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In flow activation of diol–silica with cyanogen bromide and triethylamine for preparing high-performance affinity chromatographic columns

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Abstract

A new coupling strategy using pre-packed diol–silica supports to obtain affinity columns for high-performance affinity chromatography (HPAC) is described. These columns were prepared by ''in flow'' activation in which solutions containing anhydrous solutions of CNBr and triethylamine are separately pumped to a mixer and then onto a pre-packed diol–silica column. Recycling the amino ligand to be coupled several times over the activated silica diol columns results in ligand immobilization. DNA (the *Op1 lac operator*), 6-aminohexyl-Cibacron and a peptide (melittin) were all successfully ''in flow'' coupled to freshly activated columns. Methods for CNBr activation of pre-packed diol–silica column were developed for one, two or three pump HPLC systems. The supports were successfully used for the HPAC purification of a *Lac repressor-β-galactosidase* fusion protein, alcohol dehydrogenase, and calmodulin. Columns prepared by in flow activation/coupling procedures were shown to be stable for at least 14 months. Also, in flow activated silica columns could be stored in anhydrous acetone for at least 3 months prior to coupling. Our experiments with these affinity ligand columns (DNA–silica, aminohexyl-Cibacron F3GA–silica, and melittin–silica), suggests that this is a very successful coupling protocol for producing a variety of HPAC columns.

Keywords: Stationary phases, LC; Affinity adsorbents; Diol–silica stationary phases; Cyanogen bromide; Triethylamine; Proteins

1. Introduction

The most powerful approach known for the purification of enzymes, antibodies, and other biomedically important proteins is affinity chromatography [1]. The usual carriers are polymers that contain hydroxyl groups (e.g. Sepharose, cellulose, and Sephadex) and normally, cyanogen bromide (CNBr) is employed for the activation and subsequent immobilization of amino-containing ligands [2,3]. The activation of Sepharose supports is normally done by reacting with CNBr at pH 11 [4]. A more reliable and less

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toxic way of coupling amino ligand to hydroxyl-containing supports was developed, the cyano transfer reaction [2,3].

These polymers cannot usually tolerate high pressures. Porous silica-based supports, which possess a high mechanical rigidity, would permit work at high pressures and flow-rates [1]. High-performance affinity chromatography (HPAC) can combine the specificity of affinity chromatography with the speed, efficiency and sensitivity of high-performance liquid chromatography (HPLC) for biomolecule separations [5,6]. Silica derivatization is necessary to mask strong and often irreversible adsorption of proteins to silica surfaces [1]. Also, these derivatizations allow the application of several coupling chemistries for the immobilization of ligands to the silica supports [1,5–8]. However, the reported coupling chemistries for silica supports are often complex and reactions are frequently slow and poor yields are obtained. In other cases, these reactions have other undesirable features like highly charged surfaces [9].

Hydroxyl-containing silicas (diol- and primary hydroxyl-silica) have been used for the immobilization of biologically active compounds. These reactive groups can be readily produced by first reacting silica with glycidyloxypropyl(trimethoxy)silane and hydrolyzing the resulting epoxide silica in a weak acid such as trifluoroacetic acid to produce a diol–silica. Primary hydroxyl-containing silica can be obtained by oxidizing the diol–silica with periodate, which yield a silica resin with aldehyde functions followed by reduction with sodium borohydride [1]. Several coupling strategies for immobilizations of ligands to hydroxyl-silica have been reported [7,10–12].

We recently succeeded in cyanogen bromide activation of diol–silica in the presence of triethylamine (TEA) under anhydrous conditions [13]. A batch activation procedure to obtain CNBr-activated silica was reported and gave rapid, efficient coupling and good chromatographic performance. Here, we report methods for the dynamic activation and coupling to pre-packed diol–silica columns. Activation and coupling procedures were developed for HPLC systems containing three, two, or even one pump, without exposure to toxic CNBr fumes.

2. Experimental

2.1. Materials

Unless stated otherwise, chemicals used were of the highest purity available. HPLC-grade acetone was used (Fisher, St Louis, MO, USA) and stored over 3 Å pore molecular sieves. CNBr, obtained from Sigma (St Louis, MO, USA) was stored under nitrogen in a dessicator (Drierite dessicant). TEA was from Fisher. CNBr (1.0 *M*) and TEA (1.5 *M*) solutions were prepared using anhydrous acetone immediately before and discarded immediately after use. Tryptophan solutions (10 m*M*) were prepared directly in coupling buffer (0.1 *M* $NaHCO_3$, pH 8.3, 0.5 *M* NaCl). The low-molecular-mass affinity ligand, 6-aminohexyl-Cibacron F3GA (AHCib) was synthesized by the procedure of Lowe [14] and stock solutions (10 mg/ml) prepared in phosphate buffer, pH 7.5. AHCib was then diluted with coupling buffer. The oligonucleotide $(T)_6$-αOp1 (5′-NH_2-$(T)_6$ - AATTGTATCCGCTCACAATTCCAC - 3′ where ''NH_2'' refers to the aminoethyl group added with the Applied Biosystem Aminolink reagent) was synthesized and in flow coupled to CNBr:TEA activated diol–silica as specified below. Commercially supplied pre-packed diol–silica columns (4.6× 30 mm, Macrosphere GPC 500 Å, 7 μm) or diol–silica (Macrosphere GPC 300 Å, 7 μm) were slurry packed in stainless steel columns (5×0.46 cm) using the HPLC system. For the last procedure, 1 g of diol–silica resin was suspended in 2 ml anhydrous acetone and sonicated under vacuum for 5 min. After centrifugation, the support was resuspended to 2 ml anhydrous acetone and packed into stainless-steel columns (5×0.46 cm) at 3 ml/min using anhydrous acetone as the mobile phase.

2.2. In flow activation and coupling procedures to pre-packed diol–silica columns

In flow activation and coupling experiments using pre-packed diol–silica columns were carried out as depicted in Fig. 1. In this procedure, the reagents required for activation (CNBr, TEA, and anhydrous acetone) and for ligand coupling after activation of the packed columns (coupling buffer, amino ligands

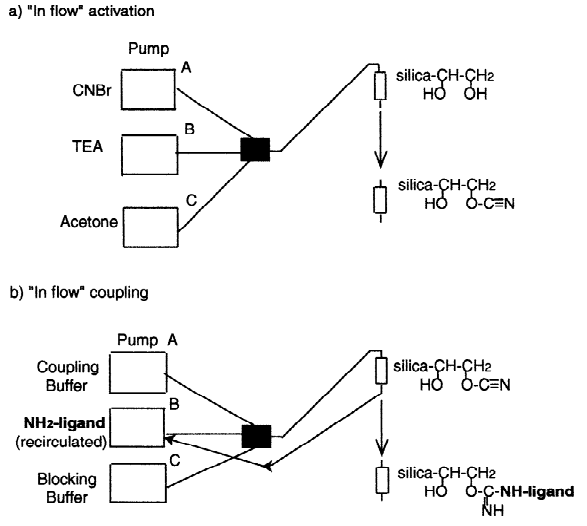

Fig. 1. Schematic diagram of the in flow activation and coupling procedure onto pre-packed diol–silica columns. Using pre-packed cartridge or slurry packed diol–silica columns, in flow activation (a) and coupling (b) using three pumps is depicted.

and blocking buffer) were delivered using three, two, or one pump on the HPLC, respectively, as described below.

2.2.1. In flow activation of diol–silica using the three pump HPLC and coupling of DNA

The experimental strategy followed for in flow activation and coupling of amino ligands to diol–silica columns using three pumps is diagramatically shown in Fig. 1. All steps were at room temperature (19 °C). A ternary HPLC system (Rainin Rabbit ternary gradient HPLC) was used to deliver solutions to a pre-packed cartridge column (3×0.46 cm, Macrosphere GPC, 500 Å, 7 μm from Alltech Associates). Briefly, 20 ml of anhydrous acetone was used to first equilibrate the column at 1 ml/min using pump C and then two pumps (A and B) were purged with CNBr and TEA solutions. Then, 1 ml of freshly prepared CNBr (1.0 *M*) and 1 ml of TEA (1.5 *M*) were delivered from separate pumps to a mixer and then on to the pre-packed column at 1 ml/min. Pumps were stopped for 3 min to allow reaction with the column. During this 3 min, pumps A and B were purged with water, and then with coupling buffer and the ligand to be coupled, respectively (Fig. 1b). The column was washed for 20 min with acetone (pump C) at 1 ml/min (Fig. 1a). All the CNBr containing solutions and washes were disposed of in a sink in the fume hood. The pre-packed, activated silica column was then subjected to in flow coupling procedure as depicted in Fig. 1b. First, the pre-packed activated column was equilibrated with coupling buffer (20 ml) and then, amino ligand solution containing 24 nmol of DNA [5′-NH_2-αOp1-$(T)_6$-3′] was recirculated overnight through the activated silica column at 0.1 ml/min. The DNA was contained in a 5-ml syringe barrel coupled to the pump inlet with a low dead volume three-way valve (normally used to purge air from these piston-type pumps). The next day, uncoupled DNA was recovered by washing the column with 50 ml of coupling buffer and the DNA collected measured by absorption at 260 nm. A total amount of 6 nmol/column was coupled using this procedure. Unreacted groups on the activated silica column were consumed using the third pump (after purging) to deliver 50 ml of blocking buffer (0.1 *M* Tris, pH 7.5, 0.5 *M* NaCl) at 0.1 ml/min. The 5′-NH_2-$(T)_6$-αOp1 DNA silica columns were made double-stranded by recirculating the respective complementary strand (5′-GTGCAATTGTGAGCGGATAACAATT-$(A)_6$). A total of 5.4 nmol of DNA was annealed by this ‘‘in flow’’ recirculation procedure or 10.8 nmol per ml of column bed volume. A DNA silica column prepared under these conditions was used for the purification of *Lac*IZ as described below.

2.2.2. In flow activation of diol–silica using two pumps and coupling of 6-aminohexyl-Cibacron F3GA

The procedure for in flow activation and coupling using two pumps is a modification of that shown in Fig. 1. First, the packed silica column (5×0.46 cm, Macrosphere GPC, 300 Å, 7 μm) was equilibrated in acetone and then the pumps were purged with freshly prepared CNBr and TEA solutions. Then, 1 ml of CNBr (1.0 *M*) and 1 ml of TEA (1.5 *M*) was pumped at 1 ml/min to the mixer and then on to the pre-packed diol–silica column. Flow was stopped for 3 min to allow activation of hydroxyl groups on the column. During this time, both pumps were rapidly purged with acetone (by pumping 50% with both

pump inlets in acetone). The column was washed with 20 ml of acetone and 50 ml of water. During this latter wash, all CNBr containing solutions and washes were disposed of down the fume hood sink. Then, 50 ml of coupling buffer at 1 ml/min was flowed through the activated column by simply changing the solvent to both pumps while pumping 50% from each; 6.8 μmol of 6-aminohexyl-Cibacron blue in 3 ml coupling buffer was recirculated at 0.1 ml/min overnight using a single pump as described in the last section. Uncoupled dye was recovered by washing with coupling buffer and quantified by absorption (620 nm). By difference, 3.7 μmol AHCib/column coupled. This corresponds to 4.4 μmol AHCib per ml of column bed volume. Unreacted groups on the column were consumed using 100 ml of blocking buffer at 0.1 ml/min. AHCib–silica columns prepared as described above were used for the purification of alcohol dehydrogenase (ADH).

2.2.3. In flow activation of diol–silica using one pump and the coupling of melittin

In this procedure, only one pump was used to separately deliver activating agents and coupling solution to a mixer and then on to a pre-packed diol–silica column. In brief, a previously packed diol–silica column (5×0.46 cm, Macrosphere GPC, 300 Å, 7 μm) was equilibrated with 20 ml of anhydrous acetone at 1 ml/min. Using a freshly prepared 10 ml anhydrous solution of CNBr (1.0 *M*) in acetone, 5 ml were pumped on to the pre-packed diol–silica column at 1 ml/min. To the remaining CNBr solution (i.e. 5 ml), 5 ml anhydrous solution of TEA (1.5 *M* in acetone) was added dropwise over the next 5 min, while the mixture was being pumped to the column. Flow was stopped (with 5 ml of CNBr:TEA mixture remaining in the reservoir) for 3 min to allow activation. During this 3 min, the pump was rapidly purged with acetone and the CNBr returned to the fume hood. The column was washed at 1 ml/min with anhydrous acetone (20 ml), water (50 ml), and coupling buffer (50 ml). The acetone and water washes were disposed of down the sink in the fume hood; 200 nmol of melittin dissolved in 3 ml of coupling buffer were recirculated through the activated column overnight at 0.1 ml/min. By absorption difference at 276 nm, a total of 28 nmol of melittin/column were coupled. This corresponds to 34 nmol peptide per ml column bed volume. Unreacted groups were consumed by washing the column with 50 ml of blocking buffer. This melittin–silica column was used for the purification of dansyl-calmodulin and bovine brain calmodulin as described below.

2.3. Chromatographic procedures

All silica columns were stored in blocking buffer when not in use. For affinity purification of *Lac*IZ (*Lac* repressor-β-galactosidase fusion protein) on DNA silica columns, we followed the same procedure used previously for DNA Sepharose columns [15]. The double-stranded DNA–silica column was equilibrated with Buffer TE0.1 (10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 0.1 *M* NaCl). Injections (10, 20 or 40 μl) of a crude bacterial extract containing the fusion protein were applied. The column was washed with 10 ml of TE0.1 at 1.0 ml/min. Bound proteins were eluted with a linear salt gradient from TE0.1 to TE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 1.2 *M* NaCl). One milliliter fractions were collected and assayed for β-galactosidase activity as previously described [16]. Alternatively, bound proteins were eluted by a step salt gradient using TE1.2 or with a linear gradient of heparin (0 to 40 mg/ml) dissolved in TE0.1.

For ADH affinity chromatography, the AHCib–silica columns prepared as described above were equilibrated with 10 m*M* sodium phosphate, pH 7.5 at 1 ml/min. Then 0.1 ml of 25 mg/ml purified ADH was injected and the column washed for 10 min. Bound proteins were eluted with 0.1 *M* sodium phosphate, pH 7.5, 0.1 m*M* NAD^+, and 0.1 *M* ethanol. One milliliter fractions were collected and assayed for alcohol dehydrogenase activity [17].

For affinity purification of dansyl-calmodulin or bovine brain calmodulin using the melittin–silica column, we used the protocol previously reported [18]. In brief, the melittin–silica column was equilibrated with Buffer A (10 m*M* sodium phosphate, pH 7.5, 1 m*M* $CaCl_2$, 0.1 m*M* EGTA). Either 50 μl of bovine brain calmodulin (1 mg/ml) or dansyl-labeled calmodulin (0.6 mg/ml) was loaded at 1 ml/min. After washing the column with 5 ml of Buffer A, calmodulin was eluted using 5 ml of

Buffer B (10 m*M* sodium phosphate, pH 7.5, 0.1 m*M* EGTA). Fluorescence (excitation at 345 nm, emission at 495 nm) or absorption (276 nm) was used to follow binding and elution.

3. Results and discussion

Handling cyanogen bromide is dangerous. Acidification can produce cyanide gas, a poison. Cyanogen bromide itself is volatile, poisonous, and can form impurities that are unstable explosives. To use this safely in a laboratory, the following precautions were taken. A single sink in a fume hood was used to dispose of CNBr solutions and these were discarded only after allowing the water to run for several minutes beforehand and afterwards to wash away any other compounds that might be present. Acetone was anhydrous and stored over activated molecular sieves [13] and pumped through the chromatograph before coupling to remove any water remaining there. Cyanogen bromide solutions in acetone were made immediately prior to use in a fume hood, kept tightly sealed whenever possible, used in the laboratory only long enough to activate columns, and any remaining solutions or waste containing CNBr was immediately returned to the fume hood and disposed of. One of the authors is very sensitive to the odor of cyanogen bromide and following these precautions allowed us to work safely with no odor of CNBr and obtain chemistry that works very reliably. The coupling procedures described here have proved safe and never failed to couple appreciable ligand, in our hands.

To characterize ligand immobilization to CNBr:TEA activated diol–silica in a dynamic process, we developed an ''in flow'' activation and coupling procedure using two HPLC systems in our laboratory. As shown in Fig. 1 and described in Section 2, activation of hydroxyl groups on the diol–silica column was carried out by separately delivering anhydrous solutions containing cyanogen bromide and triethylamine to a mixer and then to the pre-packed glycidioloxypropyl–silica (diol–silica) column. Initially, for these experiments we used a commercially pre-packed cartridge column (3×0.46 cm, Macrosphere GPC, 500 Å, 7 μm from Alltech Associates). CNBr and TEA are delivered to the pre-packed diol–silica column (Fig. 1a) and flow is stopped for 3 min to allow reaction. Excess of reagents must then be removed rapidly due to the time-dependent formation of a yellow precipitate, which can clog frits. After activation, the toxic waste should be collected in an end-capped tube and moved to a hood for safety reasons. After equilibrating the column with coupling buffer, 24 nmol of $(T)_6$-αOp1 oligonucleotide dissolved in coupling buffer is recirculated through the column for reaction. By difference with uncoupled DNA, we found that 6 nmol of DNA coupled. Remaining reactive groups were consumed by reaction with Tris. ^{32}P-labelled complementary strand DNA (Op1-$(A)_6$) was recirculated in Tris buffer and 5.4 nmol annealed.

The resulting double-stranded DNA silica column contains the operator 1 DNA element bound by *lac repressor*. Serial injections of a crude bacterial extract containing a *lac repressor β-galactosidase* fusion protein (*Lac*IZ) were applied to the column. Fig. 2a shows that this double-stranded DNA silica column binds increasing amounts of *Lac*IZ as shown by the β-galactosidase activity eluted by a linear salt gradient. The column is quite stable and gives quite similar results after 14 months of use and storage (Fig. 2b). Conductivity measurements demonstrated that *Lac repressor-β-galactosidase* fusion protein elutes between 0.7 and 0.8 *M* NaCl, which corresponds to the NaCl concentration required to also elute the same protein from DNA-Sepharose columns prepared with the same DNA [15]. These result thus show that for DNA affinity chromatography, the silica columns behave analogously to the Sepharose columns we have used previously. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the peak fractions reveal a major protein band corresponding to the known molecular mass of *Lac*IZ (M_r 155 000) (data not shown). Elution of the bound protein using a linear gradient of heparin (0–40 mg/ml) also showed that *Lac*IZ is eluted from the double-stranded DNA silica column analogous to that demonstrated for DNA-Sepharose columns [16]. Control experiments showed that commercially β-galactosidase is not retained by the double-stranded DNA silica column (data not shown). Also, purified β-galactosidase or *Lac*IZ are not retained by un-

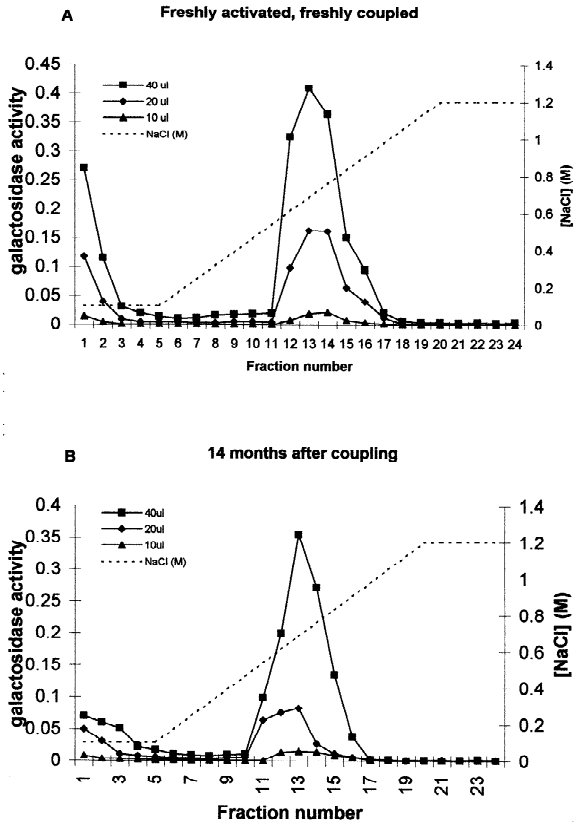

Fig. 2. Binding and elution of *LacIZ* from the silica–DNA column prepared by in flow activation and coupling of Op1 DNA binding sequence. (A) Serial injections of *Lac*IZ was carried out on the freshly prepared DNA–silica column. (B) Serial injections on the same silica column used and stored at room temperature for 14 months.

modified diol–silica columns (data not shown). Thus, DNA coupled to the silica affinity column is responsible for binding of *Lac repressor*. The non-covalent complementary DNA strand [Op1-$(A)_6$] can be removed by washing the column with hot water to make the column single-stranded and DNA re-annealed to restore the double-stranded DNA silica column (data not shown).

A modified procedure using two HPLC pumps for in flow activation and coupling was used to couple a solution containing 6.8 μmol of 6-aminohexyl-Cibacron F3GA in a previously packed diol–silica column (5×0.46 cm, Macrosphere GPC, 300 Å, 7 μm); This dye–ligand was chosen because it strongly absorbs at 620 nm where there are no interfering absorbances; 3.8 μmol AHCib coupled, which corresponds to a 57% coupling efficiency. This AHCib–silica column was used for the affinity chromatography of alcohol dehydrogenase. When monitoring elution at 280 nm, a small peak is observed in the unretained fractions and a prominent larger peak elutes after 10 min (data not shown). However, due to the strong absorption of NAD^+ at this wavelength, each fraction collected was also assayed for alcohol dehydrogenase activity as shown in Fig. 3. A small peak of unretained ADH activity elutes during the first 5 min and a larger peak is eluted by NAD^+ and ethanol after 10 min.

In other experiments, coupling AHCib to CNBr and TEA activated diol–silica using our previously reported batch conditions [13], followed by slurry-packing of columns (5×0.46 cm), resulted in the same chromatographic behavior (data not shown). Also, ADH elutes in 0.1 m*M* NAD^+ (Fig. 3), but not in 0.01 m*M* (data not shown). Thus, elution is competitive between NAD^+ in the mobile phase and the coupled dye, thought to mimic NAD^+ [14]. Also, chromatography at a slower flow-rate (0.1 ml/min) showed that slightly more ADH bound and eluted. These results suggest that effective protein–ligand interaction during HPAC may be too slow for some flow-rates.

The one pump procedure was used for the coupling of melittin. Melittin binds calmodulin with nanomolar affinity only in the presence of Ca^{2+} [19]. This peptide was successfully coupled (28 nmol/column) and used for the chromatography of dansyl-calmodulin and bovine brain calmodulin. As shown in Fig. 4, dansyl-calmodulin is retained by the column in Ca^{2+}-containing buffers and eluted in Ca^{2+}-free (EGTA) buffers as has been demonstrated before [18] for melittin–silica columns prepared using a different coupling procedure (*N*-hydroxy-succinimide ester coupling). The same result was obtained with native bovine brain calmodulin (data not shown).

In flow CNBr:TEA activated diol–silica columns can be stored for at least 3 months in anhydrous acetone as shown in Fig. 5. A column stored this way was used for the coupling of DNA (5′-NH_2-T_{18}-αOp1). After annealing with the complementary strand DNA (Op1-A_{18}), the column bound *Lac*IZ which was eluted by a linear salt gradient (0.1 to 1.2

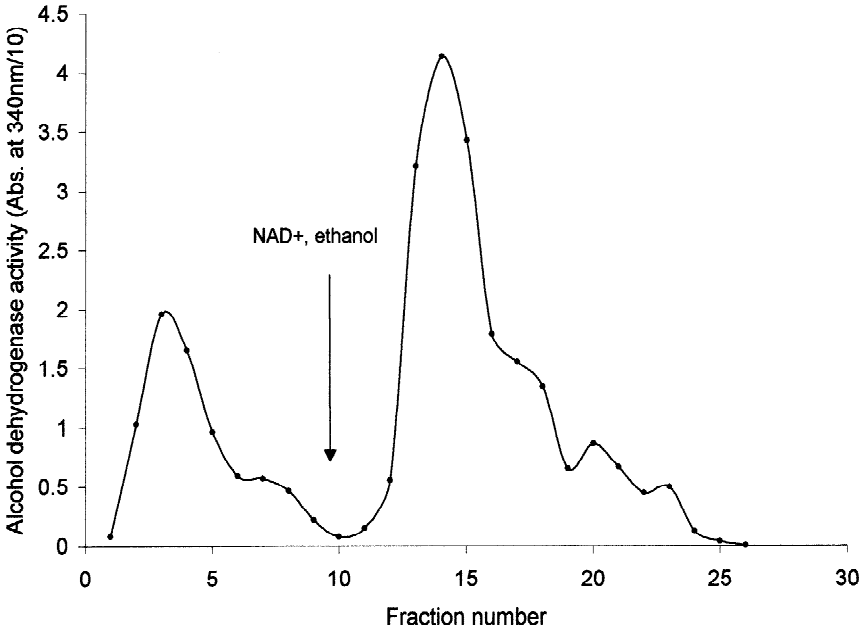

Fig. 3. Chromatography of alcohol dehydrogenase on the AHCib–silica column. The column used was prepared using the two-pump activation and coupling protocol described in Section 2. At time zero, 0.1 ml of 25 mg/ml alcohol dehydrogenase was injected onto the column equilibrated in 0.1 *M* sodium phosphate, pH 7.5. At 5 min, bound proteins were eluted with 0.1 *M* sodium phosphate, pH 7.5, 0.1 m*M* NAD^+, and 0.1 *M* ethanol. Each fraction was assayed for alcohol dehydrogenase activity [17].

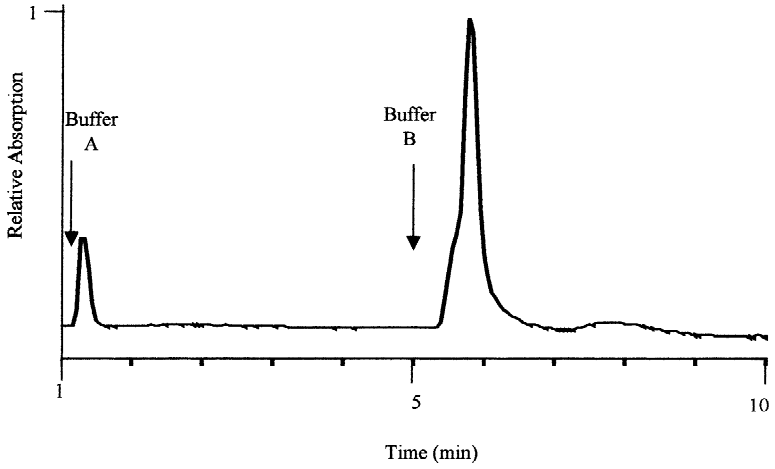

Fig. 4. Binding and elution profile of dansyl-calmodulin from the melittin–silica column. After equilibrating the column with buffer A (10 m*M* sodium phosphate, pH 7.5, 1 m*M* $CaCl_2$, 0.1 m*M* EGTA), 50 μl dansyl-calmodulin (0.6 mg/ml) was injected at 1 ml/min and the column eluted with buffer B (10 m*M* sodium phosphate, pH 7.5, 0.1 m*M* EGTA). A fluorescence detector was used to monitor chromatography.

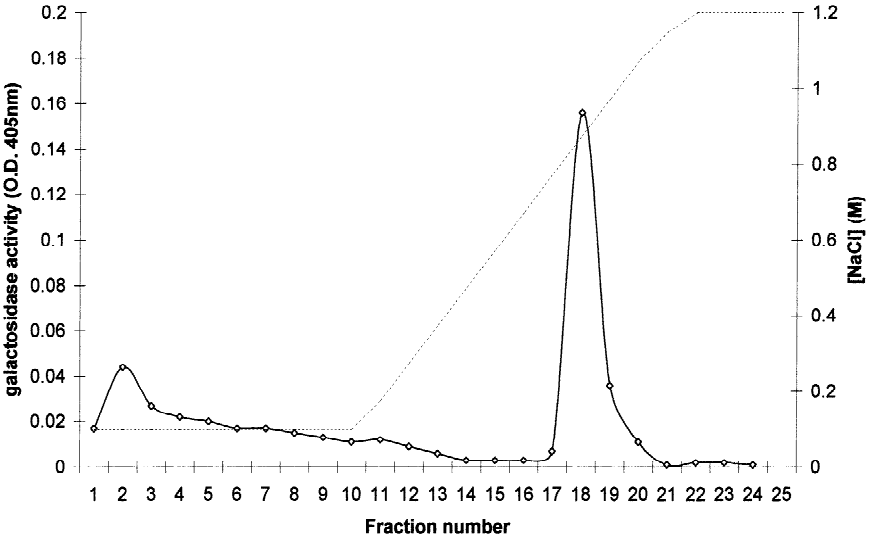

Fig. 5. Stability of CNBr:TEA activated diol–silica column stored for 3 months in anhydrous acetone. In flow CNBr:TEA activation and coupling to diol–silica (5×0.46 cm) was carried out as described in Section 2 using the two-pump procedure. The activated silica column, after rinsing with anhydrous acetone, was capped and stored for 3 months. The column was then washed with 50 ml water, 20 ml coupling buffer, and used for the coupling of DNA (5′-NH_2-T_{18}-αOp1). After annealing the complementary DNA strand (5′-Op1-A_{18}-3′), the double-stranded DNA silica column was used as described in the legend of Fig. 2 for the purification of *Lac*IZ.

M). Thus, silica columns can be pre-activated at least 3 months prior to use if stored under anhydrous conditions.

4. Conclusions

The coupling strategies described allow the use of commercially pre-packed columns. Different ligands, DNA, a dye ligand (6-aminohexyl-Cibacron), and a peptide (melittin) have been ''in flow'' coupled by these procedures. Thus, laboratories that do not have column-packing capability can use these procedures with commercially available columns for most types of ligands. Silicas of different pores and particle sizes are commercially available for a wide range of applications and most are available with a diol coating.

Since CNBr-activated Sepharose is the most widely used coupling chemistry for affinity chromatography, a large number of supports can be made by simply transferring these techniques to silica. Our results indicated that the coupling protocols should transfer to silica with little or no modification. The results indicate that other than the high-performance and resolution, elution from silica or Sepharose-based columns is quite similar. Laboratories can shift from affinity chromatography to high-performance affinity chromatography using these in flow techniques quite easily. These also suggest that the handling times would be shorter and the cost would also be lower.

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