60—CH$_2$Br—COOH</td>
<td>74</td>
<td>0.2</td>
</tr>
<tr>
<td>Sephacryl</td>
<td>54</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Direct capture efficiencies and non-specific binding of bromoacetyl Biogel P-60 (86-31) and Sephacryl (86-31) oligonucleotide supports were determined using 3.75 femtomoles of: a) complementary $^{32}$P-labeled 86-32 oligonucleotide, and b) non-complementary $^{32}$P-labeled 86-31 oligonucleotide, respectively. c) Sandwich hybridization efficiencies using these supports were obtained using 0.5 femtomole of a complementary 8600-base single-stranded DNA target (pHIVX1) and 5 femtomoles of $^{32}$P-labeled 86-83 detection probe; d) 0.5 femtomole of a non-complementary 8600-base single-stranded DNA target (pHIVX2) was used to determine non-specific binding in the sandwich assay.

Figure 2. Attachment of bromoacetyl oligonucleotides to sulphydryl-derivatized Trisacryl and Biogel supports.
provided mixed functionality supports whose anionic properties were envisaged to reduce non-specific binding.

Oligonucleotides functionalized at their 5'-termini with bromoacetyl groups were prepared by reaction of 5'-aminohexylphosphoramidate oligonucleotide derivatives with an excess of bromoacetic acid N-hydroxysuccinimide ester. The phosphate-to-bromoacetamide transformation was estimated to be 60—70% based on polyacrylamide gel analysis of the reaction products.

Optimal attachment efficiencies were obtained when the coupling of the oligonucleotide derivatives to the sulphydryl supports was performed at pH 9.0 in TEAP buffer. The coupling efficiencies to the Trisacryl, P-200 and P-300 supports were in the 18—43% range (Table 3). Further, the reactions were very reproducible and provided nearly quantitative end-attachment of capture sequence. The significantly lower efficiency of attachment to the P-2 support was surprising since the thiol concentration on the matrix was in vast excess of the oligonucleotide concentration in the reaction.

For determination of coupling efficiencies discussed above, the reactions were carried out in 1.5 ml Eppendorf tubes with 25 pmoles of bromoacetyl oligonucleotides and 50 mg of sulphydryl-polyacrylamide support. While the Biogel supports remain in suspension under the reaction conditions, the large particle size Trisacryl support was observed to settle. Scallop to gram quantities of Trisacryl for coupling reactions resulted in more efficient mixing of reactants and in higher coupling yields than the value reported in Table 3. This was shown using a hybridization assay in which 50-mg quantities of a representative Trisacryl-oligonucleotide support was titrated against increasing amounts of 32P-labeled complementary oligonucleotide. Figure 3 shows that the substitution level of the capture probe is minimally 6—8 pmoles per 50 mg of Trisacryl, which corresponds to 25—30% yield in the coupling reaction.

### Table 3. Substitution levels and attachment efficiencies for thiol polyacrylamide supports

<table>
<thead>
<tr>
<th>Support</th>
<th>μmoles NH₂</th>
<th>μmoles SH/g</th>
<th>% 86-31-Br attached</th>
<th>% 86-31-PO₄ attached</th>
<th>% end attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. P-2-SH</td>
<td>500</td>
<td>49.4</td>
<td>5.0</td>
<td>0.2</td>
<td>96</td>
</tr>
<tr>
<td>2. P-200-SH</td>
<td>500</td>
<td>70.3</td>
<td>41.0 ± 0.1</td>
<td>0.5</td>
<td>99</td>
</tr>
<tr>
<td>3. P-300-SH</td>
<td>500</td>
<td>52.2</td>
<td>43.0 ± 0.3</td>
<td>1.7</td>
<td>98</td>
</tr>
<tr>
<td>4. P-300-SH</td>
<td>500</td>
<td>45.9</td>
<td>31.1 ± 0.2</td>
<td>0.5</td>
<td>98</td>
</tr>
<tr>
<td>5. Trisacryl-SH</td>
<td>12.3</td>
<td>18.0 ± 0.7</td>
<td>0.4</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-COOH</td>
<td></td>
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</tbody>
</table>

Biogel and Trisacryl supports were transamidated with hydrazine or ethylenediamine, and then reacted with N-acetylmorpholine thiolactone. In selected cases, residual hydrazine or amine groups were capped with succinic anhydride. The functional group densities of the Biogel matrices are presented in μmoles per gram, while the thiol substitution levels of Trisacryl are based on wet gram weight of resin. The sulphydryl supports were reacted with bromoacetyl-derivatized oligonucleotides and coupling and end-attachment efficiencies were determined as described in Table 1. The coupling efficiencies are reported as the mean value ± 1 standard deviation.

**DISCUSSION**

The end-attachment of the capture oligonucleotide to the solid support via a suitable alkyl spacer was considered to be an essential requirement to ensure that a bead-based sandwich hybridization system approaches the hybridization kinetics of homogeneous solution hybridization (7). The enhanced reactivity of thiols towards α-halocarbonyl compounds suggested an
Table 4. Hybridization characteristics of thiol-polyacrylamide supports

<table>
<thead>
<tr>
<th>Immobilized support</th>
<th>Direct capture (%) of oligonucleotide</th>
<th>Sandwich hybridization (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>86-32^a</td>
<td>86-31^b</td>
<td>TAS RNA product comp^c</td>
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<tr>
<td>1. P-2</td>
<td>71.0</td>
<td>0.1</td>
<td>2.9</td>
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<td>2. P-200</td>
<td>87.8</td>
<td>0.4</td>
<td>18.0</td>
</tr>
<tr>
<td>3. P-200</td>
<td>91.9</td>
<td>0.4</td>
<td>8.6</td>
</tr>
<tr>
<td>4. P-300</td>
<td>88.4</td>
<td>0.6</td>
<td>24.0</td>
</tr>
<tr>
<td>5. P-300</td>
<td>75.0</td>
<td>0.6</td>
<td>16.0</td>
</tr>
<tr>
<td>6. Trisacryl</td>
<td>66.5</td>
<td>0.4</td>
<td>17.0</td>
</tr>
<tr>
<td>7. Trisacryl</td>
<td>70.0</td>
<td>0.1</td>
<td>17.0</td>
</tr>
<tr>
<td>8. Sephacryl</td>
<td>63.0</td>
<td>0.2</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Hybridization characteristics of sulfhydryl-Biogel and sulfhydryl-Trisacryl supports containing 86-31 capture oligonucleotide were determined in direct capture of: a) complementary ^32P-labeled 86-32 oligonucleotide; and b) non-complementary 86-31 oligonucleotide to ascertain non-specific binding; c) sandwich hybridization efficiencies were estimated using 0.5 femtomole of a TAS amplification-generated 214 base length HIV-1 5' region RNA transcript (2) and 5 femtomoles of ^32P labeled probe 86-83 oligonucleotide; d) A non-complementary TAS product (0.5 femtomole) was used as a negative control. A Sephacryl support, containing 86-31 capture sequence, was used for comparison.

Attractive approach by which thiol or bromoacetyl groups of suitably derivatized oligonucleotides and polyacrylamide supports might be reacted to furnish exclusively end-attached nucleic acid affinity supports. Our initial approach of coupling thiol-oligonucleotides with bromoacetyl-polyacrylamide supports was found to be variable in coupling efficiencies (Table 1, entries 3–5), presumably due to the susceptibility of the thiolated oligonucleotide to air oxidation during the course of the reaction. However, the converse reaction of bromoacetyl-oligonucleotides with thiol-polyacrylamide supports proceeded with reasonable and reproducible yields (Table 3). Essentially all the oligonucleotides were end-attached to the matrices using either strategy.

The end-attachment characteristic of these nucleic acid affinity matrices enable them to capture complementary oligonucleotide targets with very high efficiencies (Table 2, 4). In addition, non-specific binding on the supports was observed to be lower when anionic residues were introduced on the surface of the matrices. However, these supports did not behave predictably in sandwich hybridization reactions. For example, it was hypothesized that the highly crosslinked P-2 support (exclusion volume, 2000 daltons) would have essentially all of the capture sequence attached on the surface, and hence available for hybridization. The small pore size was expected to be beneficial in reducing non-specific binding as it would prevent inclusion of the target or detection sequence during the sandwich hybridization reaction. While P-2 support performed particularly well in direct capture of short oligonucleotides, it was very inefficient in sandwich hybridization reactions with a 214 base length RNA target (Table 4).

Higher sandwich hybridization efficiencies were obtained with increasing pore sizes of the supports and a significant reduction in non-specific binding was noted for the macroporous Trisacryl support (Table 4). The correlation of large pore size with improved hybridization properties is consistent with reported studies on polysaccharide supports (6,13). The best sandwich hybridization characteristics were obtained with the Trisacryl support (Figure 4), a very hydrophilic polymer which differs from the Trisacryl support. A TAS amplification-generated 214 base length HIV-1 5' region RNA transcript was used as target in hybridization reactions with 87–83 capture oligonucleotide and 86–31 detection oligonucleotide. A 10:1 ratio of detection probe to target was maintained for each target concentration. An identical concentration range of non-complementary TAS product was used to estimate the non-specific binding. 'Signal/Noise' was expressed as the ratio of ^32P counts obtained with complementary and non-complementary TAS products. Shaded bar: Trisacryl; Solid bar: Sephacryl.

Figure 4. Sandwich hybridization of RNA transcripts with Trisacryl and Sephacryl supports. A TAS amplification-generated 214 base length HIV-1 5' region RNA transcript was used as target in hybridization reactions with 87–83 capture oligonucleotide and 86–31 detection oligonucleotide. A 10:1 ratio of detection probe to target was maintained for each target concentration. An identical concentration range of non-complementary TAS product was used to estimate the non-specific binding. 'Signal/Noise' was expressed as the ratio of ^32P counts obtained with complementary and non-complementary TAS products. Shaded bar: Trisacryl; Solid bar: Sephacryl.
structurally from the Biogel resins in having secondary amides which carry 2-hydroxy-1,3-propane diol groups.

Trisacryl-oligonucleotide supports which are stored for extended periods of time (~1–2 years) at 4°C and used without the prehybridization step show very similar sandwich hybridization efficiencies as the same beads that had been subjected to a prehybridization step. This strongly suggests that leaching of oligonucleotides from the supports does not occur during storage. Inclusion of dextran sulfate in hybridization reactions using Trisacryl supports was essential and a final reagent concentration of 10% in the reaction buffer was found to be the most suitable for optimal sandwich hybridization efficiencies. Further, the efficiency of a one hour single step hybridization using Trisacryl support is similar to the two-step hybridization procedure which was used in this study (data not shown). Finally, Trisacryl-oligonucleotide supports are readily adaptable to a column format, and have proved useful in differential bead-based sandwich hybridization assays (28) for mutational analysis of zidovudine-resistant human immunodeficiency virus type 1.

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