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Journal of Chromatography A, 971 (2002) 95–104

JOURNAL OF CHROMATOGRAPHY A

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Cyanogen bromide activation and coupling of ligands to diol-containing silica for high-performance affinity chromatography Optimization of conditions[1]

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Received 23 January 2002; received in revised form 25 June 2002; accepted 27 June 2002

Abstract

To obtain silica supports for high-performance affinity chromatography, a method of preparing CNBr-activated diol–silica under anhydrous conditions was developed. Activation of the silane-derived hydroxyls with cyanogen bromide and triethylamine was optimized and demonstrated to efficiently couple several amino ligands (tryptophan, 6-aminohexyl-Cibacron Blue, and DNA). Sonication and vacuum degassing, a procedure used to remove air from the silica beads, increased activation. Coupling of an amino ligand under slightly basic conditions (pH 8.0) gave the highest yield. The linkage between the immobilized ligands and silica was stable from pH 2–10. Anhydrous acetone was the most effective solvent for activation but dimethylformamide and 2-propanol were also good choices. The high-performance affinity chromatography columns obtained by coupling sequence-specific DNA binding sequences for *Lac* repressor-β-galactosidase fusion protein were compared to affinity columns obtained by coupling the same DNA element to Sepharose beads; 5 μm silica gave the best performance.

Keywords: Silica, diol-; Affinity sorbents; Cyanogen bromide; Proteins

1. Introduction

High-performance affinity chromatography (HPAC) is a technique, which combines the specificity of affinity chromatography with the speed, efficiency and sensitivity of operations of high-performance liquid chromatography (HPLC) for the separation of biomolecules [1]. In particular, the high flow-rates and small bead diameters used in HPAC require high mechanical stability in the stationary phase. Until now, mostly polysaccharide-based solid supports have been used for affinity chromatography because of the simplicity of their activation and ligand coupling, but their mechanical instability limits their use in HPAC [2]. In contrast, silica, the support commonly used for HPLC is mechanically stable but there are few activation and coupling procedures for ligands available and thus HPAC is only rarely used. It is well known that chromatography on silica supports (small uniform bead sizes of 3–5 μm in current uses) has the advantages of increased resolution and better column efficiency

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[1]The work described here was awarded a United States Patent, number 6 375 846 to L.A.J. and H.W.J.

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00964-0

over the soft gel Sepharose (40–120 μm). The column efficiency is indicated by the peak width, where broad peaks result in a smaller number of biological components that can be separated in a given time [3]. Thus, biomolecule separations using silica affinity columns should give better chromatographic performance than the commonly used soft gel Sepharose. The development of HPAC has been limited by the lack of good, easily derivatizable silica supports. Ideal supports for HPAC require hydrophilicity and minimal non-specific adsorption, efficient derivatization chemistries and the ability to tolerate different organic and inorganic solvents and rapid changes in buffer composition for efficient elution of interacting biomolecules [2].

Since the development of the cyanogen bromide (CNBr) method by Axen et al. in 1967 [4] for the coupling of amino ligands to hydroxyl-containing supports (Sepharose, cellulose, etc), affinity chromatography has progressed considerably and become the technique of choice for the purification of biological substances. The original CNBr activation, which requires the use of NaOH and pH 11, has been successfully applied to the derivatization of soft gels [5], but not to silica supports, which dissolve under alkaline conditions. Furthermore, since proteins and other biological molecules are frequently irreversibly absorbed to bare silica surfaces, silica must frequently be derivatized for use with biological samples [6]. These coatings would also be stripped off by the harsh conditions of CNBr activation. CNBr activation of the silanols on the surface of quartz [7], glass, and various ceramics [8] has been reported, however, again some of the material is dissolved by the alkali and derivatives of the surface, desirable for biological applications, were not used.

However, alternative activation chemistries for coupling of ligands to silica derivatives have been reported in the literature. These activation procedures requires first the derivatization of the silica silanols with 3-glycidyloxypropyltrimethoxisilane [9], which provides intermediate products (epoxy–, diol–, aldehyde– and hydroxyl–silica) that could be used for immobilization of ligands and also mask strong and often irreversible adsorption of proteins to silica surfaces [9]. For example, coupling to epoxy–silica has been reported but it is of low reactivity and best suited for strong nucleophiles and coupling at high pH [9]. Diol–silica can be activated with carbodiimidazole, but coupling capacity was quite low due to the possible formation of a stable five-membered ring carbonate [10]. More recently, dihydrazide-activated silica supports were also reported by oxidizing diol-bonded silica and reacting with oxalic or adipic dihydrazide [11]. Also, the aldehyde function of silica obtained after periodate oxidation of diol–silica seemed to be a suitable derivative for the attachment of ligands. However, the Schiff base formed is very unstable and must be reduced. In many cases, reduction causes a loss of biological activity [9,12,13] although a recent study found this to be a good chemistry for DNA coupling [14]. Finally, reduction of aldehyde–silica leads to primary hydroxyl–silica, which can be activated, with several reagents (e.g., chloroformates [15], tresylchloride [16,17], and carbodiimidazole [10]). Another activation chemistry for coupling ligands to silica was reported based upon a reactive *N*-hydroxysuccinimide ester derivative of silica [18]. However, the final product of this activation chemistry yields affinity supports with a high negative charge and resulting cation-exchange properties [14]. In general, all reported activation procedures based on silica supports require complex chemistries and in many cases reactions are slow and poor yield is obtained.

A more reliable and less toxic way of producing CNBr-activated Sepharose was developed by Kohn and Wilchek [19,20]. This procedure uses triethylamine (TEA) dissolved in 60% acetone instead of NaOH as the ''cyano-transfer'' reagent. The activation reaction of polysaccharides with CNBr and TEA has been reported to yield the same cyanate ester groups formed upon reaction of alkoxide ion with CNBr [19,20]. Subsequently, the CNBr cyano-transfer method employing TEA was successfully applied for the coupling of immunoglobulins to quartz surfaces [7].

It is expected that the high concentrations of aqueous TEA used for activation of Sepharose would be detrimental if used for the CNBr activation of silica. However, in an unrelated study from our laboratory it was found that if silica is reacted under anhydrous conditions, organic bases do not dissolve it or have any apparent adverse effects. In that particular study, an anhydride was successfully reacted with aminopropyl–silica in anhydrous

pyridine [18]. Thus, we reasoned that if we were to use cyanoethyl transfer in an anhydrous organic solvent, we might be able to activate diol–silica or hydroxyl–silica for the effective coupling of amino ligands. In our search for an efficient HPAC carrier, we report here the successful application of CNBr activation chemistry to diol–silica by developing ways to avoid the detrimental aqueous alkaline activation protocols, by using anhydrous conditions.

2. Experimental

2.1. Materials

Unless stated otherwise, chemicals used were of the highest purity available. Acetone (HPLC grade from Fisher, St Louis, MO, USA) was stored over 0.3 nm pore molecular sieves. CNBr was obtained from Sigma (St Louis, MO, USA) and TEA from Aldrich (St Louis, MO, USA). Tryptophan (Trp) solutions (10 m*M*) were prepared directly in coupling buffer (0.1 *M* sodium hydrogencarbonate, 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 et al. [21] and stock solutions (10 mg/ml) prepared in 10 m*M* phosphate buffer, pH 7.5. AHCib was then diluted with coupling buffer when used for coupling experiments to activated diol–silica.

2.2. Modification of the silica surfaces with silanes and preparation of diol-containing silica

As shown in Fig. 1, diol-bonded silica was prepared by a modified procedure of that originally described by Larsson et al. [1]. The base silica, either Machery–Nagel Polygoprep, 50 μm, 300 Å pore or Nucleosil 7 μm, 300 Å pore, was used for the synthesis of diol–silica and all solvents were of the highest anhydrous grade. Ten grams of silica as well as all glassware to be used was dried overnight at 150 °C. A volume of 100 ml of toluene was added and distilled to constant boiling (110 °C); this required about 10 ml be distilled. To the remaining 90 ml, 10 ml of glycidyloxypropyltrimethoxysilane (Aldrich) was added and the mixture refluxed with

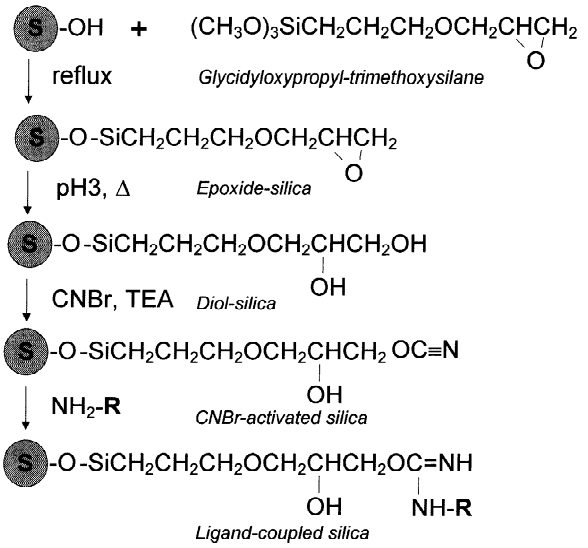

Fig. 1. Schematic of activation. Derivatization of silanol groups on silica to produce epoxide–silica was performed by a modification of the procedure as described by Larsson, et al. [9]. The names used for the various silicas produced are given.

overhead stirring for 4–5 h. The modified silica (epoxy–silica) was washed with toluene, acetone, and water. This was resuspended in 100 ml water, titrated to pH 2.8 with trifluoroacetic acid, and heating for 2 h in a boiling water bath with overhead stirring to convert the epoxide to the corresponding diol. The diol–silica was washed with water, acetone, and diethyl ether and dried under vacuum. Polygoprep diol–silica prepared in this way had an average of 120 μmol diol/g silica as determined by triplicate measurements using the periodate oxidation method [1]; the nucleosil support was similar and measured 130 μmol diol/g silica in a single determination. In other experiments diol–silica was that commercially supplied from Alltech Associates (Macrosphere GPC, 300 Å, 7 μm, and Platinum Exmere 300 Å, 5 μm).

2.3. Activation of diol–silica groups with CNBr and TEA

To covalently couple NH_2 ligands to diol–silica, we used the procedure diagramatically shown in Fig. 1. For either diol–silica prepared as described above or from commercially sources, 1 g silica was placed

in a 15 ml polypropylene tube (Sarstedt). About 5 ml anhydrous acetone was added to suspend the resin and the mixture sonified under vacuum for 5 min. After centrifugation, the supernatant was removed and the silica transferred to a 50 ml beaker to which enough anhydrous acetone was added to again suspend the diol–silica. The diol–silica/acetone mixture was equilibrated at −15 °C by placing the beaker and reagents in methanol and adding dry ice chips. CNBr (1 *M*) and TEA (1.5 *M*), both solutions freshly prepared using anhydrous acetone, were also equilibrated to the same temperature. Subsequently, 1 ml CNBr was added to the diol–silica/acetone slurry followed by dropwise addition of 1 ml TEA for a period of 3 min with constant shaking. After activation, the silica was filtered on a coarse sintered glass funnel and washed three times with ice-cold water (50 ml), 100 ml of coupling buffer (0.1 *M* $NaHCO_3$, pH 8.3, 0.5 *M* NaCl), and excess buffer filtered away to yield a semi-dry cake. These wash procedures were required to remove excess of reagents and acetone from the activated resin which interfere with absorption measurements.

2.4. Coupling of NH_2 ligands to the CNBr:TEA-activated carrier

Semi-dried CNBr:TEA-activated silica was then transferred to coupling buffer containing the respective NH_2 ligand to be coupled (tryptophan, AHCib or DNA solutions) and the mixture constantly mixed using a wheel rotator overnight at 4 °C. Uncoupled NH_2 ligand was recovered after washing the support with coupling buffer by centrifugation. The supernatant collected was used to determine the amount of ligand immobilized by difference. Remaining reactive groups on the activated silica were consumed by overnight incubation with blocking buffer (0.1 *M* Tris, pH 8.0, 0.5 *M* NaCl) on a tube rotator. Control activation and coupling experiments used diol–silica previously sonified under vacuum for 5 min in anhydrous acetone. Mock activation at −15 °C were performed by adding anhydrous acetone (in place of CNBr reagent) plus TEA solutions for 3 min. Following this, the mock activated resin was washed as described before and transferred to a coupling solution.

2.5. Immobilization of amino ligands at different pH

After activation as described, semi-dried CNBr:TEA activated diol–silica was incubated with different pH coupling buffer solutions containing 10 m*M* tryptophan. Coupling was allowed to occur overnight on a wheel rotator at 4 °C. As a control, semi-dried acetone:TEA mock-activated diol–silica was incubated with coupling buffer solutions at different pH containing tryptophan. Uncoupled amino ligand recovered by washing-filtration procedures was used for spectrophotometric determination of tryptophan (absorption at 272 nm).

2.6. Stability of the bond formed between silica and the amino ligand

For these experiments, dried AHCib–silica, obtained by coupling AHCib to CNBr:TEA-activated diol–silica as described, was incubated for 2 h in coupling buffer solutions of different pH (from pH 2 to 10) with frequent mixing. After centrifugation, AHCib–silica was neutralized by washing and incubation overnight in 0.1 *M* $NaHCO_3$, pH 7.0, 0.5 *M* NaCl, followed by resuspension in 25% glycerol. As a control, dried (pH untreated) AHCib–silica was incubated in 1 ml of 25% glycerol solution. Spectrophotometric determination of the AHCib–silica at 620 nm of each pH treatment with respect to control was recorded to calculate the amount of silica–AHCib remaining.

2.7. Kinetics of hydrolysis of reactives groups on CNBr:TEA-activated diol–silica

To study the consumption of reactive groups on the activated resin, semi-dried activated resin was incubated with water for its hydrolysis. For these experiments, freshly activated silica was immediately filtered and washed to remove excess reagents. Semi-dried activated diol–silica was resuspended to 50:50 silica:water. Remaining reactive groups at different times were determined by taking out an aliquot equivalent to 100 mg of activated diol–silica and incubated with a coupling solution containing tryptophan as the amino ligand. Coupling was then allowed to occur for 16 h. Uncoupled amino ligand

recovered by washing-filtration was collected and used for absorption measurements.

2.8. Chromatographic procedures

To compare the chromatographic behavior of the silica affinity columns with the conventional Sepharose affinity columns, the αOp1–$(T)_{18}$ DNA sequence [5′–NH_2–$(T)_{18}$–AATTGTATCCGCTCACAATTCCAC–3′] [22] was immobilized to both supports. Diol–silica from Alltech Associates (Platinum Exmere, 300 Å, 5 μm) and 50 μm Polygoprep diol–silica we synthesized were used for these experiments. For coupling of αOp1–$(T)_{18}$ to CNBr-activated Sepharose, the cyanoethyl transfer procedure in 60% acetone of Kohn and Wilchek was used [23]. The αOp1–$(T)_{18}$ DNA–silicas and –Sepharose were made double stranded by adding the complementary strand [5′–GTGCAATTGTGAGCGGATAACAATT–$(A)_{18}$] to the DNA-bound support. All coupling and annealing experiments resulted in virtually identical amounts of double-stranded DNA sequence (16 nmol/g support). For affinity purification of *Lac*IZ (*Lac* repressor-β galactosidase fusion protein) on both DNA–silica and –Sepharose columns, previously reported procedures were used [22]. The DNA–silica support was slurry-packed into stainless-steel columns (5×0.46 cm) and the DNA–Sepharose was packed into plastic columns (5×0.5 cm, BioRad) at 3 ml/min using buffer TE0.1 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 0.1 *M* NaCl) as the mobile phase. Crude extract (10 μl) containing *Lac* repressor-β-galactosidase fusion protein (*Lac*IZ) were loaded onto each affinity column. The columns were washed with 10 ml TE0.1. Finally, bound proteins were eluted by using a 10 ml linear salt gradient from TE0.1 to buffer TE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1.2 *M* NaCl). The flow-rate was 1 ml/min and 1 ml fractions were collected.

3. Results and discussion

In an alternative application of the cyano transfer method [19], we investigated the activation of diol–silica under anhydrous conditions by CNBr and TEA. The success of activation was demonstrated by the immobilization of amino ligands (tryptophan, 6-aminohexyl-Cibacron FG3A and DNA). Table 1 shows that diol–silica, activated under anhydrous conditions by the cyano-transfer method, couples tryptophan. The CNBr:TEA-activated diol–silica coupled with an efficiency of 43–52% and 23–28 μmol tryptophan per gram silica.

Table 1
Trp coupling to diol–silica

Coupling procedure	Trp coupled (nmoles per 0.1 g diol–silica)	% Coupling
(1) Mock Diol–silica (1) Sonication (2) Acetone (3) TEA	N.D.[a]	N.D.
(2) Activated Diol–silica (1) Sonication (2) CNBr (3) TEA	2614±263.6[b]	48

[a] N.D., none detected.
[b] SD, $n=3$.

Activation with CNBr and TEA has been reported to result in the immobilization of immunoglobulins on quartz surfaces containing only silanol groups [7]. In contrast, we found that CNBr and TEA activation of silanol groups present in unmodified base silica resulted in no detectable coupling of amino ligands (data not shown). This failure of coupling to unmodified silica resulted even after either incubation with HCl and sonication under vacuum for 5 min to increase the presence and availability of OH groups (data not shown). The most likely explanation of these results is that only a small amount of coupling occurs with silanols, detectable with immunological techniques but not by the methods used here which are designed to detect micromole or larger amounts.

The degree of activation obtained by different relative amounts of CNBr and TEA was determined. The effectiveness of CNBr and TEA activation of diol–silica was assayed by the coupling of tryptophan. As is shown in Fig. 2, higher amounts of CNBr and TEA increase the amount of tryptophan coupled. Addition of 1.5 mmol CNBr plus 2.3 mmol TEA during a 3-min reaction gave the maximum amount of tryptophan immobilized into the solid

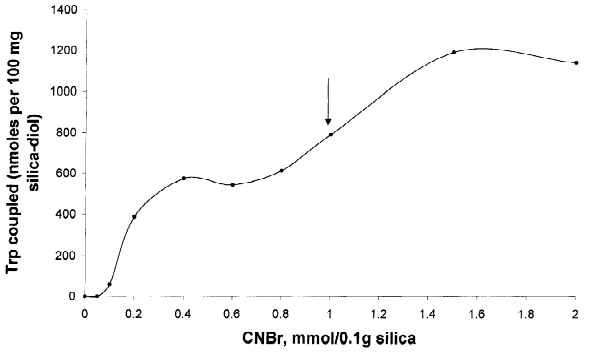

Fig. 2. Titration of diol-containing silica with different amounts of CNBr and TEA. To 100 mg diol–silica previously sonicated in anhydrous acetone were added different amounts of CNBr and TEA solutions to give different amounts of CNBr keeping the CNBr:TEA ratio constant at 1:1.5. Semi-dried activated resin after washing was incubated with tryptophan in coupling buffer overnight on a wheel rotator. Uncoupled tryptophan was collected and quantified by absorption at 272 nm. The arrow shows the standard conditions used in most experiments.

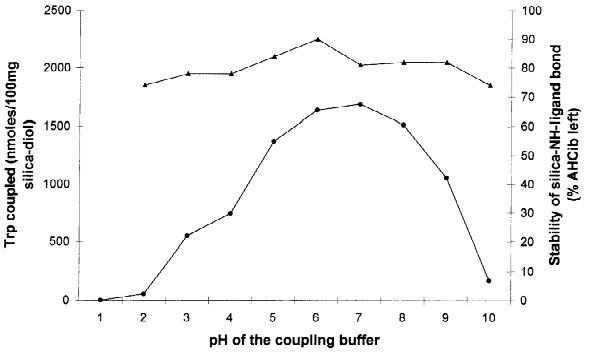

Fig. 3. Effects of pH on coupling and stability of amino ligands. One hundred milligrams of CNBr:TEA-activated diol–silica was incubated with tryptophan solutions dissolved in coupling buffer of each pH shown. The coupling reaction was performed overnight and uncoupled amino ligand collected and measured (circles). Using silica–AHCib coupled as described in Experimental procedures, we also tested the stability of the silica–ligand bond formed. One hundred milligrams silica–AHCib was incubated with coupling buffer solutions of different pH for 2 h. Then, each silica–AHCib was washed by centrifugation and resuspended in 25% glycerol solutions and the absorption at 620 nm of pH-treated silica was divided by that of untreated AHCib–silica to express the amount of AHCib remaining as a percentage of untreated control (triangles).

support (1.2 μmol tryptophan per 100 mg diol–silica with a 46% coupling efficiency). At these higher amounts of CNBr:TEA, a yellow colored precipitate forms that interferes with both filtering and washing of the activated resin as well as with absorption in the ultraviolet range for determining the amount of ligand coupled. At 1 mmol CNBr+1.5 mmol TEA and 3 min reaction (see arrow) no precipitate formed while 66% as much coupled; these conditions were thus chosen for further studies since it gave high activation of a 100 mg diol–silica and minimal interference. We used these same amounts for activation of from 0.1 to 1 g. of diol silica.

In order to determine the optimal coupling conditions, we coupled tryptophan to CNBr-activated diol containing silica under various pH conditions. As is shown in Fig. 3 (lower line), immobilization of amino ligands to CNBr:TEA-activated diol–silica was found to be pH-dependent. Tryptophan coupled best between pH 6 to 9 while tryptophan at pH below 6 or higher than 9 was not coupled effectively. The low coupling capacity for tryptophan obtained at high pH is likely due to the detrimental effects of basic pH on the silica, while at low pH (pH$<$6), it is probably due to the poor nucleophilicity of tryptophan's amino group. In an attempt to investigate the stability of the bond formed between the amino ligand and the activated diol–silica, we used the AHCib–silica and subjected it to different pH buffer conditions for 2 h. Spectrophotometric determination shows that most of AHCib remain on the silica support (Fig. 3, upper line). By comparing the AHCib–silica incubated with solutions of pH between 6 to 8 with AHCib–silica control, we found that between 84 and 90% of the AHCib still remains coupled to the silica. Thus, for 2 h exposure the coupled ligand is stable over a wide range encompassing neutrality. In contrast, at pH 3 as well as pH 11 about 26% of AHCib has been lost suggesting that the bond formed is not stable at extremes of pH (Fig. 3).

Other parameters such as reaction time of diol–silica with CNBr and TEA solutions, temperature of activation, and time required for coupling were also investigated. Our results indicated that addition of 1 ml CNBr (1.0 *M*) plus 1 ml TEA (1.5 *M*) during a 2–3 min reaction gave appreciable activation of 100 mg diol–silica. Reaction times longer than 3 min resulted in a yellow precipitate side-product that interferes with ligand coupling measurements. As the

temperature of the activation reaction is lowered to −15 °C, higher coupling reaction of tryptophan was found than when using higher temperature (0 °C). This short reaction time and low temperature are the same conditions found for the activation of hydroxyl groups on Sepharose [20,23]. Finally, coupling tryptophan for different times (30 min to 48 h) showed that the bulk of coupling occurred in the first 6 h of incubation, resulting in 1.6 μmol/100 mg activated diol–silica and further reaction time gave little additional coupling (data not shown).

In other experiments, we investigated the effects of various anhydrous solvents for the preparation of CNBr-activated silica (Table 2). The results shown in Table 2 indicated that anhydrous acetone seems to be the best choice of these organic solvents; a coupling capacity of 2.1 μmol tryptophan per 100 mg diol–silica with a 40% coupling efficiency was obtained. Dimethylformamide and 2-propanol also gave good activation. In contrast, lower coupling capacity was obtained with dioxane. Since dioxane freezes at 11.8 °C, the reaction could not be done at −15 °C and was performed at room temperature (20 °C). This higher temperature could account for the poor results with dioxane. Our results suggest that anhydrous acetone seems to be a good choice for the activation reaction of diol–silica.

Table 2
Uses of different solvents as an effective medium for CNBr:TEA activation of diol–silica[a]

Diluent solvent	Trp coupled (nmol/0.1 g Si–diol)	% Coupling
Acetone	2107±455	40
Dimethylformamide	1939±122	37
2-Propanol	1649±153	32
Dioxane	530±100	10

[a] For each activation reaction, 0.1 g diol–silica was activated in each solvent in duplicate. Each resin–anhydrous solvent mixture was then sonicated for 5 min under vacuum and after centrifugation resuspended in 2 ml of each respective solvent. Activation of diol–silica were done by adding 1 ml CNBr (1 *M*) plus 1 ml triethylamine (1.5 *M*) during a 3 min period. Each CNBr and TEA solution was prepared in acetone, dimethylformamide, 2-propanol or dioxane, respectively. All activation reactions were performed at −15 °C, except for dioxane, which was performed at room temperature (20 °C) to avoid freezing of the reaction mixture. After activation, resin was washed with ice-cold water followed by coupling buffer and semi-dried activated resins were added to a coupling solution containing 5230 nmoles Trp. Coupling was overnight on a wheel rotator at 4 °C. Unreacted Trp recovered by washing in the centrifuge and the washes combined for absorption 272 nm measurements. As a control, 0.1 g Si–diol were mock activated in each solvent and gave no coupling (data not shown). The results represent duplicate experiments.

To further test these optimized conditions, we used a commercially supplied diol–silica (Alltech, Macrosphere GPC 300 Å, 7 μm) and the above parameters to determine how much low-molecular-mass ligands (tryptophan and 6-aminohexyl-Cibacron) could be coupled to 100 mg CNBr:TEA-activated diol–silica. The results shown in Table 3 indicated that the coupling capacity obtained depends on both the initial amount and on the type of low-molecular-mass amino ligand used. By using tryptophan as the amino ligand, an average amount of nearly 1.4 μmol per 100 mg diol–silica could be coupled, whereas 6-aminohexyl-Cibacron FG3A resulted in a larger amount, 1.7 μmol per 100 mg diol–silica. These results suggest that the presence of an aminohexyl spacer arm on AHCib may have favored coupling. Mock activation without CNBr resulted in no detectable coupling of either low-molecular-mass ligands (data not shown).

To study the stability of the activated silica, after activation the silica was suspended in water and portions were withdrawn at various times for tryptophan coupling. The result of these experiments is shown in Fig. 4. The hydrolysis of reactive groups showed rather complex kinetics and suggests the presence of two phases to hydrolysis. Up to 4 h, the reaction appears first order with a half-time equal to 3.6 h; at later times (5–48 h), the hydrolysis reaction is slower with a half-time of 8.3 h. Forty-eight hours after diol–silica activation (about six half times), the remaining reactive groups for coupling amino ligands are almost completely consumed in water. This slow hydrolysis allows 3–4 μmol of amino ligand coupling per gram of silica even 5 h in water after activation (Fig. 4). In other attempts to obtain pre-activated silica resin that could be stored in the wet state for future use, we incubated the CNBr:TEA-activated diol–silica with a storage solution (acetone:dioxane:water=60:35:5). After 24 h storage, activated silica coupled an average of 166 nmol tryptophan per 100 mg of silica (only 12% coupled in a triplicate experiment) compared to the same batch used for immediate immobilization (1.4 μmol tryptophan per 100 mg of silica). This same storage

Table 3
NH_2 ligand coupling to CNBr:TEA-activated diol–silica[a]

Ligand	Amount used (nmol)	Ligand coupled (nmol/0.1 g Si–diol)	% Coupling
Trp	188	75	40
	250	166	66
	500	386	77
	750	500	67
	1000	632	63
	2500	1075	43
	5000	1392	28
	10 000	1195	12
AHCib	277	37	13
	554	69	13
	1108	342	31
	1662	494	30
	2216	662	30
	4432	1745	39

[a] Activated semi-dried resin was added to coupling solution containing the amounts of NH_2 ligand (Trp or AHCib) shown for its coupling reaction overnight on a wheel rotator at 4 °C. A control reaction was done by adding acetone plus TEA solution during activation. Unreacted NH_2 ligand was recovered by centrifugation and quantified by absorption (272 nm for Trp and 620 nm for AHCib).

solution has been reported to be an adequate solution to preserve the CNBr:TEA-activated Sepharose for subsequent coupling uses for several months [23]. The non-anhydrous conditions (5% water) present on this storage buffer may cause hydrolysis of the reactive groups such as that found in Fig. 4.

However, other attempts to obtain stable CNBr:TEA-activated silica were successful when anhydrous acetone was used for storage. CNBr:TEA activation of diol–silica followed by washing and storage in anhydrous acetone showed that these activated resins couple 1.2 μmol tryptophan per 100 mg silica even 15 days after activation. Analytical determination of cyanate esters [19,24] on these stored resins showed an amount of 1.6 μmol per 100 mg silica. These results suggest that these reactive species are probably responsible for coupling of amino ligands to CNBr:TEA-activated silica as has been demonstrated for CNBr-activated polysaccharides [19,24]. In contrast, CNBr-activated silica stored dry lost much of its ability to couple tryptophan during a period of 3 days. Only 0.59 μmol of tryptophan per 100 mg of silica is obtained when CNBr-activated silica is dried and stored for 3 days prior to coupling, in contrast to 1.2 μmol/100 mg coupled immediately after activation. Assays for cyanate ester showed 0.5 μmol/100 mg of silica is present after 3 days of storage in a dry state. In conclusion, storing CNBr-activated silica on anhydrous acetone (in a wet state) allows future use for coupling of amino ligands.

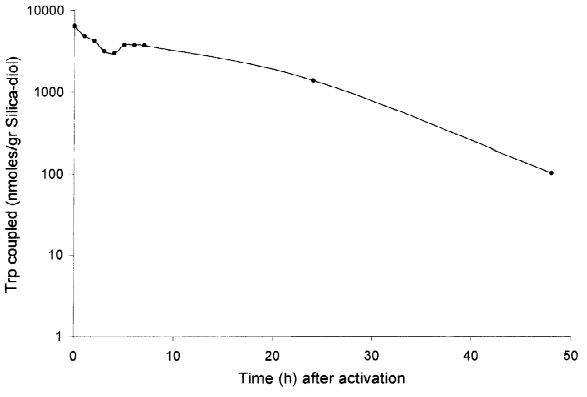

Fig. 4. Kinetics of hydrolysis of reactive groups on CNBr:TEA-activated diol–silica. Freshly activated CNBr–silica was washed and suspended in water. Aliquots containing 100 mg of silica were taken at each time period shown and after equilibration with coupling buffer, tryptophan was added and allowed to couple overnight on a wheel rotator.

To compare the chromatographic behavior of the silica affinity columns with the Sepharose affinity columns, we coupled 5′–NH_2–αOp1–$(T)_{18}$ DNA to

diol–silica and to Sepharose beads. As shown in Fig. 5, *Lac* repressor-β-galactosidase fusion protein bound and eluted from all affinity columns studied when using an increasing salt concentration gradient. Peak width is less (and resolution highest) with the smallest diameter silica. The larger diameter silica also gave a slightly narrower peak and Sepharose gave the worst performance. Conductivity experiments indicated that the salt concentration (0.7–0.8 *M* NaCl) required to elute the bound proteins is the same for each of the three affinity chromatography experiments. Thus, these results suggest that the elution behavior of CNBr–TEA-activated silicas and CNBr–TEA-activated Sepharose are quite similar.

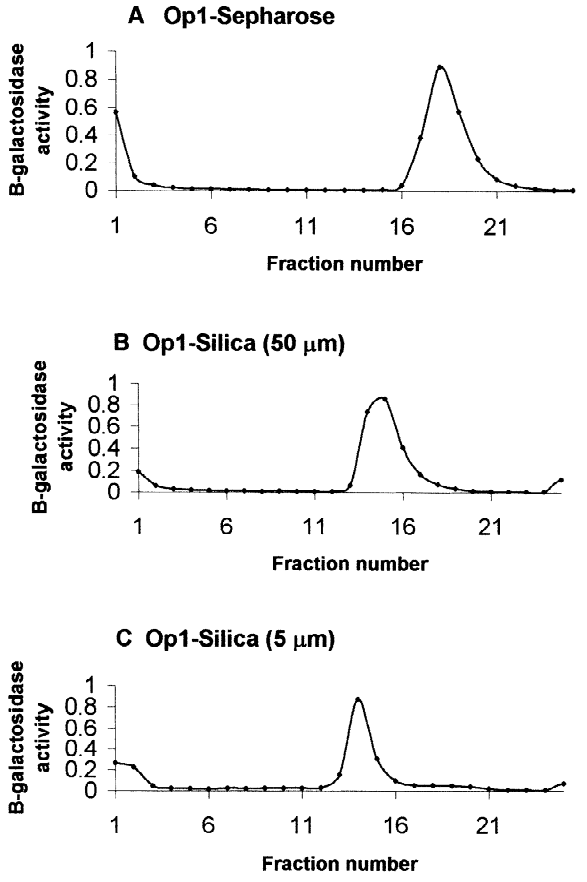

Fig. 5. Affinity chromatography of *Lac* repressor β-galactosidase fusion protein to DNA–silica and DNA–Sepharose columns. DNA (5′–NH_2–αOp1–$(T)_{18}$–3′) was coupled to silica beads as described in Experimental procedures and to Sepharose beads as described by Kohn and Wilchek [19,22]. Annealing with (5′–Op1–$(A)_{18}$–3′) resulted in double-stranded DNA columns with 16 nmol/g of resin for all three supports. A volume of 1 ml syringe column (DNA–Sepharose) or 1 ml stainless steel (DNA–silica) column was packed for each experiment. For each column, 10 μl of crude bacterial extract containing *Lac* repressor-β-galactosidase (LacIZ) were loaded and 10 ml buffer TE0.1 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 0.1 *M* NaCl) used to wash and remove contaminant proteins. Elution of bound proteins was by using a 10 ml linear salt gradient between buffer TE0.1 and buffer TE1.2 (10 m*M* Tris, pH 7.5, 1 m*M* EDTA, and 1.2 *M* NaCl). All runs were at 1 ml/min and 1 ml fractions collected. Aliquots (50 μl) of each fraction collected were assayed for β-galactosidase activity (absorption at 405 nm).

4. Conclusions

In conventional CNBr activation [4,5], the NaOH used would destroy HPLC silica. An alternative method [19] avoided NaOH and substituted the organic base, TEA and used 60% acetone. This combination would also be detrimental to HPLC silica but would be somewhat milder. The problem is that silica dissolves in aqueous bases. In previous work [18], we had found that the organic base, pyridine, could be used for prolonged reactions with silica without dissolving it as long as the conditions were anhydrous. This suggested that performing CNBr activation, much as had been done by Kohn and Wilchek [19], except using strictly anhydrous conditions, we might obtain CNBr activation of hydroxyls on silica while preserving its chromatographic performance.

In this work, a new method for the preparation of high-performance affinity chromatography supports based on CNBr activation of diol–silica was developed. The success depends upon anhydrous conditions, which allows the use of base (TEA) without adverse effect. We have demonstrated with simple chromatographic systems the CNBr activation of vicinal diol-containing silica and its subsequent coupling of several amino ligands (tryptophan, aminohexyl-Cibacron FG3A, and DNA).

Activation can be done in a variety of solvents as long as they are anhydrous. Acetone worked best of those tested. Ligand coupling in aqueous buffers occurs best over pH 6–8 and the covalent linkage is also most stable over this same pH range. Coupling capacities as high as 28 μmol/g of silica were obtained with tryptophan. The small bead diameter

silicas (5 μm) give sharper peaks and thus higher resolution than is obtainable with Sepharose, but otherwise we have found that the silica columns behave chromatographically very similar to Sepharose-based columns (Fig. 5 and data not shown).

Since CNBr-activated Sepharose is the most commonly used support for affinity chromatography, the huge number of the supports already reported in the literature can now be readily adapted to HPAC with the new activated silica reported here. Since our experiments show that elution is similar for columns prepared from CNBr-activated Sepharose and silica, it is likely that such affinity methods can now be adapted to the HPLC with only minor alteration. The advantage would be higher resolution and higher throughput.

Next, we will investigate whether this chemistry can be performed inside pre-packed HPLC columns.

Acknowledgements

The excellent technical assistance of F. Darlene Robinson is greatly appreciated. This work was funded by the NIH (GM43609).

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