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Sequence-Specific Purification of DNA Oligomers in Hydrophobic Interaction Chromatography Using Peptide Nucleic Acid Amphiphiles: Extended Dynamic Range

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Abstract

We present improvements on a previously reported method (Vernille JP, Schneider JW. 2004. *Biotechnol Prog* 20(6):1776–1782) to purify DNA oligomers by attachment of peptide nucleic acid amphiphiles (PNAA) to particular sequences on the oligomers, followed by their separation from unbound oligomers using hydrophobic interaction chromatography (HIC). Use of alkyl-modified HIC media (butyl and octyl sepharose) over phenyl-modified media (phenyl sepharose) reduced the elution time of unbound DNA while not affecting the elution time of the PNAA/DNA complex. Modifying the alkane tail length for PNAA from C₁₂ to C₁₈ increased slightly the retention of PNAA/DNA duplexes. By combining these two refinements, we show that sequence-specific purifications of DNA oligomers 60 bases in length or more can be achieved with high resolution, even when the PNAA alkane is attached to the center of the target strand. The insensitivity of the PNAA/DNA duplex binding to choice of HIC media appears to be due to a surface-induced aggregation phenomenon that does not occur in the case of untagged DNA. We also report on the use of batch HIC as an adequate predictor of elution profiles in linear gradient HIC, and its potential to considerably reduce purification times by applying step gradients.

Keywords

hydrophobic interaction chromatography; DNA separation; peptide nucleic acid; affinity separation

Introduction

Synthetic DNA oligonucleotides are indispensable molecular biology tools, and have recently garnered considerable interest as therapeutic agents (Stephenson and Zamecnik, 1978; Tamm, 2006). As more scientific and commercial avenues for oligonucleotides emerge, it will be essential to control product purity in terms of both length and sequence. Undesired side products of solid-phase DNA oligomer synthesis generally arise from inefficient monomer coupling to the growing oligomers. To address this, a capping step is

included in the synthetic scheme to block unreacted terminal groups from further extension. Shorter, “failure sequences” result from the capping and must be isolated from the full-length product.

For shorter oligomers, failure sequences can be separated from target sequences by RP-HPLC methods. A common approach to improving product resolution, and extending it to longer oligomers, is to retain a lipophilic protecting group, such as 4,4'-dimethoxytrityl (DMT, or trityl), on the target sequence for improved resolution in RP-HPLC or other forms of purification (Andrus and Kuimelis, 2000; Warren and Vella, 1994). The DMT group can then be removed after separation under acidic conditions. Recently, purification of long (50–100 base) oligonucleotides has been reported using highly non-polar fluororous protecting groups in solid-phase extraction (Pearson et al., 2005). However, single-base resolution is typically not feasible with these methods of purification, and none address sequence-specificity. This can be particularly problematic for applications that require highly pure oligomers such as molecular cloning. Incomplete capping as well as inefficient deprotection during synthesis can result in *n*-1 oligomers that still contain the purification handle. In a representative study, clonable inserts were prepared from purified 123- and 126-base synthetic oligonucleotides and it was determined that half of the resulting copies contained errors, which were ultimately attributed to the latter stages of chemical synthesis (Hecker and Rill, 1998).

We have been pursuing an alternative approach that purifies DNA oligomers by hybridization of a DNA-binding peptide (peptide nucleic acid, PNA) with an attached alkane tail (Vernille et al., 2004). Attachment of these PNA amphiphiles (PNAA) provides a handle for the removal of target oligomers from complex solutions. Since sequence recognition is required for tagging, this approach can exclude sequence errors as well as failure sequences. The use of PNA for this application is preferable to DNA due to the marked increase in stability and sensitivity towards base mismatches of a PNA/DNA duplex compared to its DNA/DNA counterpart (Egholm et al., 1993; Ratilainen et al., 1998, 2000).

A previous report described our initial efforts to separate DNA oligomers from mixtures using PNAA (Vernille and Schneider, 2004). PNAA molecules were found to efficiently bind target DNA sequences in solution, and the attachment of the PNAA gave rise to strong adsorption of the PNAA/DNA duplex to phenyl-substituted media in hydrophobic interaction chromatography (HIC). Near-quantitative recovery of target DNA was realized using an equimolar amount of PNAA and 5 min room-temperature incubation. However, resolution between unbound and PNAA/DNA duplex was marginal for oligomers 60 bases in length. Here, we improve the resolution substantially by increasing the PNAA lipid tail length while investigating the effect of three commercially available HIC stationary phases on retention behavior. By judicious choice of PNAA tail length and HIC stationary phase ligand, we have developed a gentle yet powerful, sequence-specific technique to separate oligonucleotides from complex mixtures over a significant range of oligomer lengths. Separations can be performed in either column-based HIC, or in microtiter plates in a batch mode.

Materials and Methods

PNAA Synthesis

Unless otherwise stated, all reagents, buffers, and salts used are HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA). Details of the PNAA synthesis and chemical structures are available elsewhere (Vernille, 2004; Vernille and Schneider, 2004; Vernille et al., 2004). Briefly, PNAAs were synthesized using a solid-phase peptide synthesis scheme with Fmoc/Bhoc-protected PNA monomers (Applied Biosystems, Foster City, CA) and Fmoc-protected PAL-PEG-PS resin (Peptides International, Louisville, KY). Peptide synthesis was performed in a stepwise fashion until the desired chain length was obtained. Peptides were alkylated by attachment of lauric acid (C₁₂), myristic acid (C₁₄), palmitic acid (C₁₆), or stearic acid (C₁₈) to the N terminus to complete the synthesis. The PNAA structure “C_x-agtgatctac-E₄” is listed in the N to C direction, which corresponds to the 5′ to 3′ direction of a corresponding DNA strand. PNA bases are listed in lower case and “E” indicates a glutamic acid residue. It was found that at least two glutamic acid residues were necessary to increase PNAA solubility. Previous work in this group has shown that these modifications do not interfere with the DNA binding specificity (Vernille et al., 2004).

Synthesis products were subsequently cleaved from the resin with a 4:1 trifluoroacetic acid (TFA):m-cresol mixture and subjected to HPLC purification with a Symmetry 300 C₄ column (Waters, Milford, MA) on a Waters Delta 600 HPLC. Elution was performed at a flowrate of 10 mL/min using a 30 min linear gradient from a (water/0.1% TFA) mixture to an (acetonitrile/0.1% TFA) mixture. UV absorbance was monitored at 254 nm and all peaks above a predetermined response threshold were automatically collected using a Waters Fraction Collector II. HPLC products were then lyophilized and used without further purification. Each product was characterized on a PerSeptive Voyager STR MALDI-TOF mass spectrometer (Applied Biosystems). Observed and theoretical molecular weights were: C₁₂-agtgatctac-E₄: 3426.2 (theo, 3425.4); C₁₄-agtgatctac-E₄: 3455.0 (theo, 3453.4); C₁₆-agtgatctac-E₄: 3481.1 (theo, 3481.5); C₁₈-agtgatctac-E₄: 3510.3 (theo, 3509.5).

HIC

All DNA oligomers were purchased from Integrated DNA Technologies (Coralville, IA), dissolved in 10 mM Tris-HCl buffer (pH 8.0) and used without further purification. Calf-thymus DNA (Fisher Scientific) was sheared in a bath sonicator for 5 min to achieve a heterogeneous dsDNA population of 100–3,000 bp.

HIC experiments were performed on a 6% cross-linked agarose 0.97 mL HiTrap column (GE Healthcare, Piscataway, NJ). Three different hydrophobic ligands were tested: phenyl (20 μmol ligand/mL gel), butyl (50 μmol/mL gel), and octyl (5 μmol/mL gel). Separations were achieved on an AKTA Explorer fast protein liquid chromatography (FPLC) system (GE Healthcare). PNAA was added to DNA solution in 1.5 mL centrifuge tubes in 10 mM Tris-HCl (pH 8.0) for approximately 5 min prior to injection. A 50 μL sample loop, overfilled by 50%, was used for manual sample injection. A 5× molar excess (strand basis) of PNAA was used in all experiments to accelerate hybridization. PNAA and DNA experimental concentrations are noted with each figure. Elution was performed at a flowrate

of 1.0 mL/min using a 15 mL linear gradient from a mixture of (1.5 M $(\text{NH}_4)_2\text{SO}_4$ /10 mM Tris-HCl, pH 8.0) to a buffer of 10 mM Tris-HCl (pH 8.0), then followed by 25 mL of isocratic elution using the latter buffer. Sample elution was monitored using UV detection at 260 nm. Each column was cleaned and regenerated with five column volumes of 0.5 M NaOH and 70% EtOH between injections, and was stored in 20% EtOH at 4°C.

UV Melting Curves

UV melting curves were obtained at 260 nm for PNAA–DNA solutions in various buffers using a Varian Cary 3 spectrophotometer equipped with a thermoelectrically controlled multi-cell holder using 1 cm quartz cuvettes (Starna, Atascadero, CA). Solutions were initially heated to 85°C and held for 5 min to equilibrate. The temperature was then lowered at 1.0°C/min to 10°C and cycled between the upper and lower temperature limits several times. The melting temperature (T_m) was determined by performing a van't Hoff analysis on the each curve and averaging the resulting T_m values.

Batch Adsorption to HIC Media

Initial batch studies were performed on a MultiScreen 1.2 μm filter 96-well microplate (Millipore, Milford, MA). Each well was loaded with 25 μL of a 70% slurry of butyl-modified TosohHaas Toyopearl resin (40–90 μm size, Tosoh Bioscience, Montgomeryville, PA) and 175 μL of a solution containing $(\text{NH}_4)_2\text{SO}_4$ /10 mM Tris-HCl (pH 8.0) buffer. Prior to the experiment, the resin was gently pelleted and resuspended in starting buffer three times. Next, PNAA, DNA, or a pre-incubated mixture was added to each well and allowed to equilibrate on an orbital shaker for 30 min. The starting buffer was then removed by vacuum filtration and the filtrate analyzed for nucleic acid content by both absorbance (260 nm) and fluorescence (excitation 485/emission 538) with a microplate reader (Molecular Devices, Sunnyvale, CA). The well was then refilled with 200 μL buffer, agitated for 10 min, filtered, and analyzed. The concentration of $(\text{NH}_4)_2\text{SO}_4$ in the 10 mM Tris-HCl (pH 8.0) buffer was progressively decreased for each trial, beginning at 1.5 M and decreasing to 1.25, 1, 0.75, 0.5, 0.25, 0.1, and finally 0 M. For two-step elution, only 1 M and 0 M $(\text{NH}_4)_2\text{SO}_4$ solutions were used; the media was then washed with 20% ethanol to strip off any remaining material.

Results and Discussion

The main goal of this work was to optimize the PNAA tail length and selection of HIC media to improve the resolution of peaks arising from unbound DNA oligomers and PNAA/DNA duplexes. HIC separations rely on selective non-polar interactions between hydrophobically modified media and analytes, and as such, one may expect that the separation resolution would be maximized by using the PNAA with the longest alkanes possible, and using HIC media with the greatest extent of non-polar substitution. To maintain the solubility of PNAA, there are practical limits on the length of alkane that can be attached to PNA peptides. Additionally, commercial HIC media have only a moderate degree of non-polar modification to allow for wetting by aqueous solutions and recovery of analytes under non-denaturing conditions. With these limits in mind, we studied the HIC elution behavior of PNAA/DNA complexes with PNAA alkanes 12–18 carbons in length.

Three different commercial HIC media were investigated: phenyl, octyl, and butyl sepharose.

Figure 1 compares HIC chromatograms for the DNA oligomer 5'-(T)₂₅-GTAGATCACT-(T)₂₅-3' attached to PNAA of varying alkane length. For each run, a small sample of 5 μ M DNA and 25 μ M PNAA was prepared in 10 mM Tris-HCl buffer (pH 8.0) and injected directly into the instrument's sample loop. Binding between the DNA and PNAA is fast and nearly quantitative as judged by UV melting curves and circular dichroism spectra collected on similar samples. A linear gradient elution method was chosen, starting from a pure solution of 1.5 M (NH₄)₂SO₄/10 mM Tris-HCl and ending with 10 mM Tris-HCl buffer. Accordingly, just after injection the PNAA/DNA mixture encounters a sharp increase in the concentration of (NH₄)₂SO₄ (from 0 to 1.5 M). Elutions were performed at 1 mL/min from a column approximately 1 mL in volume.

Figure 1 shows an important effect of PNAA tail length on peak location of various PNAAs to a DNA oligomer (butyl column). The unbound DNA oligomer elutes at 10.5 mL (10.5 min) while the PNAA/DNA duplexes elute at 14.5–17 mL, depending on the PNAA alkane length. Since the binding of PNAA to DNA is not perfectly quantitative, there is some residual unbound DNA that can be observed as a shoulder at about 10–11 mL elution volume. PNAA is added in a 5 $\times$ excess over DNA, but the remaining unbound PNAA is not evident for all runs. The C₁₂ trace shows a broad peak at ca. 23–24 mL that can be attributed to unbound PNAA. In all cases, a large peak is observed following a post-elution wash using 70% ethanol, which is due to unbound PNAA. As such, the unbound PNAA in the sample binds tightly to the column and remains throughout the run. Interestingly, the impact of increasing the PNAA alkane length was modest, shifting the elution volumes from about 14.5 to 17 mL by increasing from a C₁₂ tail to a C₁₈ tail.

Figure 2 shows chromatograms from similar runs using the three different HIC packings. In each case, the C₁₈ PNAA was used. For comparison, runs containing only the DNA oligomer are superposed onto each. For each case, peaks in the pure DNA traces coincide with the shoulders in the PNAA–DNA traces, previously ascribed to a small amount of unbound DNA. Also noteworthy is the observation that the free DNA peak shifts substantially with changes in media while the PNAA–DNA peak shifts to a lesser degree. While the use of the butyl column certainly gives the best resolution between unbound DNA and PNAA/DNA duplexes, the source of the improved resolution appears to be attenuation of the strong interaction between the unbound DNA and the phenyl media when replacing the phenyl column with either the butyl column or octyl column. Interaction of the PNAA/DNA duplex is not strongly impacted by these modifications.

The stronger affinity of the unbound DNA oligomer with the phenyl media is likely due to favorable π – π interactions between aromatic groups on the analyte and on the media. While these should also be present for interactions of the PNAA/DNA duplex with the media (given that 25 overhanging DNA bases flank either side of the bound PNAA/DNA duplex), the affinity of the duplex is not increased when substituting phenyl for either butyl or octyl (Fig. 2). We believe this is due to surface-induced aggregation processes in the case of PNAA/DNA equilibration as described below.

It should be noted that the manufacturer's reported ligand densities for these three HIC media vary substantially. They report a ligand density of 50 $\mu\text{mol/mL}$ gel for the butyl, 5 $\mu\text{mol/mL}$ gel for the octyl, and 20 $\mu\text{mol/mL}$ gel for the phenyl substitution; presumably, these differences arise from the varying efficiencies of surface coupling reactions for the different sized ligands. Despite the 10-fold larger molar density of butyl ligand over octyl ligand, DNA oligomers bind tightly to the octyl-substituted media. This suggests that DNA oligomers interact with only a few ligands, located at a fair distance apart, at a time during the equilibration. HIC chromatograms run at loadings with 100–1,000 times higher concentration show breakthrough behavior, indicating that operating conditions used in this work are well within the linear range of adsorption to the media.

A summary of additional HIC chromatograms is given in Table I, tabulating the resolutions (R_s) between unbound DNA and PNAA/DNA duplexes. Values for R_s were calculated by:

$$R_s = \frac{1.18(V_2 - V_1)}{W_{1/2,2} + W_{1/2,1}} \quad (1)$$

where V_1 is the elution volume for the unbound DNA peak, V_2 is the elution volume for the PNAA/DNA duplex peak, and $W_{1/2}$ is the peak width at half its maximum value (Snyder et al., 1997). To obtain the best possible accuracy, elution volumes were taken from separate runs for the unbound DNA and the PNAA/DNA duplex. Data in Table I demonstrate that the use of the butyl column and C_{18} tail gives a significantly better resolution, enabling sequence-specific separations of DNA oligomers with lengths of 60 bases and higher using this tag-and-separate approach.

Separations of target DNA oligomers from solutions containing non-target and target DNA were also performed using these improved conditions. Figure 3 shows chromatograms for a 60-base DNA target (A), a 60-base target with two populations of 60-base DNA, each with a single-base mismatch (B), and this mixture with a PNAA selective for the 60-base target alone (C). As expected, a new peak emerged at an elution volume of 17–18 mL corresponding to the PNAA/target DNA complex. The other non-target peaks are not shifted by the presence of PNAA (data not shown). The putative PNAA/DNA peak was collected and its melting temperature ($T_m = 42.3 \times 1^\circ\text{C}$) was found to be identical to that for a PNAA/DNA duplex prepared directly in solution ($T_m = 42.7 \times 1^\circ\text{C}$).

Figure 4 has a similar set of data, this time challenging the separation with 0.1 mg/mL of calf-thymus DNA to test the specificity of PNAA for oligomeric targets in the presence of duplex DNA. Chromatograms are shown for a 60-base target (A), the target plus calf-thymus DNA (B), and the mixture of (B) with PNAA selective for the target strand (C). The calf-thymus DNA is predominantly in double-stranded form, and as such has a weaker interaction with the non-polar HIC media due to the internalization of non-polar bases within the helix of duplex DNA. The calf-thymus DNA elutes immediately at ca. 1 mL, which is the approximate column volume (0.97 mL), as reported by the manufacturer. As in Figure 3, the calf-thymus DNA is unperturbed by the presence of PNAA while the bound target shifts from 11.5 to 17 mL elution volume. Note the presence of a small amount of unbound DNA in (C) due to incomplete binding of the target. It is interesting to note that

these operating conditions of HIC can readily resolve double and single-stranded DNA irrespective of affinity tag addition.

Attempts to optimize the concentration of the $(\text{NH}_4)_2\text{SO}_4$ buffer gave some interesting results. A well-known method to assess the hydrophobicity of a HIC analyte is to measure the elution time of that analyte under isocratic conditions (Diogo et al., 2002b; Mahn and Asenjo, 2005). Elution times, recast as retention factors (k), can be analyzed within the solvophobic theory, which describes the free energy for the equilibrium distribution of the analyte between the bulk solution and the surface of the HIC media. Under conditions where electrostatic interactions between the analyte and surface can be neglected, the retention factor is given by:

$$\log k' = A + C m_s \quad (2)$$

where A and C are fitted constants and m_s is the molal salt concentration (Diogo et al., 2002a; Horvath et al., 1976). A corresponds to the change in surface area of the biomolecule on adsorption and C is the “hydrophobic interaction parameter,” a measure of the affinity of the analyte for the media. Provided that A is not a strong function of salt, a linear relationship between $\log k'$ and $(\text{NH}_4)_2\text{SO}_4$ concentration is expected if the predominant mechanism for surface interaction is equilibration of analyte with the media. A thorough study by Diogo et al. (2002a) confirmed that elution of DNA oligomers obeys Equation (2) in the range of 1–2 M $(\text{NH}_4)_2\text{SO}_4$, with poly(A) oligomers exhibiting lower values of C than poly(T) oligomers, presumably due to burial of non-polar nucleobases in secondary structures of poly(A).

In contrast, isocratic elution of PNAA/DNA duplexes shows a highly non-linear behavior. Here, retention factor (k) was calculated by:

$$k = \frac{V_r - V_0}{V_0} \quad (3)$$

where V_r is the elution volume of peak and V_0 is elution volume of DNA at 0 M salt (used as a flow reference). Data for the PNAA/DNA duplexes show retention factors that are largely insensitive to added salt between 0.1 and 0.375 M $(\text{NH}_4)_2\text{SO}_4$ and similar to that of DNA alone ($k = 0.5$ – 0.6), but at 0.5 M $(\text{NH}_4)_2\text{SO}_4$ the PNAA/DNA duplex binds tenaciously to the column and can only be removed by switching buffers or stringent 20% ethanol wash, while DNA alone continues to show negligible column interaction. This abrupt change in the elution behavior with added $(\text{NH}_4)_2\text{SO}_4$ suggests that the added buffer may do more than increase the affinity of the analyte for the media.

All experiments with gradient elution allowed PNAA–DNA binding to occur in 10 mM Tris-HCl (pH 8.0) prior to column introduction. To assess whether PNAA/DNA duplexes would form in the presence of high concentrations of $(\text{NH}_4)_2\text{SO}_4$, UV melting curves were collected on PNAA/DNA duplexes similar to the ones used in separations. As shown in Table II, melting temperatures (T_m) increased with added $(\text{NH}_4)_2\text{SO}_4$ (from 43 to 60°C), likely due to the stabilizing effect of strengthened non-polar interaction between PNAA and DNA bases along with screening of electrostatic interactions between the glutamic acid

residues of the PNAA and the DNA backbone. Since the PNAA/DNA duplexes must therefore remain intact during the separation, an alternative explanation for the non-linear retention behavior in HIC is that the duplexes form aggregates, either with themselves or with excess unbound PNAA, above some critical concentration of $(\text{NH}_4)_2\text{SO}_4$.

A surface-induced aggregation process may explain the dramatic shift in PNAA/DNA affinity for the HIC packing with added salt in the range of 0.375–0.5 M $(\text{NH}_4)_2\text{SO}_4$, and also why the elution behavior for PNAA/DNA under gradient conditions is insensitive to the type of media chosen. In this case, the predominant interaction would be between PNAA/DNA duplexes in solution and those already adsorbed to the media, not between individual PNAA/DNA duplexes and the media as presumed in the solvophobic theory. The idea of surface-driven aggregation is substantiated by observing that PNAA/DNA duplex structure is insensitive to $(\text{NH}_4)_2\text{SO}_4$ concentration in solution, as judged by circular dichroism measurements, and the absence of Rayleigh scattering in the accompanying UV spectra (data not shown).

The use of linear gradient elution is primarily for experimental convenience, and is not necessary to achieve an optimal separation. Indeed, a transition to batch mode separation (Mazza et al., 2002; Thiemann et al., 2004) has several advantages, including reduced sample size, decreased equipment requirements, as well as the capacity for high-throughput purification. Figure 5 displays elution data for equilibrium batch adsorption studies using free, butyl-modified HIC media in 96-well plates. Solutions of 5 μM of PNAA C₁₂-agtgatctac-E₄, 5 μM of a complementary DNA oligomer 5'-GTAGATCACT-3', and a mixture of both PNAA and DNA in 1.5 M $(\text{NH}_4)_2\text{SO}_4$ /10 mM Tris-HCl (pH 8.0) were prepared and individually incubated with the free HIC media in filter-based 96-well plates. Following agitation, the absorbance at 260 nm was measured. The media was then re-equilibrated with solutions containing progressively lower concentrations of $(\text{NH}_4)_2\text{SO}_4$, followed by a final rinse with 20% ethanol. The concentrations required for batch elution (Fig. 5) compare qualitatively with the gradient elution data of Figures 2–4, with unbound DNA eluting at highest $(\text{NH}_4)_2\text{SO}_4$ concentration, followed by the PNAA/DNA duplex, than the PNAA which can only be completely removed by a 20% ethanol wash. Differences in the unbound DNA elution volumes (gradient) viewed in Figures 2–4 and elution concentrations (batch) can be attributed to the increase in hydrophobic surface area in the case of the longer 60-base oligomer. The residual peak at 1.5–1.25 M $(\text{NH}_4)_2\text{SO}_4$ (Fig. 5) is presumed to be unbound DNA resulting from imperfect binding of PNAA to the target; quantitative binding can be accomplished using PNAA with positive charge. Batch HIC has proven to be an excellent predictor of a linear gradient elution for both DNA and PNAA/DNA duplex using DNA oligomers of length 10, 12, and 34 bases, although the elution profiles of DNA and PNAA/DNA duplex in batch are broader compared to gradient mode (data not shown). This peak broadening has thus far precluded the batch separation of the 60-base oligomer used in this study. However, recent experiments in this lab have shown that peak broadness is largely a function of experimental conditions rather than an intrinsic property of batch HIC; we expect that tuning of these parameters will allow for batch DNA separations of lengths equivalent to those realized with a traditional column-based approach.

DNA purification with PNAA may be further simplified, with negligible product loss, by using a two-step wash in batch HIC. Figure 6 displays capture and elution of a complementary fluorescein-labeled 11-base oligomer in the presence of 100-fold molar excess of a non-complementary 10-base oligomer. Solutions of 1 μM C₁₂-agtgatctac-E₄, 0.1 μM of a complementary DNA oligomer 5'-FluorTG TAGATCACT-3', and 10 μM non-complementary oligomer 5'-GTAGAGCACT-3' (base mismatch bolded) were incubated with butyl HIC media in 1 M (NH₄)₂SO₄/10 mM Tris-HCl (pH 8.0). Following agitation, the liquid was removed by filtration and its absorbance and fluorescence measured. Absorbance was used as an indication of total nucleic acid concentration, while fluorescence solely tracked the fate of complementary DNA by utilizing the internal fluorescein moiety. The media was then re-equilibrated with 10 mM Tris-HCl (pH 8.0) and values noted. Unbound DNA, which shows little interaction with the HIC packing, is exclusively eluted with 1 M (NH₄)₂SO₄ (Fig. 6A). PNAA/DNA duplexes show greater affinity, and are therefore retained until the 0 M wash is applied (Fig. 6B). The low fluorescence value at 1 M and the negligible absorbance value at 0 M indicates excellent binding and sequence-specificity, as well as near-total constituent separation. This process has been successfully repeated with a 34-base oligomer (data not shown). Hence, facile two-step purification with batch HIC is attainable, provided that the retention of the PNAA/DNA duplex is distinct from unbound DNA and other contaminants.

Conclusions

By screening a series of alkane modifications to PNAA and commercially available HIC media, we have identified conditions suitable for an excellent separation (resolution, $R_s = 2.08$) of DNA oligomers tagged with PNAA from untagged oligomers. This improvement over previous work is largely due to use of butyl-modified HIC media over phenyl-modified media, with a moderate contribution from the increased retention of the longer-tailed PNAAs (C₁₈). The butyl modification has a much weaker interaction with DNA than the phenyl does, and interactions of the PNAA–DNA complex are not affected by switching media. Anomalous elution behavior for the PNAA/DNA duplex was ascribed to a surface-induced aggregation phenomenon that does not occur for unbound DNA. These measurements indicate that hydrophobic tagging of DNA oligomers can be used for purifications of DNA oligomers exceeding 60 bases in length, even when the target sequence is in the center of the target strand. It was also determined that batch-mode HIC adequately predicts linear gradient elution behavior, and is a viable alternative to the traditional, column-based approach. Separation time and sample use may be further minimized by adopting a two-step purification approach.

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Figure 1.

HIC (butyl column) chromatograms of a 50 μL sample containing 5 μM of the DNA oligomer 5'-(T)₂₅-GTAGATCACT-(T)₂₅-3' and 25 μM of various PNAs C_x -agtgatctac- E_4 in 10 mM Tris-HCl (pH 8.0). The length of the PNA tail (x) was varied from 12 to 18. Under these conditions, only DNA, PNA-bound DNA, and unbound C_{12} PNA eluted during the experiment. Longer-tailed, unbound PNA was removed from the column using a 70% ethanol wash. Chromatograms are shifted to an arbitrary A_{260} value for display purposes. The dashed line represents the composition of the eluent for this gradient elution (right-side axis).

Figure 2.

HIC chromatograms of a 50 μ L sample containing 5 μ M of the DNA oligomer 5'-(T)₂₅-GTAGATCACT-(T)₂₅-3' in the presence (solid line) and absence (thick dashed line) of 25 μ M of the PNA C₁₈-agtgatctac-E₄ (each in 10 mM Tris-HCl (pH 8.0)). The HIC ligand was varied as described in the text. Chromatograms are shifted to an arbitrary A₂₆₀ value for display purposes. The thin dashed line represents the composition of the eluent for this gradient elution (right-side axis).

Figure 3.

HIC chromatograms (butyl column) of 50 μL samples containing (A) DNA target only; (B) DNA target plus mismatch DNA; and (C) PNAA plus DNA target and mismatch DNA. Target DNA: 2.5 μM of 5'-(T)₂₅-GTAGATCACT-(T)₂₅-3', Mismatch DNA: 5 μM each of 5'-(T)₂₅-GTAGAACACT-(T)₂₅-3', 5'-(T)₂₅-GAAGATCACT-(T)₂₅-3' (mismatched bases are underlined) and PNAA: 12.5 μM of C₁₈-agtgatctac-E₄. Each is dissolved in 10 mM Tris-HCl (pH 8.0). Chromatograms are shifted to an arbitrary A_{260} value for display purposes. The dashed line represents the composition of the eluent for this gradient elution (right-side axis).

Figure 4.

HIC chromatograms (butyl column) of 50 μL samples containing (A) target DNA alone, (B) target DNA plus calf-thymus DNA, (C) PNAA with both target DNA and calf-thymus DNA. Target DNA: 5 μM of 5'-(T)₂₅-GTAGATCACT-(T)₂₅-3', calf-thymus DNA: sheared sample of 100 $\mu\text{g}/\text{mL}$ and PNAA: 25 μM of C₁₈-agtgatctac-E₄. Each is dissolved in 10 mM Tris-HCl (pH 8.0). Chromatograms are shifted to an arbitrary A_{260} value for display purposes. The dashed line represents the composition of the eluent for this gradient elution (right-side axis).

Figure 5.

Batch elution profiles of PNA, DNA, and PNA/DNA mixtures with decreasing concentrations of $(\text{NH}_4)_2\text{SO}_4$. Samples containing PNA alone, DNA alone, and a mixture of complementary DNA and PNA were measured separately. Media was rinsed sequentially with solutions of decreasing concentration of $(\text{NH}_4)_2\text{SO}_4$. The absorbance of the effluent following each rinse step is plotted as fraction of the total absorbance (A/A_{total}). The wash step was performed using 20% ethanol to remove any remaining material. PNA: 5 μM of C_{12} -agtgatctac- E_4 . DNA: 5 μM of 5'-GTAGATCACT-3'. All solutions were buffered using 10 mM Tris-HCl (pH 8.0). Each bar represents the average of at least three independent experiments.

Figure 6.

Step elution profiles of DNA and PNAA/DNA mixtures. The absorbance of the effluent following each rinse step is plotted as fraction of total absorbance (**A**) or fluorescence (**B**). Each rinse (1 M, 0 M) was repeated three times, and the sum of these three rinses are reported. PNAA: 1 μM of $\text{C}_{12}\text{-agtgatctac-E}_4$. DNA: 0.1 μM of 5'-GTAGATCACT-3', 10 μM of 5'-GTAGAGCACT-3' (base mismatch is bolded). All solutions were buffered using 10 mM Tris-HCl (pH 8.0). Each bar represents the average of at least three independent experiments.

Table I

Effect of PNAA tail length and stationary phase ligand on theoretical resolution of unbound and bound DNA fractions.

PNAA alkane length # carbons	<u>Resolution (Eq. 1)</u>		
	Butyl	Octyl	Phenyl
12	1.20	0.74	0.25
14	1.27	1.19	0.44
16	1.78	1.25	0.64
18	2.08	1.50	0.84

Resolution was calculated as defined in Equation 1. Each number represents an average of three independent experiments.

Table II

Duplex melting temperatures (T_m) for PNAA/DNA duplexes in the presence of the $(\text{NH}_4)_2\text{SO}_4$ eluent.

Added $(\text{NH}_4)_2\text{SO}_4$ (M)	T_m ($^{\circ}\text{C}$)
0	44 ± 1
0.1	52 ± 1
0.25	55 ± 1
1.0	60 ± 1

All samples were prepared in 10 mM Tris-HCl (pH 8.0) with $(\text{NH}_4)_2\text{SO}_4$ added as shown. PNAA, 2 μM of C₁₂-agtgatctac-E4; DNA, 2 μM of 5'-CCGTAGATCACT-3'.