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DNA–support coupling for transcription factor purification Comparison of aldehyde, cyanogen bromide and *N*-hydroxysuccinimide chemistries

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Abstract

Purification of transcription factor IIIA on internal control region DNA coupled to aldehyde-silica is described and compared with purification on cyanogen bromide-activated Sepharose and Bio-Rad Affi-Gel-10. The Affi-Gel support results in mixed-mode chromatography; both ion-exchange and affinity modes contribute. Coupling DNA to aldehyde-silica is advantageous in that it has no ion-exchange properties and performs as well as DNA coupled to CNBr-activated Sepharose. Purification of lac repressor on aldehyde-silica, and CAAT enhancer binding protein on Affi-Gel also shows the advantages of a neutral support and the disadvantages of mixed-mode chromatography for transcription factor purification. Aldehyde-silica couples to alkylamines and to the amines of adenine, guanine, and cytosine nucleoside bases. Reaction occurs with either single- or double-stranded DNA, although it is less efficient with the latter. Overall, the results demonstrate that predominantly neutral coupling chemistries, such as aldehyde or CNBr-mediated coupling, have distinct advantages for transcription factor purification. Since the CNBr chemistry has not yet been applied to silica supports, aldehyde-silica coupling is currently the most attractive method for DNA affinity HPLC.

Keywords: Affinity chromatography; Mixed-mode chromatography; Stationary phases, LC; Aldehyde-silica; DNA; Proteins

1. Introduction

Many materials have been used in affinity chromatography as carriers. The most widely used is beaded agarose which is activated with cyanogen bromide (CNBr). Although quite stable in aqueous solutions, its thermal and mechanical stability is not adequate for some purposes such as high-pressure affinity chromatography. Silica, which has adequate mechanical stability and porosity, was considered to be an unsuitable support due to the irreversible adsorption and denaturation of certain substrates by the silanol groups on its surface. This prevented the use of silica as a carrier in affinity chromatography and in similar uses until Regnier and Noel [1] modified silica, providing glycidioloxypropylsilane-bonded phases. This modification decreased nonspecific adsorption and denaturation of proteins.

We first reported the coupling of DNA to silica for HPLC in 1990 [2]. First, silica was modified with 3-aminopropyltrimethoxysilane and then further modified with succinic anhydride to produce a carboxymethyl-silica. DNA coupling was to an *N*-hydroxysuccinimide (NHS) ester derivative of this

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silica. This coupling chemistry was attractive because it was rapid, coupled in high yield, and any excess reactive esters on the silica spontaneously hydrolyze in water to the corresponding carboxylic acid, making end-capping unnecessary. These DNA-silicas were successfully used for separations of RNA and DNA [2–6]. Excess carboxylate ions on the silica surface repel these anionic polynucleotides and thus binding of a polynucleotide was never by ionic interaction but was only dependent upon the ability of DNA-silica to hybridize with specific, complementary polynucleotide sequences. This same NHS chemistry is available on other supports such as agarose, Sepharose (Pharmacia) and Affi-Gel (Bio-Rad), and the acrylic derivative sold by Bio-Rad Labs. under the Affi-Prep name.

Recently, DNA-silicas have been used in our laboratory for a comparative study of the affinity chromatography of transcription factors. Often, transcription factors behave as polycations and cation-exchange chromatography has been used for their purification [7]. Transcription factors are usually eluted from DNA-affinity supports using salt gradients and any ion-exchange property could complicate the desired affinity chromatography.

Clearly, a neutral support would be highly desirable. Indeed, most of the purification of transcription factors has been carried out using DNA coupled to CNBr-Sepharose, using the approach originating with Arndt-Jovin et al. [8]. However, silica poses certain limitations that have so far prevented the use of CNBr activation. The strongly alkaline conditions required by many activation chemistries, including CNBr activation, would partially dissolve silica, leaving it unsuitable for chromatography. Other neutral coupling chemistries are available but have not been applied to DNA-silica. For example, Larsson et al. [9] described epoxy-, tresyl-, and aldehyde-silica coupling procedures which should all be electrostatically neutral. Here, we explored two neutral coupling chemistries for DNA and contrast the results to those obtained with the NHS chemistry.

2. Experimental

2.1. Synthesis of aldehyde-silica

The procedure is modified from that originally described by Larsson et al. [9]. Silica (Machery–Nagel Polygoprep, 50 μm, 300 Å pore) was chosen in this size to more fairly compare it to the low-pressure chromatography supports, Sepharose and Affi-Prep.

2.1.1. Glycidyloxypropyl-silica

Silica (10 g) and all glassware were dried overnight at 150°C and all solvents were anhydrous. After cooling to 50°C, 100 mL toluene was added and distilled until constant boiling (110°C) toluene distilled over for ~10 mL. Glycidyloxypropyltrimethoxysilane (10 mL) (Aldrich) was added and the mixture refluxed with overhead stirring for 4 h. The silica was then washed with 50 mL each of toluene, acetone, and water.

2.1.2. Glycidioloxypropyl-silica

The above silica was converted into the corresponding diol by suspending in 100 mL water, titrating to pH 2.8 with trifluoroacetic acid, and heating for 2 h in a boiling water bath with overhead stirring. The silica was washed with water, acetone, and diethyl ether and dried under vacuum. Silica prepared in this way had an average of 131 μmol diol/g silica determined using the method in Ref. [10].

2.1.3. Aldehyde-silica

Using the method of Larsson et al. [9], 10 g diol-silica was suspended in 167 mL of 90% acetic acid and 8.3 g $NaIO_4$ was added. The mixture was stirred for 1 h at room temperature and the resulting aldehyde-silica was washed with 50 mL each of water, ethanol, and diethyl ether and dried under vacuum.

2.2. Coupling of DNA

2.2.1. Coupling of ICR (Internal Control Region) to aldehyde-silica

ICR oligonucleotide (5′-$NH_2CH_2CH_2$-GAAGCCAAGCAGGGTCGGGCCTGGTAGTACTTGGATGGGAGAC) (100 nmol) was ethanol precipitated from TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 7.5), washed with 70% aqueous ethanol, dried, and dissolved in 5 mL of 0.5 *M* sodium phosphate, pH 6.5.

To this was added 1 g aldehyde-silica and 5 mL of 0.2 *M* $NaCNBH_3$. The mixture was shaken at 60°C for 16 h to allow DNA coupling. $NaBH_4$ (1 mL, 1 *M*) was added and shaking was continued for an additional hour at 60°C to reduce excess aldehyde to the corresponding alcohol. The silica was then washed with 10 mL portions of the sodium phosphate buffer, water, and stored in TE0.1 (10 m*M* Tris, 1 m*M* EDTA, 0.1 *M* NaCl, pH 7.5). These reaction conditions were found not to modify the ultraviolet spectrum of DNA and resulted in the coupling of 18.2 nmol ICR DNA/g silica. To make the DNA double-stranded, the complementary strand (25 nmol αICR, 5′-GTCTCCCATCCAAGTACTA-CCAGGCCCGACCCTGCTTGGCTTC) was added and the mixture was heated to 95°C for 5 min and then allowed to cool slowly to room temperature. The silica was then washed three times with 5 mL TE0.1 and packed into a column.

2.2.2. Coupling of $Op1T_{18}$ to aldehyde-silica

$Op1T_{18}$-silica [5′-$NH_2CH_2CH_2(T)_{18}$-AATTGTT-ATCCGCTCACAATTCCAC] was prepared using a method similar to that of ICR except for a few modifications. The coupling reaction was carried out in an airtight septum vial and the hydrogen generated was released intermittently (about once an hour for the first 4 h and then once at the end of coupling) using a syringe needle to prevent pressure build up. $Op1T_{18}$ (28 nmol) was coupled per gram of silica when 50 nmol of $Op1T_{18}$ was used. Control silica was activated and blocked similarly to $Op1T_{18}$-silica except that no DNA was added.

2.2.3. CNBr-Sepharose and Affi-Prep 10

ICR DNA was coupled to CNBr-Sepharose and Affi-Prep 10 using the protocols suggested by the manufacturers, Pharmacia and Bio-Rad, respectively. CNBr-activated Sepharose was prepared as previously described [11]. For both supports, 50 nmol of ICR DNA was added per gram of suction dried support. The coupling buffer was 0.1 $NaHCO_3$, 0.5 *M* NaCl, pH 8.3 for both, coupling was for 16 h (Sepharose) or 8 h (Affi-Prep 10). End-capping was with 0.1 *M* Tris, 0.5 *M* NaCl, pH 8 (Sepharose) or 0.5 *M* 2-ethanolamine in coupling buffer (Affi-Prep) for 2 h. For either chemistry, 80–90% of the DNA consistently coupled. αICR was annealed as described above for silica.

2.3. Chromatography

One milliliter tuberculin syringes outfitted with polypropylene frits were used as columns and were slurry packed. For all columns in Fig. 2 the same flow-rate (0.25 mL/min), fraction size (1 mL) and salt gradient was used. The buffer used was 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4), 5 m*M* $MgCl_2$, 50 μ*M* $ZnSO_4$, 10% glycerol, and 5 m*M* dithiothreitol (DTT). Buffer A was 50 m*M* KCl and buffer B was 1.0 *M* KCl made up in this buffer. The gradient consisted of 40 min constant buffer A, followed by a linear gradient to buffer B at 120 min, followed by constant buffer B until 160 min. The sample was the transcription factor IIIA 7S ribonuclearprotein particle provided as a generous gift by Dr. William Taylor of our department. To prepare TFIIIA, 1 mg/mL 7S particle was made 0.1 mg/mL RNase A and dialyzed overnight into buffer A. For each chromatogram, 25–75 μL of dialyzed TFIIIA was loaded.

The green fluorescent protein–CAAT enhancer binding protein (GFP-C/EBP) chimeric fusion protein was expressed in bacteria and purified as previously described [12]. The self-complementary oligonucleotide EP18 (5′-$NH_2CH_2CH_2$GCAGATT-GCG CAATCTGA-3′) was annealed by heating to 95°C, cooling to 80°C over 20 min, followed by a linear decrease to 20°C over 16.7 h, and finally to 4°C over 2 min. Coupling of 50 nmol of the annealed oligonucleotide to Affi-Prep 10 used the protocols suggested by the manufacturer, Bio-Rad. For the experiment in Fig. 4, 1 mL columns were loaded with 0.1 mL of a crude bacterial extract of GFP-C/EBP and washed with 10 mL of TE0.1 and eluted with 10 mL of TE1.2 (TE containing 1.2 *M* NaCl). The flow-rate was 0.3 mL/min. One milliliter fractions were collected.

Lactose repressor-β-galactosidase fusion protein (LaclZ) was produced as described earlier [13] from *Escherichia coli* clone BMH-72-19-1 which was a generous gift from Dr. David Levens (Laboratory of Pathology, National Cancer Institute, Bethesda, MD, USA). Crude LaclZ (50 μL) was loaded onto the $Op1T_{18}$ column. The column was washed with 15 mL TE0.1 and then eluted with 15 mL of the same

buffer containing 1.2 *M* NaCl. The flow-rate was 0.3 mL/min and 1 mL fractions were collected.

2.4. Slot-blot TFIIIA assay

A 0.1 mL portion of each chromatographic fraction was blotted onto nitrocellulose using the Bio-Rad slot-blot apparatus. The blots were blocked in TTBS (20 m*M* Tris–HCl, pH 7.5, 0.5 *M* NaCl, 0.2% Tween 20) with 10 mg/mL bovine serum albumin (BSA) overnight and washed three times with TTBS/BSA (1 mg/mL BSA) and incubated for 1 h at room temperature with 1:1000 diluted antibody for TFIIIA (polyclonal; a gift from Dr. William Taylor of our department) in TTBS/BSA. After washing the nitrocellulose membrane three times with TTBS/BSA, immunoblots were incubated for 1 h with 1:1000 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG. Finally, blots were again washed three times with TTBS (containing 1 mg/mL BSA). The blots were developed with a 1:100 dilution each of 30 mg/mL nitroblue tetrazolium (NBT) (in 70% dimethylformamide) and 15 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (in 100% dimethylformamide) in buffer AP (0.1 *M* $NaHCO_3$ and 1 m*M* $MgCl_2$, pH 9.8).

2.5. Fusion protein assay

GFP-C/EBP protein-containing fractions were determined by fluorescence using an excitation wavelength of 395 nm, an emission wavelength of 515 nm, and a bandpass of 16 nm. LaclZ-containing fractions were assayed for β-galactosidase activity as described previously [14].

3. Results

Fig. 1A and B show schematic presentations of aldehyde and NHS-coupling chemistries, respectively. The figure also gives the nomenclature for some of the derivatives discussed. Fig. 2A–C show slot-blot assays of the chromatography fractions obtained from the separation of transcription factor IIIA (TFIIIA) on three different supports coupled to the ICR DNA sequence. The three supports were CNBr-activated Sepharose 4B superfine (Fig. 2A), aldehyde-silica (Fig. 2B), and Bio-Rad's Affi-Prep 10 support (Fig. 2C), which uses the same NHS-ester coupling chemistry we had previously used with silica [2]. Good results were obtained with both DNA-Sepharose and -silica (using the new aldehyde coupling); a small amount of the detectable TFIIIA flows through each column in early fractions but most is retained and elutes during the salt gradient. Increasing salt concentration is known to reduce transcription factor–DNA binding and has usually been used to elute such DNA affinity columns [15].

The results with the Bio-Rad support were different. Most of the TFIIIA bound to the column but failed to elute (Fig. 2C). To investigate this further, Affi-Prep 10 support to which no DNA was coupled but was otherwise treated the same (i.e., a negative control) was used. This control support also bound TFIIIA and the TFIIIA did not elute with the gradient normally used (data not shown).

When the DNA-coupled Affi-Prep support was eluted at higher salt concentrations (Fig. 3, same gradient as Fig. 2 except 1.5 *M* KCl in the limit buffer), TFIIIA does elute. The same behavior was also observed with the negative control lacking DNA (data not shown). Thus, the binding to the Affi-Prep column under conditions which elute other DNA supports is because of the ion-exchange properties inherent in this NHS-carboxylic ester coupling chemistry. When this same chemistry was used with silica, we found similar results with lac repressor (data not shown). Again, we found that this transcription factor bound to columns whether or not DNA was attached and eluted at the same salt concentration from either column. Thus, this behavior is not unique to a particular transcription factor or to a particular support but derives from the charged groups that are a consequence of this coupling chemistry. Presumably, when water reacts with the NHS ester, it produces a high concentration of carboxylate anions, producing the cation-exchange property.

This mixed-mode (ion-exchange and affinity chromatography) behavior can be quite confusing if not appreciated. We have already described results with TFIIIA (Figs. 2 and 3) and lac repressor. Fig. 4 shows the results obtained with the CAAT enhancer binding protein (C/EBP) on a control Affi-Prep column (squares) or one which contained coupled

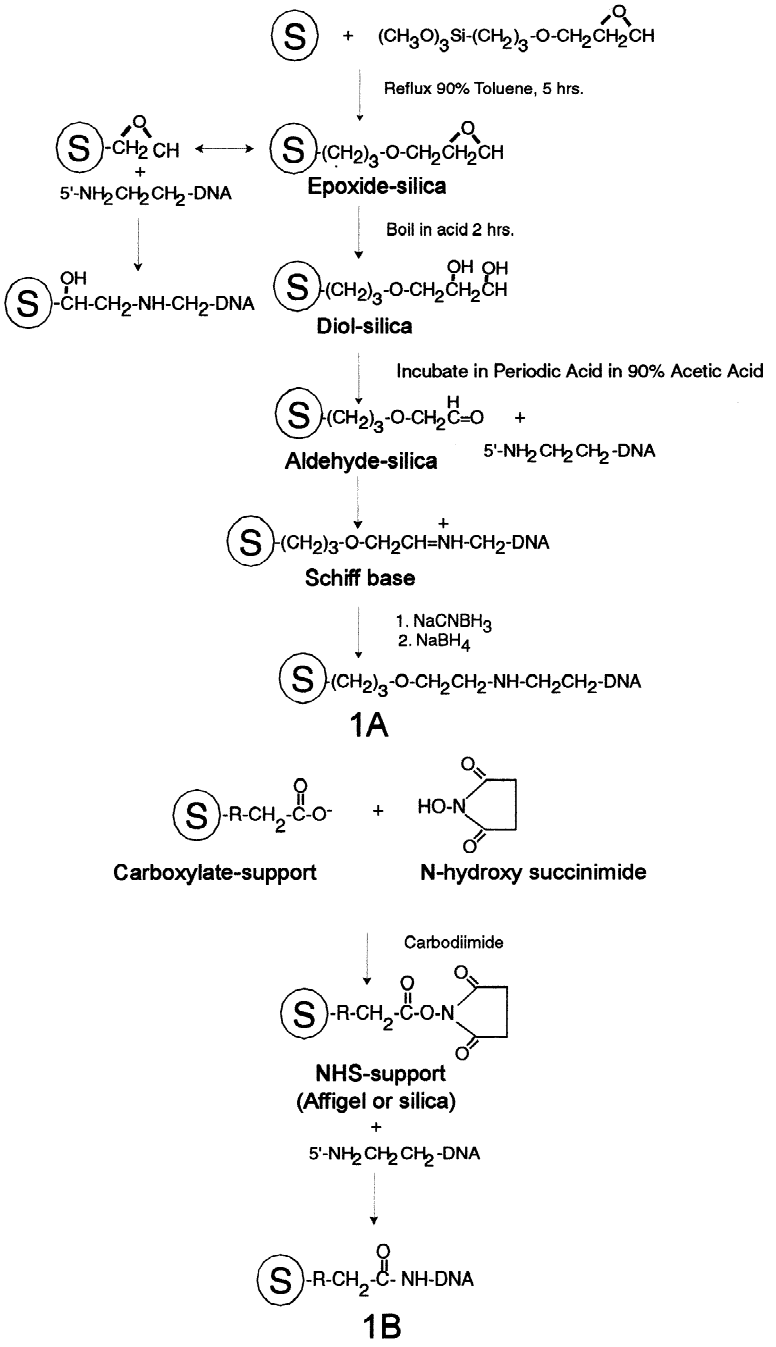

Fig. 1. Schematic presentation of aldehyde, epoxide (A) and NHS-coupling (B) chemistries.

CAAT element (circles). For this experiment, C/EBP sequences were fused to the green fluorescent protein and expressed in bacteria [12] to facilitate detection; a crude bacterial extract containing this fusion protein was applied to the columns. This result shows that C/EBP, a basic leucine zipper motif

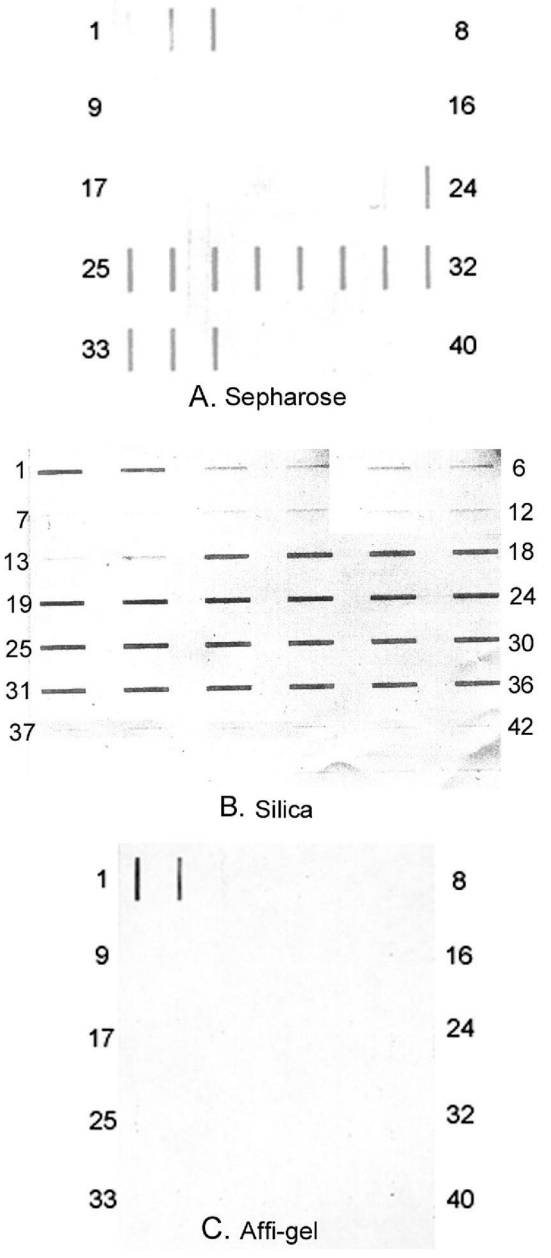

Fig. 2. Slot-blot assays of the chromatographic fractions obtained from the separation of transcription factor IIIA (TFIIIA) on three different supports: (A) CNBr-activated Sepharose 4B superfine, (B) aldehyde-silica and (C) Bio-Rad's Affi-Prep 10 support.

protein, also binds by charge (to the control) and by affinity and charge (to the DNA column) and that elution can be quite similar.

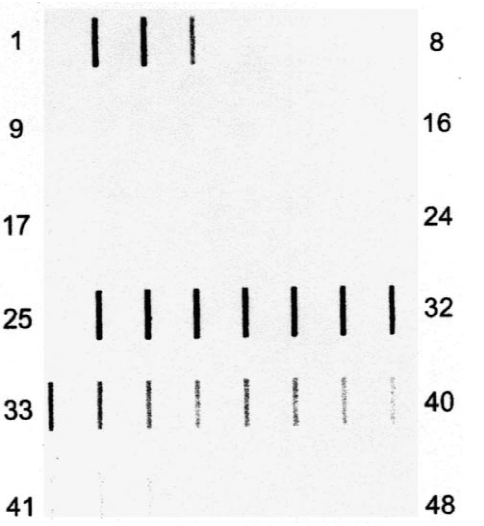

Fig. 3. TFIIIA elutes from the Bio-Rad Affi-Prep 10 support at higher salt concentration.

Fig. 5 shows the results obtained with the LaclZ on $Op1T_{18}$-silica prepared by the neutral aldehyde chemistry of Larsson et al. [9]. It is clear that $Op1T_{18}$-silica retains more than half of the LaclZ loaded which can be seen eluting as a single sharp peak with TE1.2. Most of the LaclZ loaded on the control silica is unretained and eluted in the flow-through fractions, indicating that LaclZ is retained by $Op1T_{18}$-silica because of the highly specific interaction between LaclZ and the Op1 DNA.

To determine whether the aldehyde coupling was able to couple to the nucleic acid base amines (on A, C, and G) and whether this coupling occurred in single- and double-stranded DNA, the experiment

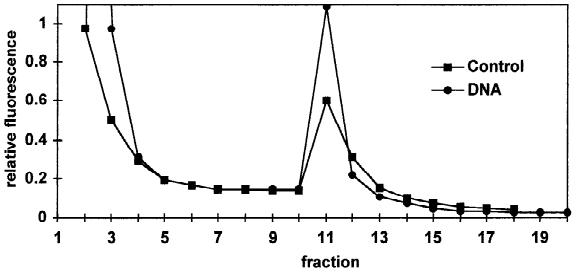

Fig. 4. C/EBP binds to the DNA on Affi-Prep support by both charge and affinity. Elution of C/EBP from DNA-containing Affi-Prep column (circles) and control Affi-Prep columns (squares).

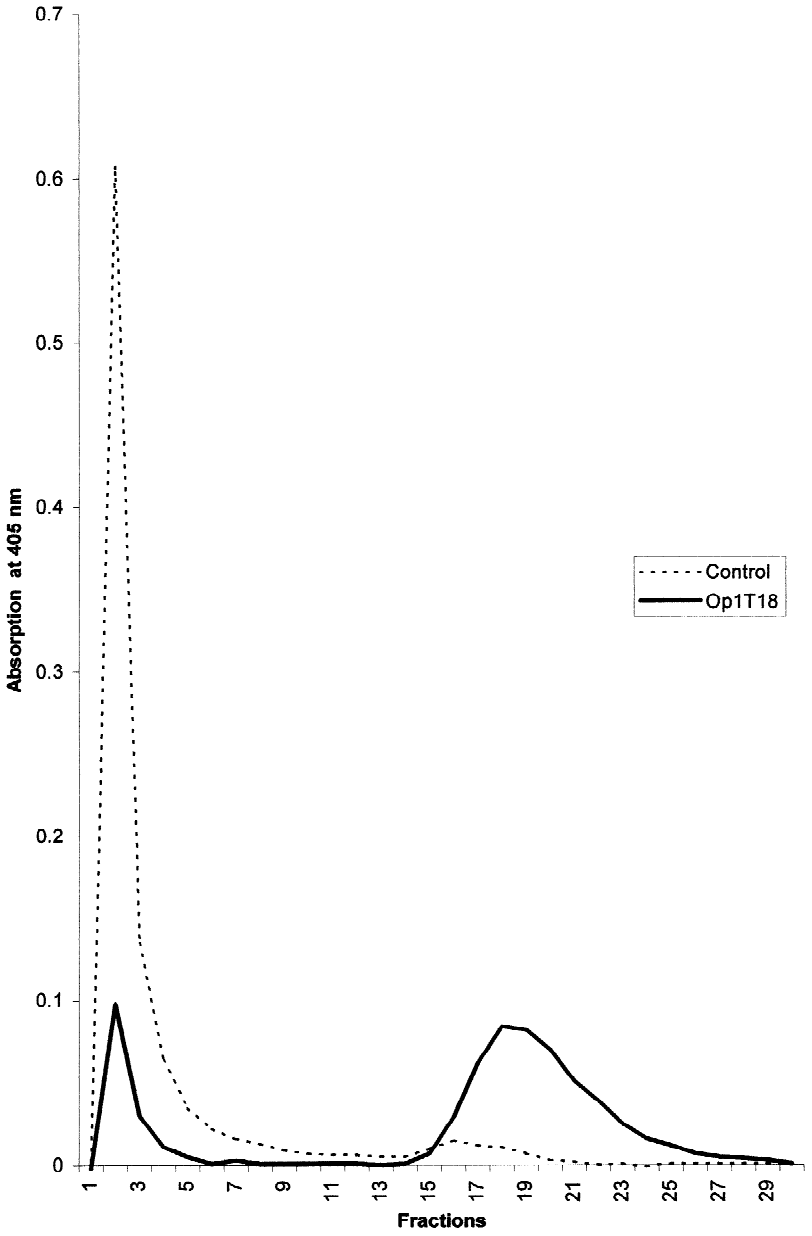

Fig. 5. LaclZ binds to $Op1T_{18}$-silica because of specific interactions between LaclZ and the Op1 DNA. Elution of LaclZ from $Op1T_{18}$-silica (———) and control-silica (- - -) columns.

shown in Table 1 was performed. The lac operator 1 DNA ($Op1T_6$, TTTTTTMTTGTTATCCGCTCAC-MTTCCAC) sequence was synthesized either without or with a 5′-aminoethyl moiety. This was either used single-stranded or slowly annealed to the complement strand (αOp1, GTGGAATTGTGAGC-GGATAACAATTAAAAAA) to make a double-stranded DNA. There are thus five combinations: (1) single-stranded $Op1T_6$, with and (2) without an aminoethyl, (3) the single-stranded complement $\alpha Op1T_6$ and two duplex DNAs, (4) not containing or (5) containing a 5′-aminoethyl on one strand.

Table 1
Aldehyde coupling occurs by modifying nucleotide bases

DNA	nmol DNA strand/g silica
(1) NH_2-ethyl-Op1, single-stranded	19.4±4.7
(2) Op1, single-stranded	21.2±2.2
(3) αOp1, single-stranded	33.0±4.1
(4) Op1:αOp1, double-stranded	38.2±13.4
(5) NH_2-ethyl-Op1:αOp1, double-stranded	37.7±1.5

After coupling, the amount of DNA coupled was determined by ashing the silica in $Mg(NO_3)_2$, determining the amount of inorganic phosphate released, and dividing the result by the number of phosphate present in the DNAs using a previously published procedure [5].

The results reveal that the presence or absence of a 5′-aminoethyl group has little effect upon coupling. It can, however, provide a site for coupling since we have found that oligothymidylic acid does not couple to aldehyde-silica but the same oligonucleotide with a 5′-aminoethyl does couple well (data not shown). Reaction with aldehyde-silica requires a primary or secondary amine, lacking in thymidylic acid, and this probably explains the need for the aminoethyl group in this case. Amines are present on A, C, and G bases and can provide sites of reaction in other DNAs. αOp1 couples better than does Op1. Op1 has 17 A, C or G within its sequence while αOp1 has 24 of these including a string of six sequential A's; this larger amount of nucleophiles and in locally high concentrations may contribute to the better coupling of αOp1. The data also suggest that the formation of duplex DNA inhibits coupling moderately. If no protection (from aldehyde coupling) of nucleotide bases occurred in duplexes, coupling of the double-stranded DNA should simply be the sum of the coupling of each strand. For Op1:α-Op1, 54.2 nmol strand should couple (21.2+33) but, in fact, only 38.2 nmol was found, indicating that some coupling sites are inaccessible in the duplex.

4. Discussion

Silica has been successfully used as a carrier for biologically active ligands in high-performance affinity chromatography (HPAC). The mechanical rigidity and high porosity of this material permits work at high pressures and high flow-rates and improved performance for purifying biologically active compounds [9]. The most prevalent carrier used in HPAC had been silica modified with the glycidyloxypropyl moiety, epoxide-silica. The carrier is problematic in that it suffers from the inherent low chemical reactivity of epoxides at near neutral pH values. Therefore, long reaction times and alkaline reaction conditions were needed for efficient coupling to epoxides. Moss et al. coupled DNA covalently via epoxides to cellulose utilizing oxirane 1,4-butanediol diglycidyl ether to activate cellulose and link DNA. The optimal conditions for the latter reaction included use of a dehydration technique whereby DNA and activated cellulose were allowed to react on a glass slide in 0.1 *M* NaOH [16]. Such highly alkaline conditions would be detrimental to silica and a more neutral pH with accompanying slow reaction would result in poor yield. Diol-silica, obtained after hydrolysis of this epoxide silica, has also been used for the immobilization of biologically active compounds [17]. Activation of hydroxyl groups was accomplished with carbodiimidazole, but the resultant coupling capacity was quite low. Recently, dihydrazide-activated silica supports were made by oxidizing diol-bonded silica, and reacting with oxalic or adipic dihydrazide [18], although the uses of this chemistry to couple DNA are yet to be explored. The aldehyde-silica, obtained after periodate oxidation of the diol, is a more suitable derivative for the attachment of ligands because aldehyde groups are sufficiently reactive for coupling to proteins at acidic to neutral pH in good yield via a Schiff base. The Schiff base form is unstable and must be reduced. In many cases, reduction of this bond causes a loss of biological activity [17,19,20]. This chemistry had not been used for DNA coupling, and whether reduction would adversely affect the DNA was not known. Here, we find that the ultraviolet spectrum of the DNA was unaffected by reductive coupling (see Experimental), indicating no gross changes have occurred in the DNA. Furthermore, the DNA is functionally active since it still binds transcription factors (Figs. 2 and 5). Reduction of aldehyde-silica leads to primary hydroxyl-silica, thus a neutral silica results after reduction, explain-

ing the lack of detectable ion-exchange properties [17,21].

There is an important advantage of aldehyde-silica over agarose which is activated with cyanogen bromide. The cyanogen bromide activation has the disadvantage that coupling of amines results in the formation of N-substituted iso-urea bonds which lack adequate stability for some uses. Such stability results in the leakage of ligands and after some time columns of such material acquire ion-exchange properties which interfere with the biospecificity. In contrast, the reduced Schiff base produced by aldehyde coupling is a secondary amine and should be quite stable. Secondary amines are also cationic at low pH but these amines are not a property of the coupling chemistry per se but rather derive from the ligand coupled. Since only small amounts of DNA ligand are usually coupled (here, less than 50 nmol/g) these contribute minimally to the charge properties of the support.

Coupling schemes which introduce large amounts of charged groups can result in mixed-mode chromatography. While mixed modes may be beneficial to some separations, this behavior can sometimes be confusing if appropriate controls are not performed. Aldehyde-silica represents an attractive alternative for coupling DNA since it has no ion-exchange properties and performs about as well as DNA coupled to CNBr-activated Sepharose, a commonly used support. We have also found coupling of DNA to aldehyde-silica to be much more rapid than coupling to another neutral support, glycidyloxypropyl-silica, in agreement with observations of Larsson et al. [9] with other ligands.

The affinity support based on stable aldehyde chemistry displays high specificity and combines high binding and loading capacity, and good reproducibility. The same columns had been used for at least 30 elutions with crude cell extracts with no change in its chromatographic behaviour (data not shown). The aldehyde chemistry does have its disadvantage though — evolving H_2 gas during coupling representing a fire hazard and provisions must be made to prevent high pressure from occurring in sealed containers. However, aldehyde-silica demonstrates the clear advantage of neutral coupling chemistries for some forms of DNA-affinity chromatography and, unlike CNBr activation, it can be used with HPLC silicas. Regardless of the coupling chemistry chosen, a chemistry that does not itself contribute charge properties would be preferred.

Acknowledgements

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