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# Affinity chromatography using enzymatically synthesized nucleotide-containing DNA binding polymers

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## Abstract

A series of nucleotide-containing polyphenols has been synthesized by a simple, two-step enzymatic method. The binding properties of these synthetic polymers to complementary oligonucleotides have been evaluated using a commercially available oligo(dT)cellulose column. Complementary synthetic nucleosides were retained on this column to a greater extent than non-complementary synthetic nucleosides. These results suggest that the synthetic nucleosides prepared via this two-step enzymatic approach may have application as affinity matrices.

## Introduction

Recent interest in oligonucleotides as promising therapeutic and diagnostic agents has resulted in a number of new synthetic strategies to synthesize nucleotide analogs, which are stable chemically and towards nucleases. Chemical and enzymatic methods have been developed (Uhlmann & Peyman 1990, Overberger & Chang 1995, Mag *et al.* 1997, Liu & Orgel 1995) to modify the sugar moieties, the phosphodiester linkages (Noe *et al.* 1995), and nucleoside units of oligonucleotides. Most of these synthetic methods involve multiple chemical steps and result in poor selectivity. Our laboratory previously reported a simple, two-step enzymatic method to synthesize a thymidine-containing polymer (Wang & Dordick 1998). We anticipated that this enzymatic approach would be general and not limited to specific nucleosides or the phenols.

Polyphenols represent a diverse set of polymers that can be made to contain a wide array of side-chain structures and chemistries that give rise to many functional properties. Polyphenolic nucleic acid-containing polymers might be useful if they have affinity for specific natural nucleotides through their interaction based on complementary via hydrogen bonding. An enzymatic route of synthesis offers a number of advantages over alternative chemical approaches including the facile generation of phenolic oligomers and polymers without the need for formaldehyde-based coupling (Garcia & Pizzi 1998), and the ability to selectively control the reactivity and substrate specificity of the system. Similarly, combinatorial generation of nucleotide-containing polyphenols might be performed based on such exquisite reaction control. In the current study, our original method (Wang & Dordick 1998) has been extended to several other 2′-deoxypolynucleotides. The binding properties of these nucleotide-containing polymers to complementary oligonucleotides have been studied and their immobilization and application as affinity matrices have been explored.

## Materials and methods

### Materials

Lipase from *Candida antarctica* (Novozym 435) was obtained from Novo Nordisk (Bagsvaerd, Den-

mark) and soybean peroxidase was obtained from Enzymol International (Columbus, OH). Nucleosides, *p*-hydroxyphenylacetic acid, and oligo(dT)cellulose were obtained from Sigma. Polystyrene (SM2) beads were obtained from BioRad. All solvents, trifluoroethanol and polystyrene standards were obtained from Aldrich and were dried prior to use by storing over 0.4 nm molecular sieves for at least 24 h.

*Synthesis and characterization of monomers*

The synthesis of the trifluoroethyl ester of *p*-hydroxyphenylacetic has been described previously (Wang & Dordick 1998). Acylation of nucleosides was performed in 100 ml of nearly anhydrous acetonitrile containing 25 mM nucleoside, 25 mM trifluoroethyl ester of *p*-hydroxyphenylacetic acid, and 10 mg $ml^{-1}$ lipase from *Candida antarctica*. The mixture was stirred magnetically at ca. 250 rpm at room temperature and 40 $\mu l$ aliquots were periodically removed for analysis. The progress of acylation was followed by reversed-phase HPLC ($C_{18}$, 250 × 4.6 mm) using a mobile phase of water:acetonitrile (3:2, v/v). The conversion of the trifluoroester was used to determine progress of the reactions. After 24 h, the reaction mixtures were filtered, and the solid residues were washed extensively with acetonitrile. The acetonitrile-soluble product was purified on a silica gel flash column with the mobile phase consisting of $CH_3CN$ for the T, U, dU, dC monomers and ethyl acetate: methanol (4:1, v/v) for the dA and dG monomers, respectively, yielding white solid powders upon drying.

*T monomer*: $^1$H-NMR (DMSO-$d_6$, $\delta$, ppm): 11.30 (1H, s, NH of the thymidine), 9.30 (1H, brs, phenyl-OH), 7.04, 6.68, 2.06 (2H, s, 2′ H of sugar). $^{13}$C-NMR (DMSO-$d_6$, $\delta$, ppm): 171.5 (C=O of ester), 163.7 (C=O of base), 156.3, 150.4 (C-4 of base), 135.8 (C-6 of base), 130.3, 124.2, 115.0, 109.8 (C-5 of base), 83.7 (C-1′ of sugar), 39.4 (C-2′ of sugar), 70.3 (C-3′ of sugar), 83.8 (C-4′ of sugar), 64.2 (C-5′ of sugar). FAB-MS: 376 ($M^+$), 225 ($M^+$-acid)

*U monomer*: $^1$H-NMR (DMSO-$d_6$, $\delta$, ppm): 11.43 (1H, s, NH of the uridine), 9.38 (1H, brs, phenyl-OH), 7.49 (1H, d, aromatic H in uridine), 7.13 (2H, d), 6.77 (2H, d), 5.80 (1H, d, aromatic H in uridine), 3.72 (2H, brs, $-CH_2-$ in sugar). $^{13}$C-NMR (DMSO-$d_6$, $\delta$, ppm): 171.3 (C=O of ester), 162.8, 156.1, 150.4, 140.4, 130.2, 124.1, 115.1, 102.0, 88.1 (C-1′ of sugar), 69.6 (C-2′ of sugar), 72.4 (C-3′ of sugar), 81.1 (C-4′ of sugar), 63.9 (C-5′ of sugar).

*dA monomer*: $^1$H-NMR (DMSO-$d_6$, $\delta$, ppm): 9.35 (1H, brs, OH group in acid), 8.27 (1H, s, aromatic H in base), 8.16 (1H, s, aromatic H in base), 7.03 (2H, d, aromatic H in acid), 6.68 (2H, d, aromatic H in acid), 6.36 (1H, dd, H-1′ in ribose), 3.40 (2H, s, $-CH_2-$ of the acid). $^{13}$C-NMR (DMSO-$d_6$, $\delta$, ppm): 171.5 (C=O group of ester), 130.2 (C-3,5 of phenyl group), 124.2 (C-4 of phenyl), 115.1 (C2,6 of phenyl), 84.0 (C-1′ of sugar), 83.7 (C-4′ of sugar), 70.7 (C-3′ of sugar), 64.2 (C-5′ of sugar), 39.3 (C-2′ of sugar).

*dC monomer*: $^1$H-NMR (DMSO-$d_6$, $\delta$, ppm): 9.40 (1H, brs, OH group in acid), 7.50 (1H, d, aromatic in nucleoside), 7.04 (2H, d, aromatic H in acid), 6.67 (2H, d, aromatic H in acid), 6.16 (1H, dd, H-1′ of sugar moiety), 5.80 (1H, d, aromatic in nucleoside), 3.20 (2H, s, $-CH_2-$ of the acid). $^{13}$C-NMR (DMSO-$d_6$, $\delta$, ppm): 171.6 (C=O group in acid), 168.6 (C-2 in cytidine), 159.8 (C-4 of the cytidine), 156.3 (C-1 of the phenyl), 144.8 (C-6 of the cytidine), 130.4 (C-3, 5 of phenyl), 124.4 (C-4 of phenyl), 115.3 (C2, 6 of phenyl), 97.5 (C-5 of the cytidine), 84.8 (C-4′ of sugar), 86.2 (C-1′ of sugar), 73.5 (C-3′ of sugar), 67.3 (C-5′ of sugar), 42.2 (C-2′ of sugar).

*dG monomer*: $^1$H-NMR (DMSO-$d_6$, $\delta$, ppm): 10.63 (1H, s, N3-H in guanosine), 9.34 (1H, s OH in acid), 7.80 (1H, s, N1-H in guanosine), 7.04, 6.67, 6.12 (1H, dd, H-1′ of sugar moiety), 5.40 (brs, NH2 in guanosine), 3.33 (2H, s, $-CH_2-$ of the acid). $^{13}$C-NMR (DMSO-$d_6$, $\delta$, ppm): 171.5 (C=O of the ester), 156.7 (C=O in guanosine), 156.2 (C-1 of phenyl), 153.7, 151.1, 135.1, 130.3, 124.2, 116.7, 115.1, 82.3, (C-1′ of sugar), 84.0 (C-4′ of sugar), 70.5 (C-3′ of sugar), 64.2 (C-5′ of sugar).

*dU monomer*: $^1$H-NMR (DMSO-$d_6$, $\delta$, ppm): 11.35 (1H, s, NH of nucleoside), 9.35 (1H, brs, OH of phenyl), 7.44 (1H, dd, aromatic H in base), 7.05 (2H, dd), 6.70 (2H, dd), 5.62 (1H, dd, aromatic H in base), 6.13 (1H, t, H-1′ of sugar), 3.30 (2H, s, $-CH_2-$ of the acid). $^{13}$C-NMR (DMSO-$d_6$, $\delta$, ppm): 171.3, 162.9, 156.3, 150.3, 140.2, 130.2, 124.2, 115.1, 101.9, 84.0, 83.7 (C-4′ of sugar), 70.1, 64.1 (C-5′ of the sugar). FAB-MS: 363 $(M+H)^+$, 107(acid-COOH)$^+$

*Synthesis and characterization of polymers*

Nucleotide-containing polymers were synthesized from the T, U, dA, and dU monomers. Peroxidase-catalyzed polyphenol synthesis was performed in 10 ml aqueous buffer (50 mM sodium phosphate buffer, pH 7, containing 50% (v/v) acetonitrile to improve monomer solubility), containing 40 mM acy-

lated nucleoside monomer and 1 mg ml$^{-1}$ peroxidase. One milliliter of 0.5 m $H_2O_2$ was added over 4 h with stirring. A reaction was also performed with *p*-hydroxyphenylacetic acid as the monomer. The polymers precipitated as they formed and at the end of the reaction (12 h) unreacted reagents and enzyme were washed from the polymers with distilled water. Polymer molecular weights were determined by gel permeation chromatography using a Waters HR 5E styrogel column, connected to a Wyatt minidawn, light scattering detector (Wyatt Technology, CA) and Waters 410 refractometer. Dimethylsulfoxide was used as eluant with polystyrene (MW 2000 – $4.0 \times 10^5$) as molecular weight standards. Thermogravimetric analysis was performed on a TGA 2950 instrument (DuPont Instruments, Wilmington, DE).

*Binding specificity assay*

Binding specificity was determined on an oligo(dT)-cellulose column. Oligo(dT)cellulose (500 mg) was packed into a column (5 cm × 1 cm) and washed with 3–5 bed volumes of 0.1 M NaOH, followed by equilibration buffer (10 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA and 0.5 m NaCl) until the pH value of eluant was 7.5. PolydA, polyU, and polyHPAA [poly(hydroxyphenylacetic acid] were each dissolved in equilibration buffer. Sample solution (200 $\mu$l) was applied to oligo(dT)column and eluted with wash buffer (10 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA and 0.1 M NaCl). Eluant (200 $\mu$l/tube) was collected and the UV absorbance (polydA and polyU at 260 nm, polyHPAA at 230 nm) was measured. An additional study of binding capacity on synthetic polynucleotide analogs was undertaken. Synthetic polyT (20 mg) was dissolved in 2 ml dimethylsulfoxide and mixed with polystyrene beads (2 g) to prepare polyT coated beads. The mixture was shaken at room temperature for one day (250 rpm). The polyT coated polystyrene beads were dried and extensively washed by distilled water. The UV measurement of the effluent showed that all the polyT added was bound to the polystyrene beads. The column was packed (4 × 1 cm) and eluted with wash buffer. No nucleotides were found in the eluant. The natural nucleotides (T10, A10, and U10) were then loaded onto this column, following the identical procedure described above for the oligo(dT)cellulose column.

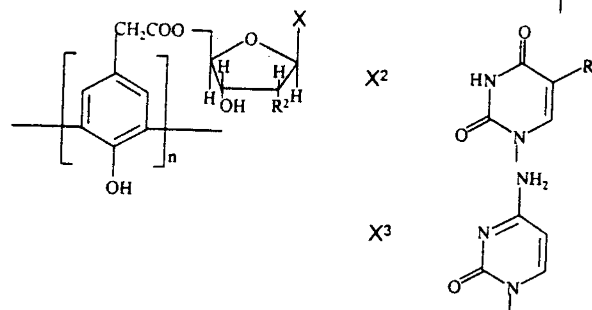



*Fig. 1.* Structure of synthetic nucleotide polymers. The base X has the structures: $X^1$, $R^2$ = H, $R^3$ = H, $R^4$ = $NH_2$ for polydA and $R^2$ = NH, $R^3$ = $NH_2$, $R^4$ = O for polydG; $X^2$, $R^1$ = H, $R^2$ = H for polydU and $R^1$ = H, $R^2$ = OH for polyU and $R^1$ = $CH_3$, $R^2$ = H for polyT; $X^3$, $R^2$ = H for polydC.

## Results and discussion

The structures of synthetic nucleoside polymers that have been prepared are shown in Figure 1. High regioselectivity of lipase-catalyzed acylation was confirmed by $^{13}$C-NMR (Rich & Dordick 1997), and indicated that with all nucleosides, acylation occurred almost exclusively at the 5′-OH to give the 5′-*p*-hydroxyphenylacetate. Conversions to the nucleoside ester were ca. 70% (based on conversion of the trifluoroethyl ester of of *p*-hydroxyphenylacetic acid), with the exception of the dC and dG monomers, which gave slightly lower yields (Figure 2). The isolated yields of the polynucleosides were: polyA, 37%; polydU, 39%; polyT, 33%; polyU, 50%. The time course of polyT synthesis showed more than 80% T monomer was polymerized by peroxidase (data not shown). The polymers had the following molecular weights: PolydA 185 kDa, polyT 217 kDa, polydU 616 kDa, and polyU 284 kDa. Thermogravimetric analysis showed that all the polymers are thermally stable to 200 °C.

Oligonucleotide analogs with improved stability toward nuclease degradation that retain specific hybridization with complementary counterparts may have potential uses as affinity matrices. Various methods can be used to measure the binding specificity of polynucleotides (Ludwick *et al.* 1986). Fortunately, the strong ultraviolet absorption of purine and pyrimidine bases in our synthetic polynucleotide analogs makes ultraviolet spectroscopy a useful tool to mea-

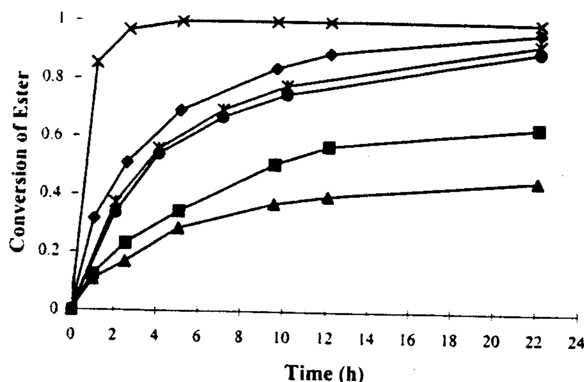
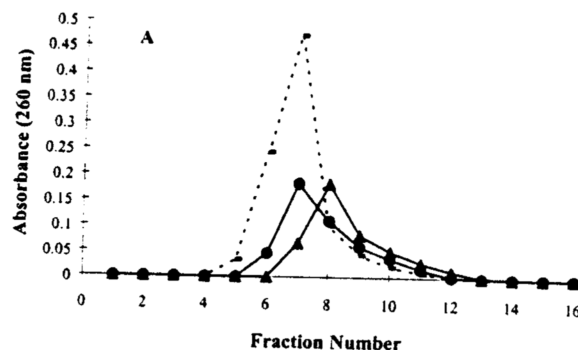



*Fig. 2.* Lipase-catalyzed acylation of nucleosides (♦) 2′-deoxyadenosine; (■) 2′-deoxycytidine; (▲) 2′-deoxyguanosioe; (×) 2′deoxyuridine; (<) thymidine; and (●) uridine.



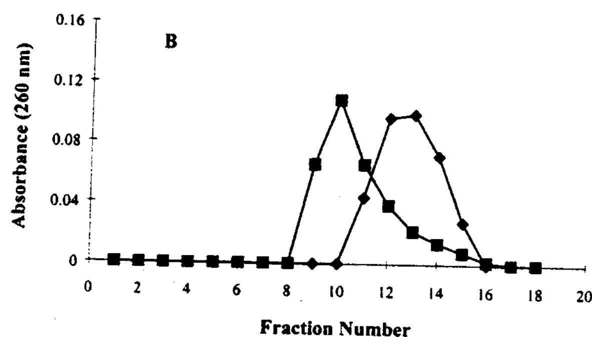




*Fig. 3.* Binding specificity of synthetic and natural polynucleotides to an oligo(dT)cellulose column. (A) Synthetic polynucleotides polydA (▲) and polyU (●) are shown by solid line. PolyHPAA(poly *p*-hydroxyphenylacetic acid) used as a control is shown as the dotted line. (B) Natural polynucleotides polydA (♦) and polyU (■) eluting from oligo(dT)cellulose column.

sure the binding specificity. To that end, we evaluated the binding specificity of our polynucleoside analogs toward an oligomeric natural nucleoside bound, a commercially available oligo(dT)cellulose packing. This material has high binding capacity for complementary nucleoside oligomers and polymers, and has been used for purification of polyadenylated mRNA (Aviv & Leder 1972, Thanki *et al.* 1978).

Saturated polydA, polyU, and polyHPAA (poly(hydroxyphenylacetic acid)) (in 200 $\mu$l 10 mM Tris/HCl buffer, pH 7.5 containing 1 mM EDTA) were added to the cellulose column separately. Effluent was collected in fractions and the absorbance (polydA and polyU at 260 nm, polyHPAA 230 nm) was measured. The polyHPAA was used as a control polymer that lacked nucleoside pendent groups. Differences in the affinity of these polymers for the oligo(dT)cellulose column was observed (Figure 3A). PolydA, which complementarily binds to the oligo(dT) pendent in the column, showed the highest binding capacity with the column and the greatest retention volume. PolyHPAA had the weakest interaction with the column and the lowest retention volume. Thus, the polyphenol backbone contributes only weakly, if at all, to binding. Under identical conditions, natural nucleotides afforded similar results on the oligo(dT)cellulose column as the synthetic polynucleotide analogs (Figure 3B).

An additional study of binding capacity on synthetic polynucleotide analogs was then undertaken. While the same selectivity (preference for complementary nucleotide containing polymer) of binding was obtained as that observed on the natural oligo(dT)cellulose column, the resolution (i.e., separation between complementary and non-complementary nucleotide containing polymers) on the polyT coated

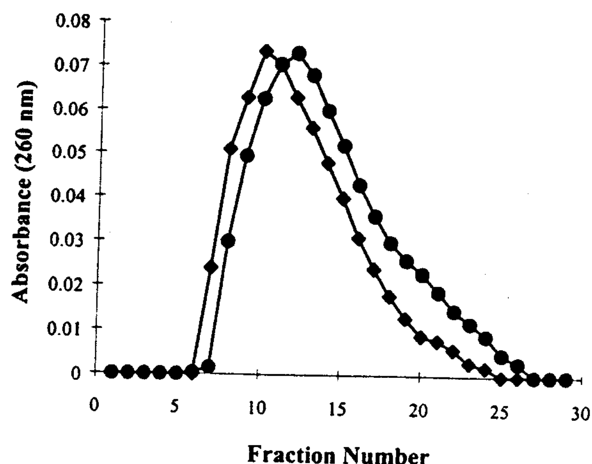



*Fig. 4.* Binding assay of natural polynucleotides polyU (♦) and polydA (●) on a polystyrene column coated with synthetic polyT analogs.

beads was poor. This may result from hydrophobic interaction between the polystyrene resin and polydA or polyU, which reduces the importance of the specific hydrogen bonding interaction. Despite the modest resolution between polydA and polyU, the results shown in Figure 4 demonstrate that these new synthetic polymers have the same hydrogen bonding selectivity as their natural nucleotide counterparts.

In summary, a simple bi-enzymic methodology has been developed to synthesize polynucleotide analogs. This approach can be used to generate polymers that exhibit specific binding ability to their complementary counterpart. In addition, the enzymatic route is highly regioselective affording substitution only at the 5′ position of the sugar moiety in nucleosides. This methodology currently is being explored for use in generating new, stable matrices for the bioseparation of nucleosides and their derivatives.

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