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AFFINITY CHROMATOGRAPHY WITH TRIAZINE DYES IMMOBILIZED ONTO ACTIVATED NON-POROUS MONODISPERSE SILICAS

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SUMMARY

Non-porous monodisperse silicas with a particle diameter of 2.1 μm were modified with different silanes for immobilization of various triazine dyes including Procion Red HE3B, Procion Red MX5B, and Cibacron Blue F3GA. Lactate dehydrogenase and malate dehydrogenase from different species and aldehyde reductase from rat brain were purified by affinity elution using the substrate of the enzyme and NADH. With Cibacron Blue F3GA the selectivity for NADH-dependent enzymes was higher than with the two Procion dyes. The utility of these immobilized triazine dye systems on non-porous silica supports for the rapid separation of Cohn Fraction III plasma proteins, including plasminogen, is also described.

INTRODUCTION

Biospecific and biomimetic affinity chromatography are well known techniques for the purification of biopolymes. The high selectivity of the stationary phase for a single component, or a class of related components with similar ligand binding properties, is one of the characteristics of this method. In recent years there has been a pronounced trend to utilize mechanical stable matrices such as chemically modified silicas in place of the conventional soft polymer gel systems in most areas of affinity chromatography. For example, affinity adsorbents based on porous microparticulate silica have been successfully applied in the high-performance liquid affinity chromatographic purification and analysis of a variety of biologically active substances including serine proteases[1], oxidoreductases[2], lectins[3] and immunoglobulins[4]. Improvement in separation speed has been achieved with these silica based affinity media over conventional soft gel affinity media. However, the enhancement in separation performance is typically not as high as with chemically modified porous silicas in the bioaffinity mode as seen with, for example, the corresponding reversed-phase mode[5].

Several factors underlie this observation. First, chemical modification of silicas

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used as affinity media in isolation of biomolecules can represent a level of technical difficulty not experienced with soft gel matrices[6]. Porous silica matrices, in common with their soft gel counterparts are often very heterogeneous with poorly defined surface compositional characteristics, and exhibit polydispersity in terms of particle size and pore size distribution. These effects can cause low mass recoveries in the separation process with concomitant effects on the bioactivity. Furthermore, the kinetics of the adsorption and desorption of the affinity complex can be a very slow process with all porous media due to resistance to mass transfer effects in the intraparticulate boundary and pore. These effects are manifested as a dependency of peak dispersion and peak capacity of the analytes on the pore size of the base materials[7]. These effects are most noticeable when using silicas with pore diameters from 6 to 50 nm as the parent matrices for the biospecific affinity separation of large proteins and can result in significantly tailed peaks[4]. This problem is still apparent with porous particles of less than 10 μm and has been attributed to kinetic effects associated with diffusion of solute molecules into the pores of the silica.

Phillips *et al.*[8] have described the use of non-porous glass beads with particle diameter > 10 μm to eliminate the diffusional effects due to the pore structure. In 1984 we have described[9] a new support material based on 1.5 μm non-porous monodisperse silica beads which exhibited many characteristics of a very efficient stationary phase for use in high-performance liquid chromatography (HPLC). Decreasing particle diameters from 10 μm to 1.5 μm or to smaller values increases the geometrical surface area within a unit column. From both theoretical and practical considerations non-porous particles of small particle diameter should thus have advantages especially for very rapid analytical and micropreparative affinity chromatography[9,10]. In this paper we describe the use of 2.1 μm non-porous silica using immobilized triazine dyes as affinity adsorbents in the fractionation of several enzymes present in biological extracts.

EXPERIMENTAL

Materials

The chromatographic matrix was a non-porous, monodisperse silica with 2.1 μm particle size previously developed in our laboratories. These silica particles are commercially available from E. Merck (Darmstadt, F.R.G.) under the tradename Monospher. The surface area of this silica matrix is 2.5 $m^2 g^{-1}$ calculated with the BET III equation using nitrogen as adsorbate[11]. This value is higher than the theoretically calculated geometrical surface area (1.38 $m^2 g^{-1}$) assuming a true solid density of 2.2 $g ml^{-1}$. The concentration of silanol groups on the surface is *ca.* 8 $\mu mol\ m^{-2}$ evaluated by the method from Holík and Matějková[12] using ^1H NMR spectroscopy.

Chemicals

3-Aminopropyltriethoxysilane (APS) and 3-mercaptopropyltrimethoxysilane (MPS) were obtained from ECA (Steinheim, F.R.G.). Glycidoxypropyltrimethoxysilane (Glymo) was obtained from Ventron. Procion Red MX5B and Procion Red HE3B were a gift from ICI (Melbourne, Australia). Cibacron Blue F3GA was obtained from Serva (Heidelberg, F.R.G.). Rabbit muscle dehydrogenase, streptokinase and pig heart malate dehydrogenase, lithium lactate, oxalacetate, sodium

malate, sodium pyruvate, NAD, NADH, and NADPH were obtained from Sigma (Sydney, Australia). Cohn Fraction III from human blood was a gift from the Commonwealth Serum Laboratories (Melbourne, Australia).

Preparation of activated silicas

All silicas were dehydrated and activated under reduced pressure at 10 Pa and 433 K for 12 h. The reaction procedure was carried out after cooling the silica to room temperature. In case of derivatisation of the silica in toluene, dry (sodium wire) and freshly distilled solvent was added to the silica under vacuum, in order to allow the solvent to wet completely the surface structure of the non-porous particles. In the case of chemical derivatisation of the silica in water, the suspension was held under vacuum (10 Pa) and sonicated in order to achieve solvent penetation of the surface. The silane was added to the suspension and the reaction carried out as described below.

Aminopropyl-silica. 0.19 g 3-aminopropyltriethoxysilane was added to 20 g silica suspended in 80 ml toluene. The reaction mixture was heated under reflux for 24 h under anhydrous conditions. The modified silica was suspended and centrifuged (8000 *g*) several times in toluene and then in chloroform in order to remove reaction components. The derivatised silica was stored under anhydrous conditions until used.

Diol-silica. An amount of 0.42 g glycidoxypropyltrimethoxysilane was added to 20 g silica suspended in 80 ml water pH 3.5 (adjusted with 0.1 *M* nitric acid). The reaction vessel was evacuated to 10 Pa, sonicated in an ultrasonic bath for 10 min and then heated to 363 K for 3 h with stirring essentially as described by Regnier and Noel[13]. After cooling, the derivatised silica suspension was neutralized by suspension in water and centrifuged (8000 *g*) several times. Subsequently the diol-silica was suspended and centrifuged 3 times in toluene and chloroform, in that order.

Mercaptopropyl-silica. An amount of 0.34 g 3-mercaptopropyltrimethoxysilane was added to 20 g silica suspended in 80 ml water pH 3.5 (adjusted with 0.1 *M* nitric acid). The reaction conditions and washing procedure were the same as described above.

Immobilization of triazine dyes

The modified silicas with immobilized diol-, mercapto-, or amino-group functionalities were suspended in 100 m*M* sodium carbonate, pH 8, containing the triazine dye according to a procedure described by Small *et al.*[14] for agarose based gels. The reaction was carried out at both 295 K and 333 K. The immobilized dye matrices were washed by centrifugation in an Eppendorf centrifuge and suspended in 50 m*M* sodium phosphate pH 7.0 several times until the washing supernatants were colorless.

Preparation of cell-free rat brain extracts

This extract was prepared by homogenization of rat brain, followed by centrifugation of the homogenate at 100 000 *g* for 1 h. The supernatant was then subjected to fractionation with solid ammonium sulphate to a final concentration of 4.5 *M* at 277 K. Resolved fractions were dialysed overnight against 10 m*M* sodium phosphate pH 8.0 at 277 K. Insoluble proteins were removed prior to injection by filtration through 0.22 μm sterile filters. The protein concentration in the soluble fractions was ca. 2 mg ml^{-1} as determined by UV adsorption at 280 nm. The solution was injected on the column without further preparation.

Preparation of Cohn III solution

An amount of 100 mg freeze dried Cohn Fraction III was dissolved in 2 ml 0.1 *M* sodium phosphate pH 7.0, centrifuged at 10 000 *g* and filtered through a 0.22-μm filter. This solution was diluted 10 times to a final concentration of about 2 mg ml^{-1} protein with 10 m*M* sodium phosphate (5 ml), in order to decrease the salt concentration prior to loading directly onto the dye affinity columns.

Lactate dehydrogenase (LDH) assay[15]

The reacion mixture contained the following reagents in a total volume of 3 ml: 2.83 ml 0.1 *M* sodium phosphate pH 7.0, 0.1 ml sodium pyruvate (2.5 mg ml^{-1}), 0.05 ml sodium NADH (10 mg ml^{-1}), 0.02 ml of the enzyme diluted in the buffer. The reaction was initiated by the addition of the enzyme solution to the cuvette and monitored continuously at 340 nm at room temperature using a Varian UV spectrophotometer.

Malate dehydrogenase (MDH) assay[15]

The reaction mixture contained the reagents following in a total volume of 3 ml: 2.83 ml 0.1 *M* sodium phosphate pH 7.5, 0.1 ml oxalacetate (2 mg ml^{-1}), 0.05 ml sodium NADH (10 mg ml^{-1}), 0.02 ml of the enzyme diluted in the buffer. The reaction was initiated as described above for the LDH assay and monitored at the same conditions.

Aldehyde reductase assay

The assay for aldehyde reductase was based on the method described by Turner and Hryszko[16]. In brief, 2.5 mg NADPH and 3.85 mg pyridine-3-aldehyde were dissolved in 20 ml 0.1 *M* sodium phosphate, pH 7.2. The reaction was initiated by adding 0.03 ml of control or test samples to 2.97 ml of the mixture described above and monitored at room temperature at 340 nm using a Varian UV spectrophotometer.

Thrombin, kallikrein and plasminogen microassay

Assays for these enzyme activities were performed at 310 K in 50 m*M* Tris–HCl, 50 m*M* sodium chloride, pH 7.5. The chromogenic substrates S 2238, S 2302, and S 2251 (Kabi Vitrum, Stockholm, Sweden) were used to measure thrombin, kallikrein and plasminogen activities respectively[17]. Assays were carried out as a microassay technique, using procedures developed in these laboratories[18], in microtitre plates with 25 μl sample, 25 μl of 1 m*M* substrate solution and 50 μl of buffer. In the case of the plasminogen assay 5 μl of Streptokinase (10 000 units ml^{-1}) and 45 μl of buffer were added to 25 μl of sample and incubated for 10 min in order to activate the plasminogen. Substrate was added to initiate the reaction. The rate of *p*-nitroaniline release was determined by measuring the change in optical absorbance at 405 nm (A_{405}) using a Titretech Multiscan reader.

Chromatographic procedures

Chromatographic and column packing was carried out using a Beckman 344 HPLC system. All triazine immobilized silicas were packed into stainless-steel columns (40 mm × 6 mm I.D.) containing sintered metal frits with 0.5 μm pore sizes. The columns were filled using the downward slurry technique in 2-propanol–toluene (2:3,

v/v) at constant flow-rate. During column packing the maximum pressure reached was 40 MPa. All columns were equilibrated in 10 m*M* sodium phosphate, pH 7.0, containing 0.5 *M* sodium chloride. To avoid the possibility of protein adsorption on the stainless-steel frits, the column surface and the affinity silica matrix, a solution of bovine serum albumin (BSA) (1 mg ml^{-1}) in buffer, pH 7.0, was continuously pumped through the column overnight in order to saturate these binding sites.

On-line monitoring of enzyme activities

When specific elution was carried out with substrate and cofactor, enzyme elution was monitored at 340 nm. An increase in absorbance at 340 nm indicated the presence of the eluted enzyme due to the conversion of NADH to NAD.

RESULTS

Three different reactive dyes (Procion Red HE3B, Procion Red MX5B and Cibacron Blue F3GA) were immobilized onto 2.1 μm non-porous silica chemically modified with either APS, MPS or diol ligands. To examine the influence of temperature on dye immobilization, parallel coupling reactions were carried out at 296 K and 333 K. A porous silica phase, the Merck Si 60 diol with a particle size of 20–30 μm, was used to compare the immobilization yields and binding capacities of porous and non-porous supports. The amount of silica-based immobilized dye of stationary phases with nominally equivalent surface area per ml gel (2.5 $m^2 g^{-1}$) was estimated by measuring the UV absorption at 615 nm for Cibacron Blue F3GA and 546 nm for the Procion Red dyes before and after coupling to the activated silica supports. Measured ligand densities were higher when the reaction was carried out at 333 K rather than 295 K. All subsequent immobilization procedures for the chromatographic matrices were carried out at the higher temperature. Ligand content was highest with the APS-modified silica (Table I). However, the dye–silica linkage was not stable and continuous leaching of dyes from the column occurred when using the APS-modified silica support. Low ligand densities were observed when dyes were immobilized to the diol-modified silica (Table I). Similar results were obtained when using commercially available Merck diol-silica Si 60 and dye immobilization procedures described in the

TABLE I

AMOUNT OF DYES BOUND AT 333 K TO NON-POROUS SILICAS WITH DIFFERENT FUNCTIONALITIES

Functionality	*Procion Red MX5B* (μg ml^{-1})	*Procion Red HE3B* (μg ml^{-1})
Aminopropyl $-(CH_2)_3-NH_2$	1200	920
Mercaptopropyl $-(CH_2)_3-SH$	630	300
Diol $-(CH_2)_3-CH_2-\underset{\displaystyle OH}{\underset{\vert}{CH}}-\underset{\displaystyle OH}{\underset{\vert}{CH_2}}$	<100	<100

experimental section of this paper or the immobilization procedure of Small *et al.*[14]. This finding was surprising since it is not concordant with the reported observations of the Lund workers. Immobilization of dye to MPS-modified silicas resulted in a stable dye–silica linkage with no leaching of dye evident after washing with 2500 column volumes of buffer (10 m*M* sodium phosphate, 500 m*M* sodium chloride, pH 7.2). All studies described below were consequently done using dye immobilized to MPS-modified silicas.

From preliminary experiments it was apparent that careful selection of the equilibration buffer (10 m*M* sodium phosphate pH 8.0) was necessary for the retention of the dehydrogenases LDH and MDH to the dye affinity supports. With buffers at or below pH 7.0, non-specific interaction between most proteins from the tissue extract and the immobilized dyes was evident. In these lower pH cases these proteins were indiscriminately bound in the adsorption step. Furthermore the amount of adsorbed enzyme was very sensitive to changes in salt concentration of the loading buffer. Salt concentration, larger than 20 m*M* at pH 8.0 decreased the amount of bound enzyme. Sensitivity of sample loadability to changing salt concentration has previously been shown to be much less responsive with porous particles of surface areas $> 50\ m^2\ g^{-1}$ and lower pore diameter. The sensitivity of loadability to varying salt concentrations reflects the relatively low association constants of the solute–dye affinity complexes compared to other protein ligand affinity complexes[19].

Using partial purified enzyme fractions, rabbit muscle LDH and pig heart MDH

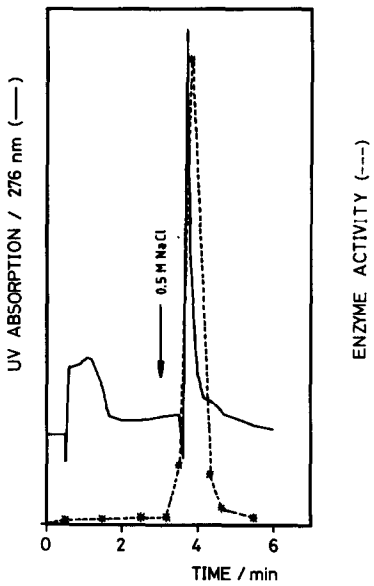

Fig. 1. Elution of rabbit muscle lactate dehydrogenase (LDH) on Procion Red MX5B immobilized on non-porous silica. Injection volume, 1 ml; sample concentration, 40 $\mu g\ ml^{-1}$ in 10 m*M* sodium phosphate pH 8.0 + 8 m*M* ammonium sulphate (from storage solution); temperature, ambient, flow-rate, 1 ml min^{-1}; column back pressure, 7.8 MPa; detector, 276 nm; 0.05 a.u.f.s.; eluent (a) 10 m*M* sodium phosphate pH 8.0 (b) 0.5 *M* sodium chloride in (a), step gradient as indicated in chromatogram.

both adsorbed to and could be eluted from all three dye columns (Cibacron Blue F3GA, Procion Red HE3B and Procion Red MX5B) using 500 m*M* sodium chloride (Fig. 1). Specific elution of a mixture of the two enzymes from Cibacron Blue was achieved using 2 m*M* NADH and 2 m*M* lactate (LDH) or malate (MDH) in 10 m*M* phosphate buffer pH 8.0 (Fig. 2) but could not be achieved with NADH alone. This suggests LDH and MDH were bound to Cibacron Blue F3GA at both the substrate and cofactor binding sites[20]. Elution from both the Red dye columns was achieved in the same buffer with 2 m*M* NADH alone.

Aldehyde reductase, LDH and MDH from rat brain mitochondria all bound strongly to Cibacron Blue F3GA column. Rat brain mitochondrial LDH and MDH could be specifically eluted with 2 m*M* NADH and 2 m*M* lactate (LDH) or 2 m*M* malate (MDH). In particular, aldehyde reductase could be specifically eluted with 2 m*M* NADPH and 2-pyridine-aldehyde (Fig. 3). Other unrelated proteins adsorbed to the dye columns were eluted using a salt gradient from 0–500 m*M* sodium chloride. Specific elution of all enzymes could not be achieved from the Procion Red columns under the conditions described above.

Using Procion Red HE3B non-porous silica partial purification of plasminogen was also achieved directly from human Cohn Fraction III (Fig. 4). Most Cohn fraction III proteins did not bind to this matrix and eluted in the breakthrough peak. Plasminogen was bound and eluted with a step gradient of 500 m*M* sodium chloride. Since Cohn Fraction III contains a number of other protease activities, the binding behaviour of these enzyme activities was monitored using the chromogenic substrates

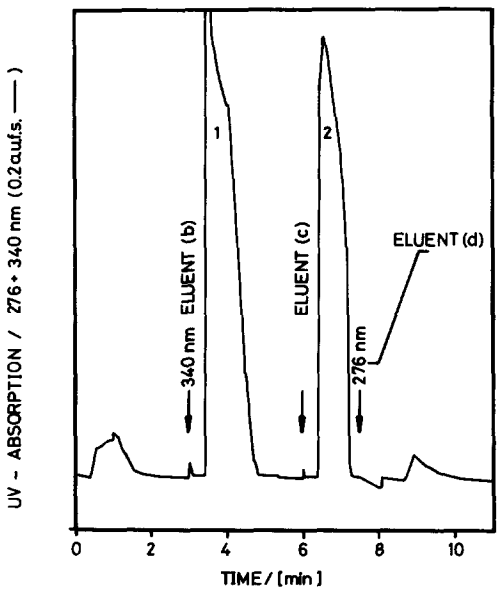

Fig. 2. Affinity elution with substrate and cofactor on Cibacron Blue F3GA immobilized non-porous silica. Injection volume, 1 ml; sample concentration, LDH (40 μg ml^{-1}) MDH (40 μg ml^{-1}) in (a); eluent (a) 10 m*M* sodium phosphate pH 8.0 (b), 2 m*M* NADH-Na + 2 m*M* lactate, (c) 2 m*M* NADH + 2 m*M* malate, (d) 0.5 *M* sodium chloride in (a); temperature, ambient: flow-rate, 1 ml min^{-1}; column back pressure, 8.5 MPa; detector, 276 nm and 340 nm; 0.2 a.u.f.s. as indicated with arrows. (1) LDH activity, (2) MDH activity.

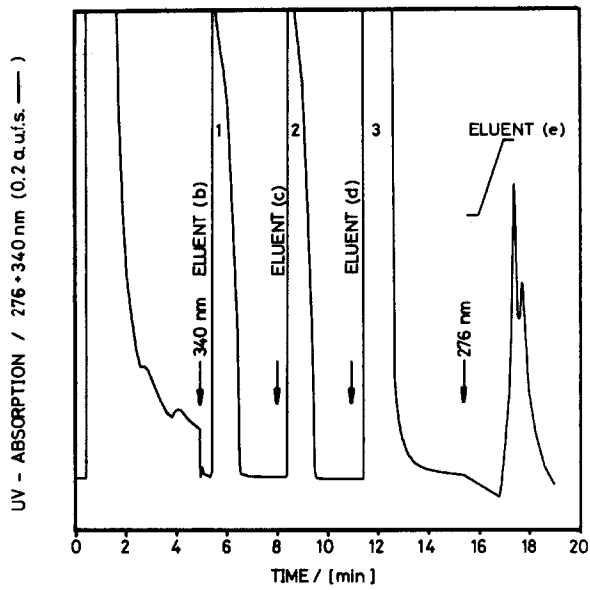

Fig. 3. Affinity elution of LDH, MDH and aldehyde reductase from rat brain mitochondria with substrate and cofactor on Cibacron Blue F3GA immobilized non-porous silica. Injection volume, 1 ml; sample concentration (>2 mg ml^{-1}) in (a); eluent (a) 10 m*M* sodium phosphate pH 8.0, (b) 2 m*M* NADH-Na + 2 m*M* lactate, (c) 2 m*M* NADH-Na + 2 m*M* malate, (d) 2 m*M* NADPH + 2 m*M* pyridine-3-aldehyde, (e) 0.5 *M* sodium chloride, eluent b, c, d, e in (a); temperature, ambient; flow-rate, 1 ml min^{-1}; pressure, 8.5 MPa; detector, 276 nm and 340 nm; 0.2 a.u f.s. as indicated with arrows. (1) LDH activity, (2) MDH activity, (3) aldehyde reductase activity.

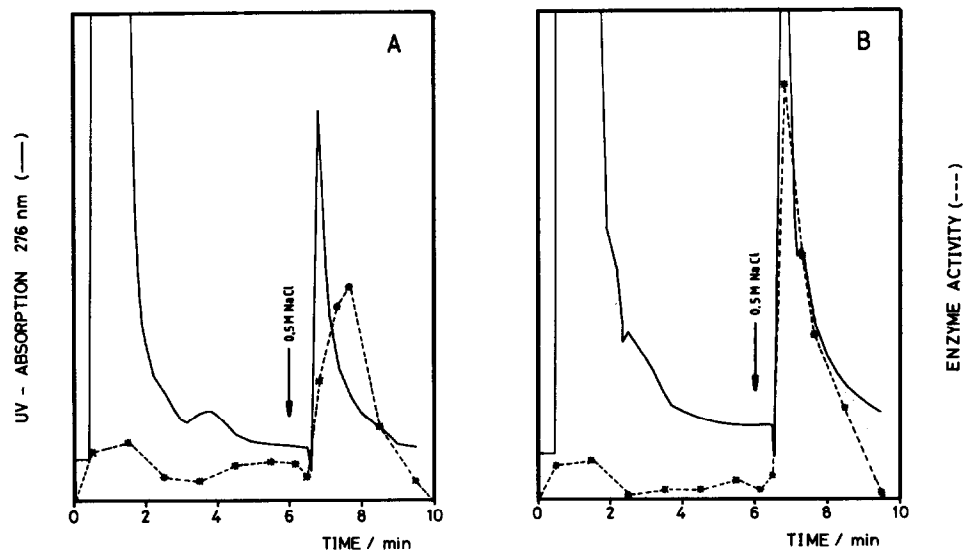

Fig. 4. Elution of plasminogen with a salt gradient on Procion Red HE3B (A) and Cibacron Blue F3GA (B) immobilized on non-porous silica. Injection volume, 1 ml; sample concentration, >2 mg ml^{-1} in 27 m*M* sodium phosphate pH 7.5; eluents (a) 10 m*M* sodium phosphate pH 7.5 (b) 0.5 *M* sodium chloride in (a); temperature, ambient; flow-rate, 1 ml min^{-1}; detector, 276 nm; 0.05 a.u.f.s.

S 2302 (kallikrein like activity) and S 2238 (thrombin like activity). Both these activities were resolved from plasminogen using this system. Higher selectivity of Procion Red HE3B for plasminogen as the silica based matrix was demonstrated and in agreement of previous findings using immobilized Procion Red HE3B-agarose[21]. Resolution of these components using Procion Red MX5B and Cibacron Blue F3GA column was also examined and similar results were obtained.

DISCUSSION

This study documents the use of non-porous monodisperse silica beads in biomimetic affinity chromatography with immobilized reactive dyes. Previously Janzen *et al.*[22] and Jilge *et al.*[23] have demonstrated the use of these particles in reversed-phase chromatography and in hydrophobic interaction chromatography. As evident from the present study the low surface area of the 2.1-μm non-porous silica particle is not a critical parameter in micropreparative separation of proteins by biomimetic affinity chromatography. High loadings in the range $>$ 0.6 nmol protein per ml immobilized dye support can in fact be achieved at low salt concentrations (10 m*M*) in the loading buffer. The necessity for high accessibility of the immobilized ligand is important in case of expensive ligands like monoclonal antibodies and other biospecific or biomimetic ligands. The capacity and stability of these new silica based materials is sufficiently higher to permit very rapid analytical and micropreparative HPLC applications. Despite the small particle size the back pressure of laboratory scale columns is in the normal operational range (8–10 MPa) with the consequence that the current generation of HPLC equipment can handle both the flow-rate and back pressure requirements.

Using the MPS immobilization chemistry introduced in this paper, immobilization of reactive dyes to silica supports can be achieved in a two step procedure. Thus microparticulate non-porous MPS silica can be used to exploit a wide variety of dye selectivities. Such support matrices may find wide applications in the rapid laboratory scale preparation of enzymes from crude extracts or in the monitoring and control of large scale bioprocess purifications involving dye affinity systems.

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