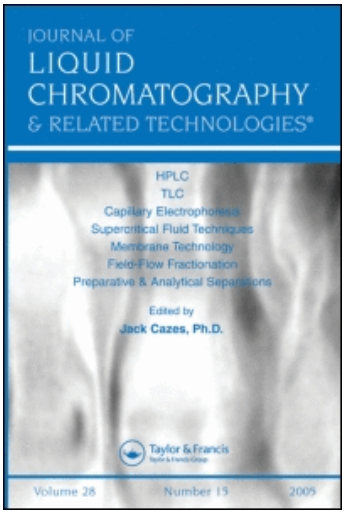

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Immobilized Porphyrins as Versatile Stationary Phases in Liquid Chromatography

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IMMOBILIZED PORPHYRINS AS VERSATILE STATIONARY PHASES IN LIQUID CHROMATOGRAPHY

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ABSTRACT

The preparation, characterization and potential liquid chromatographic applications of various porphyrin-silica stationary phases are reviewed. These new phases are synthesized by covalently linking unsymmetrical tetraphenylporphyrin (TPP) derivatives (monocarboxyl- or monohydroxyl-), as well as native protoporphyrin IX (ProP) to appropriately derivatized silica support matrices. The porphyrin-silicas may be further modified by metallation with a wide range of central metal ions (e.g., Cu^{2+}, Zn^{2+}, Ni^{2+}, Fe^{+3}, In^{3+}, Sn^{4+}, etc.) by refluxing in the presence of metal ion salts. Columns packed with either metallated or unmetallated materials exhibit exceptionally high shape selectivity in the separation of polycyclic aromatic hydrocarbons (PAHs) and fullerenes owing to

strong π-π interactions between such solutes and the immobilized porphyrin structures. Columns packed with metallated porphyrins, particularly In(III) and Sn(IV), may also be used for separation of anions, including aromatic carboxylates and sulfonates, via selective ligation reactions with the metal centers. Similar coordination chemistry can be exploited to achieve selective peptide separations through a combination of specific metal ion affinity reactions of certain amino acids (histidine, tryptophan) with given metal ion centers (i.e., Fe^{+3}, Cu^{+2}) and concomitant π-π interactions of aromatic amino acids with the immobilized conjugated macrocycle. In the area of peptide/protein separations, the metalloporphyrin-silicas may offer an attractive alternative to current immobilized metal ion affinity phases (IMAC), because of their exceptionally strong metal ion binding constants. This allows the columns to provide reproducible analytical and preparative separations without potential for metal ion contamination of the purified materials.

INTRODUCTION

The development of new stationary phases that offer unique selectivities above and beyond those afforded by conventional ODS, phenyl, ion-exchange and size-exclusion columns, represents a challenging yet very important avenue in liquid chromatography research. For example, significant effort has been placed on creating phases that can be used to preferentially retain planar polycyclic aromatic hydrocarbons (PAHs) over their less toxic non-planar homologues[1-6] so that greater resolution and hence better quantitation of these species can be achieved. In the area of anion-exchange chromatography, researchers have explored the use of different quaternary ammonium structures, varying lengths of spacer groups, and the principles of ligand exchange as a means of developing columns that offer greater selectivity.[7-11] The use of immobilized metal ions as stationary phases has also been examined with respect to selective separation of peptides and proteins, where the content of certain amino acids within the peptide/protein structure that interact strongly with the immobilized metal ion (e.g., histidine) dictates retention behavior. More recently, a number of researchers have synthesized novel stationary phases (e.g., pyrenyl(ethyl)-silica, tri(dinitrophenyl)-silica, etc.) for the specific purpose of purifying closely related fullerene structures, a rather difficult task, especially when attempting to purify large quantities of these species using mobile phases in which the fullerenes are most soluble.[12-30]

In the examples cited above, columns with radically different immobilized chemical structures have been required to achieve some level of selectivity (for the solutes mentioned) above and beyond that provided by conventional "off the shelf" stationary phases. Hence, packings designed for fullerene separations (e.g., tri(dinitrophenyl)-silica) are not likely to be useful for anion chromatography. Similarly, it is improbable that metal ion affinity columns previously reported for peptide and protein separations (metal ions tethered on immobilized iminodiacetate ligands)[31,32] could also be employed for PAH or fullerene separations. An interesting question then is whether specific chemical structures exist that when immobilized on LC supports (notably silica) could offer practical selectivities for a wide range of solute types simply by varying the operating mobile phase conditions.

One class of chemical structures that would appear to offer many possible modes of potential solute interaction for separation science is the porphyrins. Indeed, immobilized metalloporphyrins have been used previously in the development of gas and anion selective sensors, where selective coordination of the target gas/ion with a given central metal ion can result in changes in membrane potentials or optical properties of polymer films containing the immobilized porphyrin species.[33-35] The application of immobilized metalloporphyrins for batch separations has also been explored. Kokufuta *et al.* demonstrated that polystyrene particles possessing immobilized molybdenum porphyrins can be used for selective extraction of phosphate ions.[36] However, until recently, there have been no reports describing the use of immobilized porphyrins as stationary phases in HPLC systems. Herein, we summarize recently published,[37-42] as well as unpublished results regarding the preparation, characterization, and performance of various porphyrin and metalloporphyrin-silica phases. It will be shown that such phases offer unique versatility and selectivity for a wide range of liquid chromatography separations, ranging from highly shape selective separations of PAHs to reproducible separations of peptides via metal-affinity and π-π interactions.

EXPERIMENTAL

Equipment

The primary HPLC system used in these studies was composed of a Spectra Physics (San Jose, CA) SP 8700 solvent delivery system, a Spectra Physics SP 4290 computing integrator, a Kratos (Ramsey, NJ) Spectroflow 773 variable-wavelength UV-Vis detector, and a Rheodyne (Cotati, CA) Model 7010 sample valve equipped with a 20 µL sample loop. Columns were

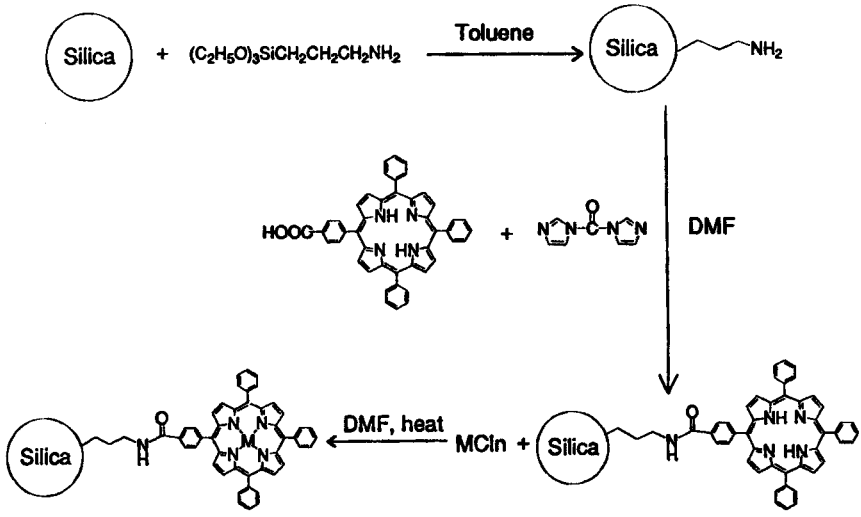

Figure 1. Synthetic scheme used to prepare metallated pCPTPP-silica.

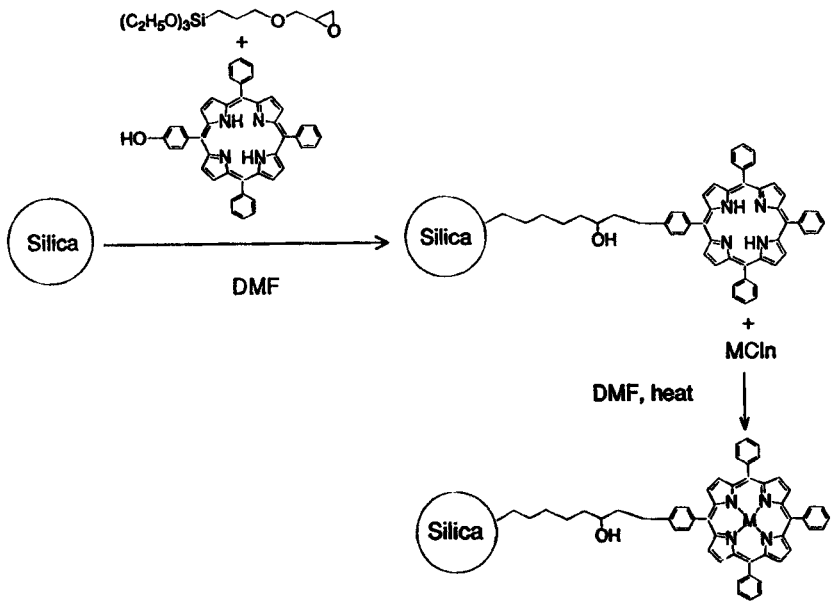

Figure 2. Synthetic scheme used to prepare metallated pHPTPP-silica.

thermostated using a Fisher Scientific water jacket (Pittsburgh, PA) connected to a Fisher Scientific Model 80 Isotemp constant temperature circulator. A second HPLC system consisting of a LKB Bromma (Piscataway, NJ) Model 2150 pump, a Hewlett Packard (Avondale, PA) 3396A computing integrator, a Rheodyne model 7125 injection valve with a 20 μL loop, and either a LKB Bromma Model 2238 Uvicord SII detector or an Anspec (Ann Arbor, MI) SM95 UV-Vis detector was also used for a portion of these studies.

Preparation of the Porphyrin-Silica Stationary Phases

Tetraphenylporphyrin (TPP) and protoporphyrin (ProP) structures were immobilized onto silica particles (10 μm) using the reaction schemes illustrated in Figures 1-3. The [5-(p-carboxyphenyl)-10,15,20-triphenyl] porphyrin (pCPTPP) based stationary phase (Figure 1) was prepared by first synthesizing the unsymmetrical monocarboxyltetraphenylporphyrin via a mixed aldehyde/pyrrole condensation reaction and then immobilizing this species covalently on aminopropyl silica gel using a carbonyldiimidazole reaction to yield a relatively stable amide linkage.[38] Briefly, the method consists of preparing amine derivatized silica gel by refluxing the silica in an aminopropyltriethoxysilane/toluene solution for 4h. The pCPTPP is then attached by activating the carboxylic acid with 1,1'-carbonyldiimidazole in dimethylformamide (DMF). The washed and dried aminated silica gel is then added with coupling being completed after 24 h. Residual amine sites on the silica gel are then acetylated by refluxing in acetic anhydride for 1.5 h.

A second route to preparation of a tetraphenylporphyrin-silica phase, and one still under study, is based on first reacting an unsymmetrical monohydroxy-TPP species (5-(p-hydroxyphenyl)-10,15,20-triphenyl] porphyrin (pHPTPP) with glycidoxypropyltrimethoxysilane (GPTS) to form a covalent ether linkage, followed by reaction of the trimethoxysilane terminal group with the surface silanols of the silica gel (Figure 2). Approximately 1.80 g of pHPTPP was added to 50 mL of anhydrous N,N-dimethylformamide (DMF). The phenol group of the porphyrin was reacted with 500 μL of GPTS in the presence of 50 μL of the catalyst[43] tri-n-butylamine by refluxing for 4 h. Porphyrin immobilization was carried out by the addition of 2 g of 10 μm, 100Å silica (Machery-Nagel, Düren, Germany) and refluxing for 4 h, followed by shaking for 16 h. The pHPTPP-silica solution was then filtered through a 10-15 ASTM sintered glass funnel and washed with subsequent aliquots of 50 mL dry chloroform, acetone, 10% acetic acid in acetone (v/v) and 50 additional mL of acetone.

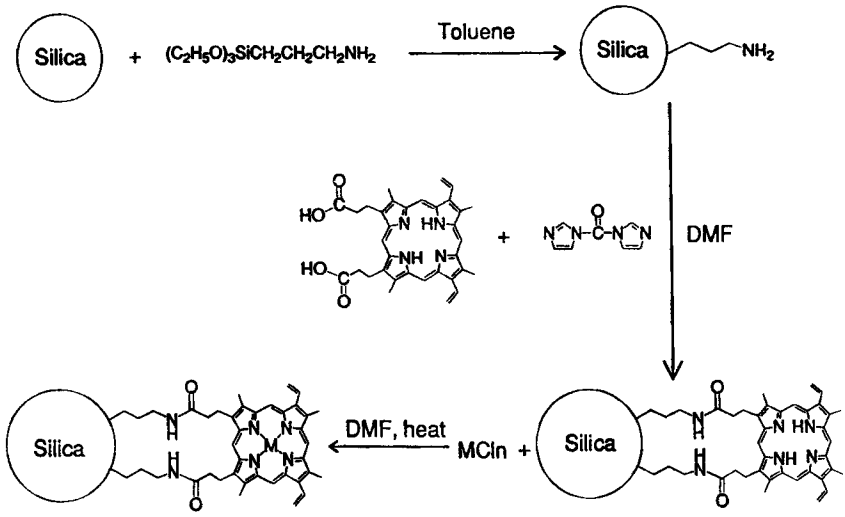

Figure 3. Synthetic scheme used to prepare MProP-silica.

The ProP-silica phase was prepared by simply activating the native carboxyl groups of protoporphyrin IX with carbonyldiimidazole, and then allowing the activated species to react with aminopropyl silica to form amide bonds at either one or both original carboxyl groups of the porphyrin structure (Figure 3). The specific conditions for this reaction are summarized in detail elsewhere.[44]

In general, metallation of all porphyrin-silicas structures can be accomplished by refluxing the porphyrin silica gel with the chloride salt of the metal in DMF for 4 h. Metallation can be confirmed by taking the UV-Vis spectra of the silica phase after dissolution in concentrated NaOH.[38]

All porphyrin-silica phases were packed by the downfill slurry method[45] under a pressure of 6000 psi into either 100 mm x 4.6 mm or 250 mm x 4.6 mm stainless steel columns using 70% 2-propanol/ 30% methanol (v/v) as the packing solvent.

The dead volume (V_0) for all columns was measured by injection of a approximately 0.5% (v/v) carbon disulfide (CS_2) solution prepared in the mobile phase.

Materials

HPLC grade toluene, *p*-xylene, and carbon disulfide as well as analytical grade chlorobenzene were obtained from Aldrich (Milwaukee, WI). Various amino acids and peptides tested as solutes were products of Sigma Chemical (St. Louis, MO). The PAHs studied were reagent grade or better and purchased from Aldrich with the exception of α,α′ -binapthyl which was obtained from ICN (Irvine, CA). The fullerene samples were produced via a carbon arc method as described elsewhere.[46,47]

RESULTS AND DISCUSSION

Preparation and Characterization of Porphyrin-Silica Phases

As illustrated in Figures 1-3, stationary phases consisting of covalently bound metalloporphyrins are prepared in essentially a two step process. The first step involves covalent attachment of the given porphyrin structure to a porous silica. The second step consists of inserting the metal ion of choice into the center of the immobilized porphyrin via refluxing in dimethylformamide containing high concentrations (e.g., 0.1 M) of metal ion salts. Immobilization of TPP structures based on either an amide (Figure 1) or ether (Figure 2) linkage have been developed. The ether linkage method, developed to simplify preparation and improve column stability, relies upon reaction of an epoxide with an monohydroxy-TPP derivative. The immobilization of ProP on silica (Figure 3) provides a means to readily assess the influence of the four phenyl groups orthogonal to the porphine ring of TPP on the observed retention behavior of given solutes (by comparison of retention on TPP- vs. ProP-silicas). At the same time, the ProP-silicas offer their own unique selectivities with respect to PAH and peptide retention (see below).

Preparation of all the porphyrin-silicas must be performed under rigorous anhydrous conditions in order to achieve reasonable yields of porphyrin surface coverage. Typically, elemental analysis (CHN) of resulting silica packings yields coverages for the TPP-silica stationary phases of around 0.4 μmol/m^2, which is approximately 10% of the total available amine sites. Preliminary experiments suggest that a significant increase in TPP coverage can be achieved (to about 0.8 μmol/m^2) using the synthetic route (Figure 2) that involves coupling the monohydroxy-TPP directly to the silica support via the glycidoxypropyltrimethoxysilane reagent. In the case of less sterically hindered ProP, coverages are much higher, generally about 2 μmol/m^2, approximately 50% of the available amine sites.

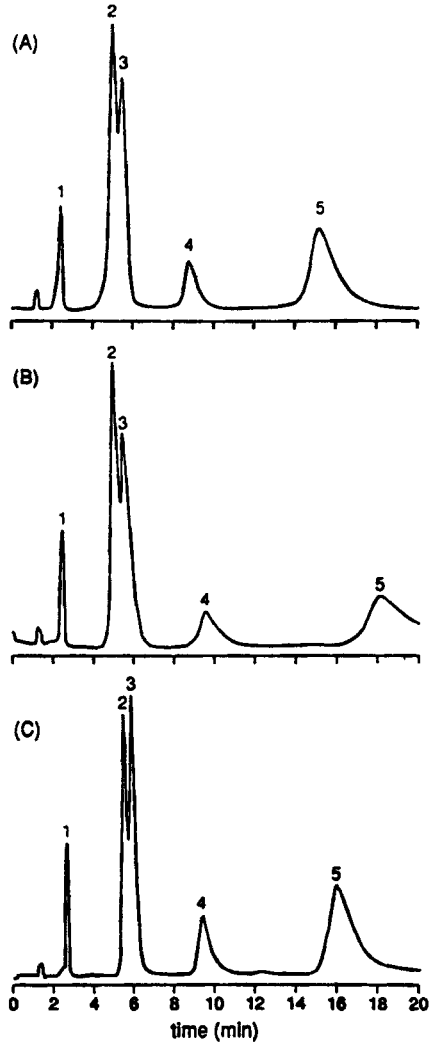

Figure 4. Typical separation of five PAHs on three different 100 mm x 4.6 mm columns packed with (A) H_2TPP-silica, (B) InTPP-silica, and (C) SnTPP-silica. Peak identity: (1) naphthalene, (2) phenanthrene, (3) anthracene, (4) pyrene, (5) chrysene. Conditions: mobile phase: 70% MeOH/30% H_2O; flow rate: 1.0 mL/min; column temperature: 25°C; detection: 254 nm (0.100 AUFS). (Adapted from Ref. 37 with permission)

Table 1

Selectivity Factors of PAH Pairs on Various Porphyrin Stationary Phases

Selectivity Factors	Stationary Phase			
	H_2TPP^a	$InTPP^a$	$SnTPP^a$	H_2ProP^b
α triphenylene/0-terphenyl	3.1	4.3	3.0	39.2
α perylene/α,α'-binapthyl	3.9	6.4	3.4	36.5
α chrysene/pyrene	1.6	1.8	1.6	2.1

[a] Conditions shown in Figure 5 legend.
[b] Conditions shown in Figure 6 legend.

Separation of Polycyclic Aromatic Hydrocarbons

The presence of a large planar aromatic system within the immobilized porphyrin structures (in the form of the porphine ring) suggests the potential application of such phases for the separation of PAHs. The ability to separate PAHs with shape selectivity for planar over non-planar forms has attracted considerable attention over recent years owing the obvious environmental significance of these compounds.[1-6] At present, polymeric ODS type phases offer a much higher degree of shape selectivity when compared to conventional monomeric ODS reversed phase columns.[1]

Figure 4 shows the typical separation of a mixture of PAHs on 10 cm columns packed with H_2TPP-, InTPP- and SnTPP-silica phases. Figure 5 summarizes the capacity factors (k') observed for a wide range of PAHs on the same three TPP-silica columns (H_2TPP-, InTPP- and SnTPP-silica). Several aromatic pairs, i.e. triphenylene/o-terphenyl, perylene/α,α'-binapthyl and chrysene/pyrene have been suggested previously as probes for assessing shape selectivity of new stationary phases.[4] Table 1 summarizes the selectivity factors for these pairs on the three TPP-silica columns. On a typical monomeric ODS stationary phase the selectivity factor ($\alpha_{triphenylene/o\text{-}terphenyl}$) ranges from 1.0 to 1.7,[6] while the value of a polymeric ODS phase lies between 2.0 and 2.7.[2] However, on the various TPP-silica phases, these values are all above 3.0. Furthermore, when the number of double bonds of the unsubstituted PAHs are plotted vs. log k' on the columns packed with TPP-silicas, a linear relationship

(not shown here) is observed suggesting that retention of the PAHs on these new columns is due primarily to a π-π interaction between immobilized TPP and the PAHs.

As shown in Figure 6 and Table 1, the retention behavior of various PAHs on columns packed with the ProP-silica phase reveals an even higher degree of shape selectivity. Indeed, the retention of PAHs on unmetallated ProP-silica columns is so strong that 100% acetonitrile must be used as the mobile phase to elute these solutes in a reasonable time frame (compared to 60% acetonitrile/40% water used for the TPP-silica work described above). There appears to be at least two factors responsible for this greatly enhanced interaction strength: first is the much greater surface coverage of porphyrin on the silica of the ProP-silicas *vs.* TPP-silicas (2-3 *vs.* 0.4 μmol/m^2); second is the potential for less steric hindrance for the interaction of PAHs with immobilized ProP compared to immobilized TPP. Indeed, for TPP, larger planar PAHs may be precluded from forming tight π-π complexes with the planar porphine ring due to the steric hindrance of the four phenyl rings perpendicular to the porphine plane. Preliminary fluorescence titration experiments (not shown here) do in fact reveal that ProP forms a charge transfer complex with perylene in acetone, while TPP does not.[48] As shown in Table 1, the much stronger interaction of the larger planar PAHs with immobilized ProP yields extraordinary shape selectivity for the three solute pairs examined.

It should be noted that retention behavior of PAHs on TPP- and ProP-silicas is not particularly sensitive to whether there is a metal ion in the center of the immobilized porphyrin or not (see Figure 4). Indeed, when the metal ion center is either in the +2 or +3 oxidation state, at least one side of the metal ion-porphine ring complex is accessible for direct interaction with neutral PAHs via π-π interaction. However, as shown in Figure 4, even when the metal ion is in the +4 oxidation state, such as Sn(IV), there is still significant interaction with the PAH solutes. Since the PAH separations are carried out under reversed phase conditions, it is likely that relatively small hydroxide anions serve as the ligands for the metal centers, and these are not large enough to block PAH π-π interactions with the planar portion of the porphyrin structure.

Separation of Fullerenes on TPP-Silicas

Since Krätschmer et al. published a method to produce fullerenes in macroscopic quantities in 1990,[49] there have been numerous reports describing new approaches to efficiently separate these novel allotropes of carbon via

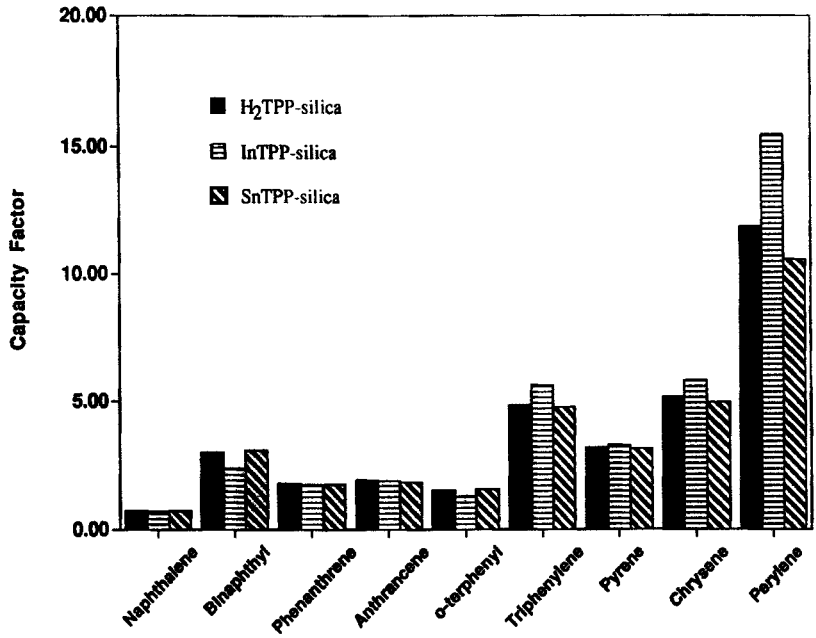

Figure 5. Capacity factors (k') of various PAHs on 100 mm x 4.6 mm columns packed with H_2TPP-silica, InTPP-silica, and SnTPP-silica columns. Conditions: mobile phase: 80% MeOH/20% H_2O; flow rate: 1.0 mL/min.; column temperature: 25°C; detection: 254 nm (0.100 AUFS).

HPLC methods. While separation of the various fullerenes using relatively weak mobile phases (e.g., hexane) is not particularly difficult on conventional columns, such as ODS phases, efforts to isolate much larger quantities of fullerenes for fundamental and applied studies requires purification with much stronger solvents. For example, the solubility of C_{60} is only 0.043 mg/mL in hexane, but 2.8 mg/mL in toluene, and 7.9 mg/mL in carbon disulfide.[28] Hence, columns that can operate using toluene or even CS_2 as mobile phases would provide the most efficient means of separating individual fullerenes on a preparative scale.

Supports with π-acidic character have shown to be well-suited for the separation of fullerenes. The tri(dinitrophenyl)-silica or "Buckyclutcher" phase developed by Welch and Pirkle,[13] in particular, was one of the first LC stationary phases to demonstrate reasonable selectivity for C_{60} and C_{70} separations ($\alpha_{C70/C60}$ = 1.5 in toluene). Kimata et al. have further demonstrated good selectivity in the separation of a variety of fullerene isomers with columns

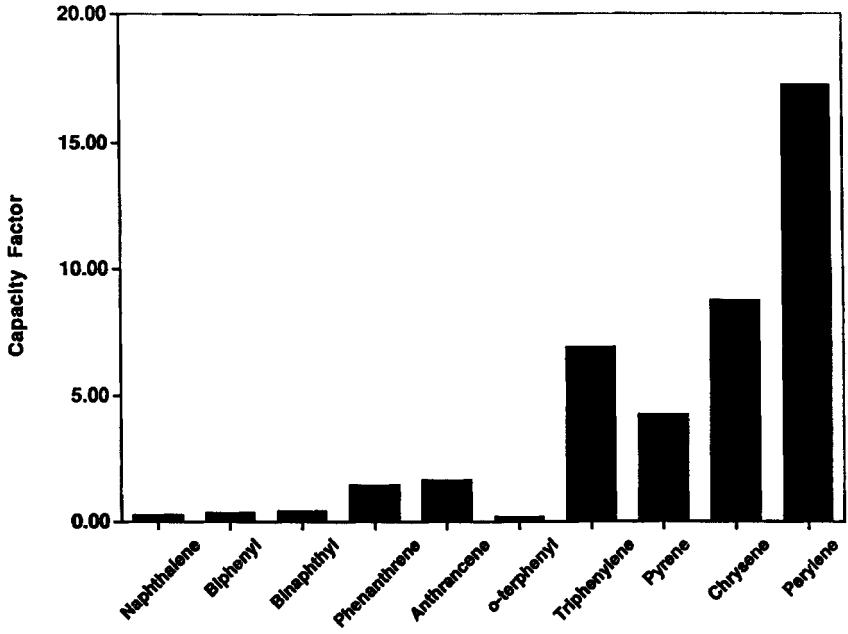

Figure 6. Capacity factors (k') of PAHs on a 100 mm x 4.6 mm column packed with H_2ProP-silica. Conditions: mobile phase: 100% acetonitrile; flow rate: 1.0 mL/min; column temperature: 25°C; detection: 254 nm (0.100 AUFS).

packed with 2-(1-pyrenyl)ethyl-silica (PYE)[21] and more recently with a 3-[(pentabrombenzyl)oxy]propylsilyl-silica (PBB) phase[50] ($\alpha_{C70/C60}$ = 1.8 and 2.5 in toluene, respectively). However, these and other stationary phases[51-63] exhibit only modest selectivity for the separation of C_{60} and C_{70} when using stronger solvents as the mobile phase.

The new TPP-silica phases described here have been shown to offer dramatically enhanced retention and selectivities for the separation of fullerenes.[39-41] As shown in Figure 7, C_{60} and C_{70} can be completely resolved using mobile phases ranging from 100% toluene to 100% CS_2 (with α values ranging from 4.3 to 1.8). Again, as in the case of PAHs, $\alpha_{C70/C60}$ values do not change appreciably whether or not the TPP structure is metallated (either with +3 or +2 metal ions). However, when Sn(IV) is the central metal ion, much poorer selectivity for the separation of C_{60} and C_{70} has been observed.[42] Since fullerene separations are performed in aprotic solvents, it is likely that the presence of larger anionic ligands (Cl^-) on both axial sites of the central metal

ion partially block π-π interactions between the fullerenes and the immobilized TPP species (behavior that is significantly different from the case of PAH separations in methanol/water mobile phases, see above).

Beyond C_{60} and C_{70}, higher molecular weight fullerenes and endohedral metallofullerenes can also be separated on columns packed with TPP-silicas. Figure 8 shows the chromatogram obtained for the separation of a fullerene mixture obtained from the Soxhlet extraction of raw fullerenes with pyridine using soot that had been produced from graphite rods containing yttrium. As shown, a fairly clean separation of $Y@C_{82}$ can be achieved in one pass through a 25 cm column packed with ZnTPP-silica. Similar results have been published previously for the separation of $La@C_{82}$.[40] As with the separation of empty fullerenes, operation of the column with a mobile phase of 25% CS_2 and 75% toluene enables purification of much larger quantities of the endohedral metallofullerenes on the TPP-silica phases than on conventional columns.

The results presented above were obtained on columns packed with TPP-silicas prepared using the monocarboxyl-TPP derivative and aminopropyl silica as the starting materials (Figure 1). Very recent results obtained with packings prepared by coupling 5-(p-hydroxyphenyl)-10,15,20-triphenyl] porphyrin to glycidoxypropyltrimethoxysilane through an ether linkage, followed by immobilization on the silica surface (Figure 2) suggest that such phases exhibit both greater selectivities and efficiencies for the separation of fullerenes. Indeed $\alpha_{C70/C60}$ values of > 6.0 in 100% toluene and > 3.0 in 100% CS_2 have been observed on these new porphyrin-silica phases.[64] Moreover, the number of theoretical plates (N) is also considerably higher for columns packed with this new type of TPP-silica phase. Since the preparation of these materials does not require use of aminopropyl-silica, it is possible that much more homogeneous stationary phases may result (i.e., for TPP-silicas prepared with monocarboxyl-TPP, incomplete acetylation of the residual amine sites could yield rather heterogeneous phases).

Regardless of which method is used to prepare the TPP-silicas, it is clear that such phases interact very strongly with fullerenes. The strength of the fullerene-porphyrin interaction can be attributed to the similar diameter of the fullerene and the TPP cavity (see Figure 9), thereby providing the opportunity for π-π interactions to occur in three dimensions. Indeed, it is likely that the fullerene π-electrons enjoy not only face to face π-π interactions with the porphyrin macrocycle, but face to edge π-π interactions with the meso phenyl rings of the porphyrin. The importance of the meso phenyl groups on the immobilized porphyrin has been confirmed by efforts to separate C_{60} and C_{70} on ProP-silicas, where only minimal selectivity for C_{70}/C_{60} has been observed.[41]

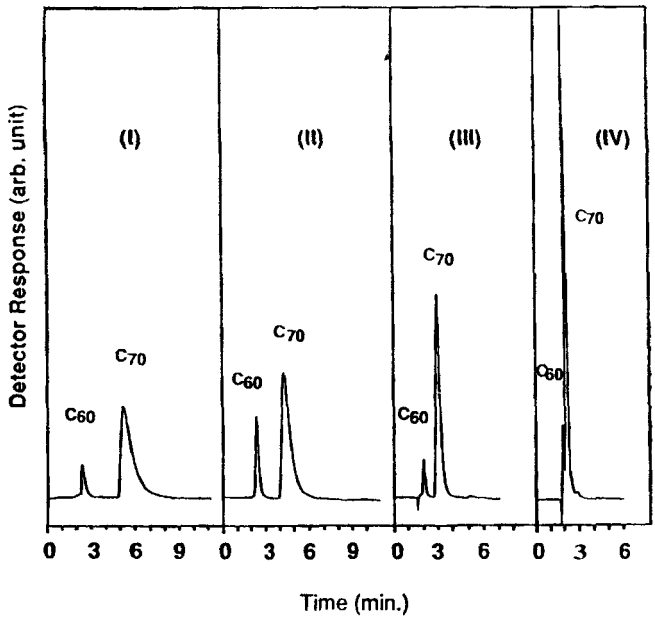

Figure 7. Typical separation of C_{60} and C_{70} on a 250 mm x 4.6 mm column packed with ZnTPP)-silica using four different mobile phases: (I) toluene, (II) *p*-xylene, (III) chlorobenzene and (IV) carbon disulfide. Conditions: flow rate: 2 mL/min; detection: UV@430 nm (0.100 AUFS); injection: 20 μL of fullerene solution in toluene; temperature: 30°C.

In general, fullerene retention on porphyrin-silica columns increases with increasing fullerene molecular weight, due to the fact that as fullerene size increases both the surface interaction area and the π-basicity of the fullerene increases. The importance of π-basicity in the retention mechanism is further supported by the evidence that La@C_{82} and Y@C_{82} are retained longer than C_{82} or C_{84}. Indeed, in such endohedral metallofullerenes, the central metal donates electrons to the fullerene π-orbitals, thereby making M@C_{82} species a stronger base than non-metallo C_{82}. Hence, although both species are exactly the same size, the metallofullerene is retained longer on the TPP-silica support.

One interesting aspect of fullerene separations on TPP-silica phases is the observed dependence of selectivity on the pore size of the silica.[42] For example, the selectivity factor for C_{60} and C_{70} was found to decrease from $\alpha = 5.6$ for a 60Å pore size silica to $\alpha = 2.6$ for a 300Å pore size silica using pure toluene as the mobile phase (with similar surface coverage of immobilized TPP).

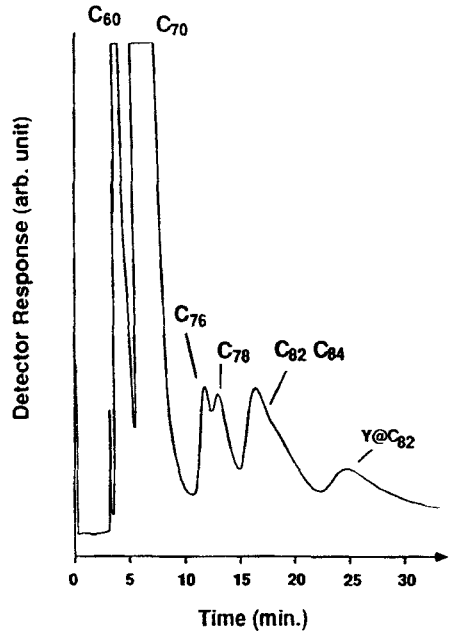

Figure 8. Chromatogram for sample of fullerene soot containing $Y@C_{82}$ on a 250 mm x 4.6 mm column packed with ZnTPP-silica. Conditions: flow rate: 2 mL/min; detection: UV@482 nm (0.100 AUFS); injection: 100 µL of fullerene solution in toluene; temperature: 25°C; mobile phase: 75% toluene/25% CS_2.

It is thought that the three dimensional size and shape of the fullerenes may allow a single fullerene solute to enjoy simultaneous interactions with more than one immobilized TPP species. Hence, the small diameter and high pore curvature of 60Å silica is likely to enhance the possibility for such simultaneous interactions compared to 300Å silica. Further studies to fully discern the role of pore size on fullerene separations are currently in progress in this laboratory.

Anion Separations on Metalloporphyrin-Based Stationary Phases

Metalloporphyrin-silica phases also exhibit unique properties as anion-exchange materials. The basis for the potential application of these phases in anion exchange chromatography can be found in previous literature reports regarding the technique termed ligand exchange chromatography.[16]

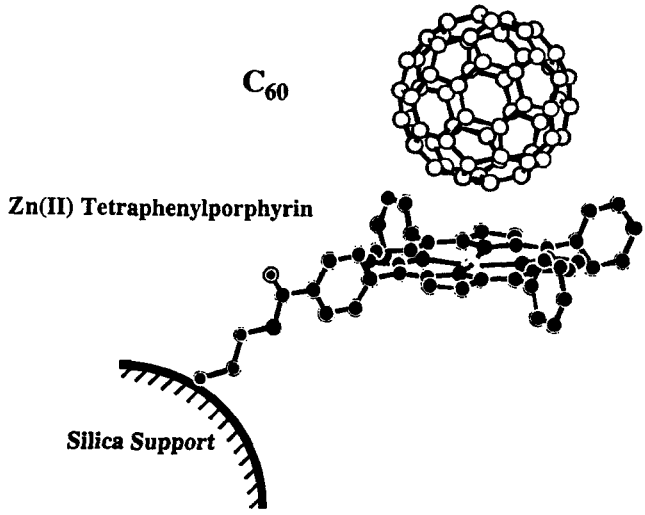

Figure 9. Molecular model of proposed π-π interaction between immobilized MTPP and C_{60}.

Ligand exchange chromatography has been defined by Davankov as a process in which complex-forming species are separated through the formation and breaking of labile coordinate bonds to a central metal ion. Anion retention in ligand exchange chromatography may occur by either an inner-sphere or outer-sphere coordination mechanism. Inner-sphere coordination involves direct interaction between the anionic ligand and the metal center of the exchanger while outer sphere mechanisms require initial tight inner sphere coordination of a solvent or other neutral ligand molecule and subsequent hydrogen bonding with anions not directly in contact with the metal center. Further, the metallic cations used in ligand exchange chromatography may be classified as "hard" or "soft."

Hard acid cations (e.g., Sn^{+4} and In^{+3}) have a large charge:radius ratio and preferentially associate with ligands containing oxygen, while soft acid cations (e.g., Zn^{+2} and Cu^{+2}) coordinate preferentially with ligands having sulfur or nitrogen electron donors. Hence, using the newly developed porphyrin-silicas with different metal ion centers that are in their +3 or +4 oxidation state, it should be possible to separate anions based on specific coordination reactions with a given metal center cation.

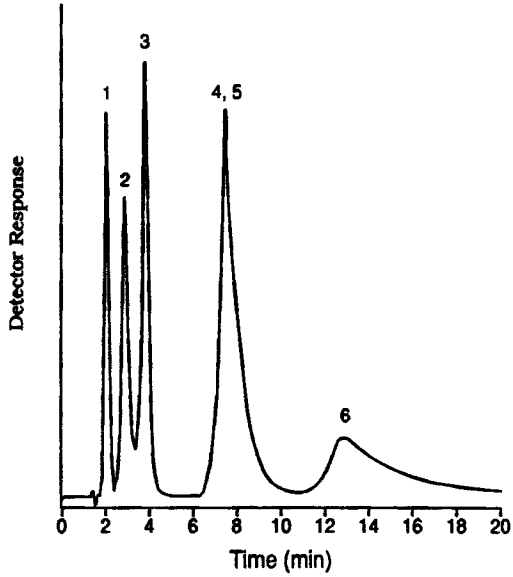

Figure 10. Typical separation of anions on 100 mm x 4.6 column packed with InTPP-silica. Peak identity: (1) iodide, (2) thiocyanate, (3) p-toluenesulfonate, (4) benzoate, (5) p-hydroxybenzoate, (6) salicylate. Conditions: mobile phase: 15% methanol/85% 10 mM acetate buffer, pH 4.5; column temperature: 25°C; detection: 220 nm (0.100 AUFS). (Adapted from Ref. 38 with permission).

Initial evaluation of the anion-exchange selectivities of columns packed with SnTPP- and InTPP-silica supports (Figure 10) was performed using inorganic (e.g., I^- and SCN^-) and organic anions (e.g., benzoate, p-hydroxybenzoate, salicylate and p-toluenesulfonate) as test solutes. Figure 10 shows the typical separation of these test anions achieved using a InTPP-silica column, and Figure 11 compares the capacity factors for these anions on both InTPP- and SnTPP-silica phases, as well as a conventional quaternary ammonium-based anion exchange column (Hamilton PRP X-100).

Figure 11 clearly illustrates that significant differences in selectivity among the three stationary phases are observed. The quaternary ammonium-based support shows little retention preference between the lipophilic inorganic anions and the aromatic anions. In contrast, the SnTPP- and InTPP-silica supports display markedly enhanced selectivity towards aromatic anions (e.g., salicylate) over the lipophilic inorganic anions, and this correlates well with the

potentiometric anion selectivities found when Sn(IV) or In(III)-based metallotetra- phenylporphyrins are incorporated in polymer membranes for the purpose of developing new anion selective electrodes.[65,66]

Differences in the chromatographic selectivity between SnTPP- and InTPP-silica phases towards aromatic carboxylates are also suggested by the data shown in Figure 11. The capacity factor of p-hydroxybenzoate on the SnTPP support is greater than that of salicylate or benzoate, while on the InTPP support, salicylate is the preferentially retained anion. The aromatic carboxylates elute as reasonably sharp symmetric peaks on the column packed with InTPP-silica, but on the SnTPP-silica column these solutes elute as broad tailing bands. The difference in peak shape on the two metalloporphyrin supports suggests slow kinetics for the dissociation of an inner sphere metal-carboxylate complex with the Sn(IV) metalloporphyrin stationary phase. Indeed, significant improvement in peak symmetry for salicylate on the SnTPP-silica support can be realized when separations are carried out at elevated temperatures (e.g., 60°C).

The InTPP-silica phase has been applied to separate a number of benzene- and naphthalene-based sulfonates.[38] While both solvophobic and/or π-π interactions between the aromatic anions and the immobilized metallo-TPP may play a role in solute retention, comparison of the capacity factors of aromatic carboxylates and sulfonates on metallated and non-metallated TPP-silica stationary phases clearly indicates that metal-ligand exchange is the primary retention mechanism for these aromatic anionic solutes on both the SnTPP- and InTPP-silica supports.[38]

Mobile phase pH has a significant impact on the retention of anionic compounds on the metalloporphyrin-silica supports. The capacity factors for a series of ten aromatic sulfonates on columns packed with SnTPP- and InTPP-silicas as a function of mobile phase pH (from pH 4.0-6.0) are tabulated in Table 2. Both metalloporphyrin supports exhibit decreased selectivity for the aromatic sulfonates with increasing pH of a 15% methanol/85% 10 mM succinate eluent. This decrease in retention occurs as a result of the very strong ligation reaction between hydroxide ions and the metal ion centers.[65] Thus, mobile phase pH, either using isocratic or gradients, can be used to optimize the separation of aromatic carboxylates and sulfonates, using higher pH values to decrease the retention of solutes that coordinate strongly with the metal ion centers.

The separation of anions on MProP-silica phases has not yet been explored, nor has there been any detailed examination of the anion exchange properties of TPP-silica phases containing metal ions other than In(III) and

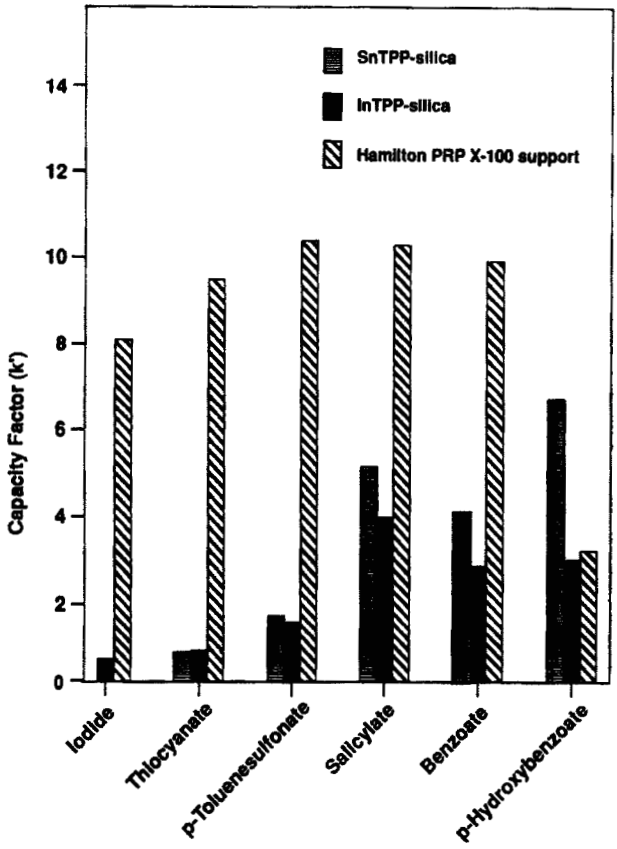

Figure 11. Capacity factors (k') of inorganic and organic anions on 100 mm x 4.6 mm columns packed with SnTPP-silica, InTPP-silica, and a Hamilton PRP X-100 support. Separations on the SnTPP and InTPP columns were carried out using a 10 mM sodium succinate, pH 5.5, eluent at a flow rate of 1.0 mL/min. These solutes were eluted from the Hamilton PRP X-100 column with 8 mM sodium carbonate, pH 11, containing 1 mM p-cyanophenol at a flow rate of 1.0 mL/min.

Sn(IV). In principle, it should be possible to use a wide range of other metal ions to achieve novel ligand exchange anion selectivities (e.g., Co(III) for selective nitrite retention[67]). Of course, only metal ions that form stable complexes with the porphyrin macrocycle in their +3 or +4 state can be used, since +2 metal centers will not require counter anions to satisfy electroneutrality conditions.

Table 2

Influence of Mobile Phase pH on the Capacity Factors of Aromatic Sulfonates on the In(III)TPP- and Sn(IV)TPP-silicas[a]

	Capacity Factor (k')									
	pH 4.0		pH 4.5		pH 5.0		pH 5.5		pH 6.0	
Aromatic Sulfonate	In(III)	Sn(IV)	In(III)	Sn(IV)	In(III)	Sn(IV)	In(III)	Sn(IV)	In(III)	Sn(IV)
Sulfanilic Acid	1.2	1.2	0.6	0.6	0.4	0.4	0.2	0.2	0.2	0.1
Benzenesulfonate	2.1	2.1	0.9	0.8	0.6	0.6	0.4	0.3	0.2	0.2
p-Toluenesulfonate	3.7	3.7	1.5	1.8	1.1	1.0	0.6	0.6	0.4	0.4
1-Naphthaleneulfonate	18.8	19.0	7.6	9.4	5.6	5.1	3.1	2.9	2.1	2.0
2-Naphthalenesulfonate	25.2	23.6	10.1	11.7	7.6	6.2	4.2	3.7	2.8	2.6
4-Amino-1-Naphthalenesulfonate	7.9	7.8	3.5	3.8	2.4	2.1	1.4	1.2	0.9	0.8
6-Amino-4-Hydroxy-2-Naphthalenesulfonate	11.5	>26.0	5.4	8.1	4.2	5.4	2.4	3.7	1.7	3.1
7-Amino-4-Hydroxy-2-Napthalenesulfonate	11.5	>26.0	5.6	12.5	4.3	8.2	2.4	8.0	1.7	5.6
1,5-Napthalenedisulfonate	9.8	10.0	2.1	2.0	0.8	0.7	0.2	0.2	0.1	>0.1
2,6-Naphthalenedisulfonate	13.1	12.4	2.7	2.5	1.2	0.9	0.4	0.3	0.2	0.1

[a] Column: 100 mm x 4.6 mm stainless steel column; mobile phase: 15% methanol/85% 10 mM succinate; flow rate: 1.0 mL/min; column temperature: 25°C; detection: 220 nm (0.100 AUFS).

Separation of Amino Acids/Peptides on Metalloporphyrin-Silicas

The concept of ligand exchange chromatography with immobilized metal ions has also been applied previously for the separation of amino acids, peptides and proteins, where given amino acid side chains (e.g., histidine, tyrosine, tryptophan)[31,32] can form coordination complexes with certain metal ions (e.g., Zn^{+2}, Ni^{+2}, Fe^{+3}). The technique, more often termed immobilized metal ion affinity chromatography (IMAC), normally utilizes supports composed of iminodiacetate ligands tethered to soft gels or silica supports.[31,32] Using the existing phases, however, IMAC has several shortcomings, particularly when it comes to performing reproducible analytical separations of peptides and proteins. Indeed, continuous metal ion leaching from current

support materials makes analytical IMAC virtually impossible (i.e., retention times change as metal is depleted from support). Since the affinity of metal ions to porphyrin structures is known to be on the order of 10^{30} M^{-1}, some 15 orders of magnitude greater than most metals bind diacetate type ligands,[68-74] it seems reasonable to expect that metalloporphyrin phases may be especially useful for reproducibly separating peptides and proteins by ligand exchange type reactions.

To examine this prospect in detail, preliminary experiments have been carried out using MProP-silicas rather than MTPP-silicas as stationary phases. The immobilized ProP structure is somewhat less hydrophobic than TPP and it also provides a phase that should have less steric hindrance for potential ligation reactions of larger peptides and protein structures. A test group of 9 amino acids was selected to be representative of the various classes of the 20 natural amino acids: i.e., L-glycine (Gly) and L-leucine (Leu) as hydrophobic amino acids; L-glutamate (Glu), L-lysine (Lys), L-serine (Ser) and L-cysteine (Cys) as charged/polar amino acids, and L-histidine (His), L-phenylalanine (Phe) and L-tryptophan (Trp) as aromatic amino acids. Table 3 summarizes the absolute capacity factors (k') measured on each of the six columns (10 cm) for each test amino acid using a 50 mM phosphate buffer, pH 7.0, as the mobile phase. As tabulated, Gly, Glu, Ser, Lys and Cys exhibit the least retention on any of the ProP-silica columns indicating that ionic interactions, in comparison to other retention mechanisms (see below), are minimal and make only a small contribution to the overall retention behavior of amino acids on the MProP-silica phases. Further, the more hydrophobic Leu and polar Ser amino acids are also not retained to any significant degree (relative to His and Trp) on any of the ProP-silica columns examined; these results suggest that the ProP-silica stationary phases do not exhibit substantial hydrophobic or hydrogen-binding character.

The retention behavior of His and Trp on the MProP columns *vs.* unmetallated ProP-silica clearly demonstrates the inherent amino acid ligand exchange selectivity of the new MProP-silica phases (see Table 3). Columns packed with FeProP-silica provide the greatest selectivity for retention of His, followed by the NiProP, CuProP, ZnProP and CdProP phases. The retention of His is due to coordination of the imidazole nitrogen with the central metal ion. It is interesting to note that Trp also exhibits extremely high affinity (much greater than His) on certain metalloporphyrin phases, e.g., CuProP, FeProP and NiProP, relative to that on the unmetallated H_2ProP-silica phase. The amino acid retention patterns shown in Table 3, however, also reveal that Trp is highly retained even on the unmetallated H_2ProP-silica phase (compared to all the other test amino acids). Further, MProP-silica phases that exhibit greatly enhanced retention of Trp also display the longest retention times for Phe,

Table 3

Capacity Factors of Selected Amino Acids on the Six Porphyrin Silical Columns[a]

Amino Acids	Stationary Phase					
	H_2ProP	FeProP	CuProp	ZnProp	NiProp	CdProP
Glycine	0.1	0.2	0.1	0.1	0.2	0.1
Histidine	1.0	14.6	4.1	2.1	7.3	1.3
Lysine	1.2	4.0	2.2	1.7	5.3	1.6
Cysteine	0.2	0.7	0.3	0.2	0.2	0.2
Serine	0.1	0.2	0.1	0.1	0.2	0.1
Phenylalanine	1.5	7.2	8.7	2.1	9.7	1.5
Tryptophan	10.5	70.7	109.0	13.9	63.2	11.2
Glutamine Acid	0	0	0	0	0	0

[a] Mobile phase: 100% 50 mM phosphate buffer, pH 7.0; flow rate: 1 mL/min; temperature: ambient; detection: 214 nm (0.100 AUFS).

although not nearly to the extent observed for Trp. These results suggest that there is a substantial contribution from π-π interactions between the immobilized porphyrin ring and the aromatic amino acids. Therefore, a combination of simultaneous metal ion ligation and π-π interactions are likely responsible for the exceptionally high selectivity toward Trp exhibited by several of the MProP-silica phases, especially CuProP-silica.

More detailed studies on the effect of organic modifier (acetonitrile) in the mobile phase (pH 7.0 phosphate buffer) on the retention of Trp, His, and Phe on the CuProP and FeProP silica phases demonstrates that increasing the acetonitrile content dramatically reduces the retention time of amino acids on both columns. In addition, lowering the pH of the mobile phase (from pH 7.0 to pH 2.5) also drastically reduces the retention of His on the FeProP-silica phase (from $k' = 14.3$ to $k' = 0.0$). At low pH, the imidazole nitrogen on His is protonated eliminating retention via a metal-ligation reaction with this nitrogen. Similar effects are observed for Trp and Lys. Since organic modifier dramatically decreases the π-π interaction and lowering pH weakens metal-ligation interactions, it is a simple matter to tune these two retention mechanisms, by choosing an appropriate pH and organic modifier content for the mobile phase (see below), to achieve the retention and selectivity desired.

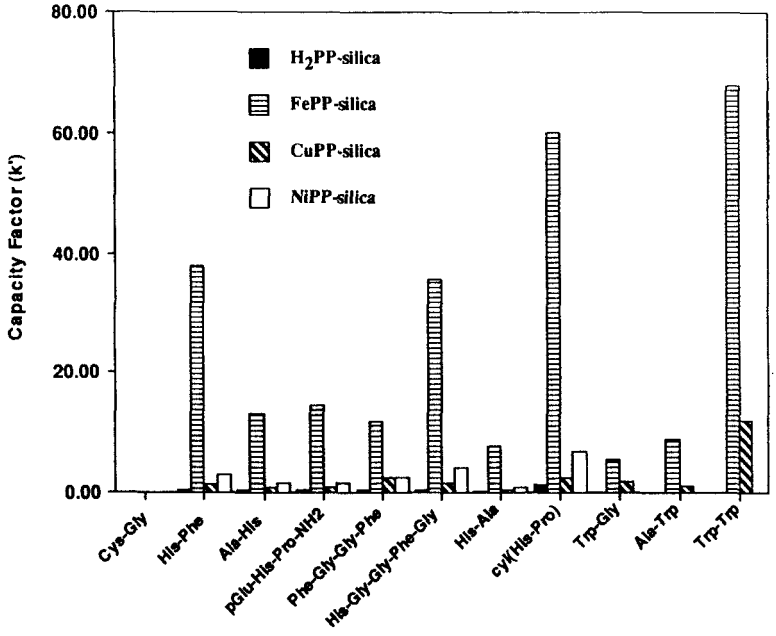

Figure 12. Capacity factors of model peptides on 100 mm x 4.6 mm columns packed with H_2ProP-silica, FeProP-silica, CuProP-silica and NiProP-silica. Conditions: mobile phase: 75% 50 mM phosphate buffer, pH 7.0 / 25% acetonitrile; flow rate: 1 mL/min; temperature: ambient; detection: UV@214 nm (0.100 AUFS).

Since the amino acids His, Trp, and Phe are all retained to varying but significant degrees on some MProP-silica phases, the retention behavior of small peptides containing these and other amino acids was also examined. Using a mobile phase of pH 7.0 phosphate modified with 25% acetonitrile (to elute all test peptides in a reasonable time while still allowing a practical evaluation of the different retention behaviors of the peptides) the capacity factors for a group of 10 test peptides on 10 cm H_2ProP-, CuProP-, ZnProP- and FeProP-silica columns are shown in Figure 12. For the most part, the results of these studies are consistent with the observed retention of individual amino acids on the same stationary phases (see above). For example, Cys-Gly elutes in the void volume on all the porphyrin columns while all peptides containing His and Trp are preferentially retained on the FeProP-silica column, presumably through metal ligation interactions. Other porphyrin columns exhibit only minimal retention of these peptides, except for Trp-Trp on the CuProP column. Even with 25% acetonitrile in the mobile phase, π-π interactions still contribute to some extent to the retention of the test peptides,

and this explains why peptides containing both His and Phe are retained longer than those with His alone. The Trp-Trp dipeptide is retained the longest on the FeProP- and CuProP-silica phases of any of the peptides tested. Overall, these results suggest that there is an enhanced cumulative interaction affinity for small peptides containing multiple amino acids that individually exhibit strong interactions with a given MProP-silica phase. This is analogous to what is observed on current IMAC phases. Indeed, polyhistidine sequences are now used routinely as N-terminus sequences of recombinant proteins so that such proteins can be retained strongly by IMAC supports, greatly simplifying the protein purification process.

Beyond unique chemical selectivities, the extremely tight binding of metal ions to the ProP-silicas represents a potentially significant advantage over conventional IMAC phases.[71-74] As stated previously, metal ion leaching from current IMAC phases is a major limitation to the use of such phases in routine HPLC peptide/protein separations. To demonstrate the inherent metal ion stability of the new MProP-silica stationary phases, the separation of two model peptides was conducted before and after extensive column washing (FeProP-silica) with 50 mM EDTA (pH 6.0) for about 30 min. As shown in Figures 13a,b, the chromatograms (using a gradient for elution; see the figure legend for exact conditions) for tryptophan-releasing hormone (TRH; pGlu-His-Pro-NH_2) and His-Phe are essentially the same before and after washing the column with EDTA. Any slight differences in retention times (see Figure 13 legend) are apparently due to small variations in the gradient profile from run to run. In another experiment, EDTA was deliberately added to the same peptide mixture at a concentration of 10 mM. Again, as shown in Figure 13c, there is no major change in the resulting chromatogram, indicating that no significant leaching of the Fe(III) within the ProP structure occurs even when a very strong chelator passes through the column (note: retention of TRH and His-Phe is largely due to retention of His via metal-ligation interaction). The reproducibility of the separation shown in Figure 13 exemplifies one of the potential advantage that metalloporphyrin-silica stationary phases may offer for peptide and protein separations via metal ion affinity.

Summary and Limitations of Current Porphyrin-Silica Phases

The initial HPLC studies with porphyrin-silica stationary phases summarized above demonstrate that very high chemical selectivities can be achieved in a variety of applications. It has been further shown that for fullerene, PAH and peptide separations, the observed retention relationship is highly dependent upon the specific structure (and metal cation) of the immobilized porphyrin. However, the superior selectivity exhibited by

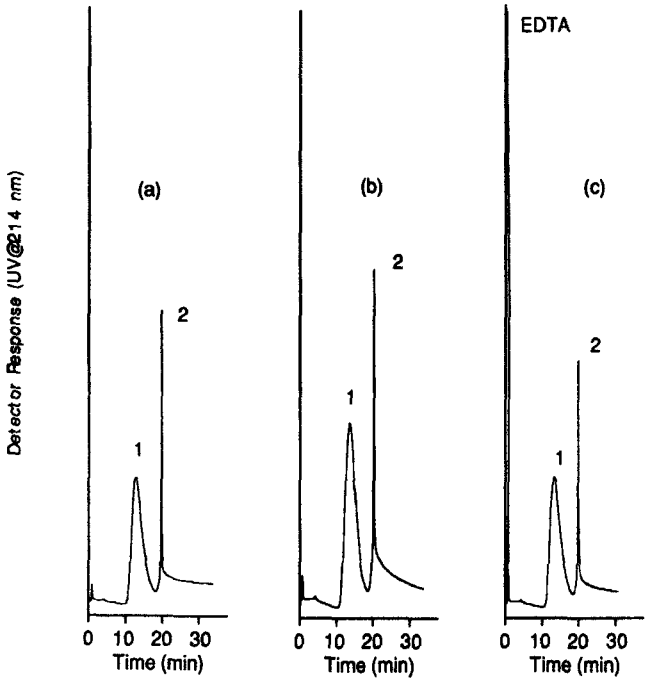

Figure 13. The effect of EDTA on the HPLC separation of TRH (1) and His-Phe (2) using a 100 mm x 4.6 mm column packed with FeProP-silica: (a) before 30 min washing with 50 mM EDTA (retention times of 1 and 2 were 12.72 min and 19.87 min); (b) after EDTA washing (retention times of 1 and 2 were 13.76 min and 20.56 min); (c) sample of peptides with 50 mM EDTA (retention times of 1 and 2 were 13.42 min and 20.24 min). Conditions: mobile phase: (A) 10% acetonitrile/90% pH 7.0 phosphate buffer and (B) 25% acetonitrile/75% pH 2.5 phosphate buffer; gradient: 0-100% B from 0-15 min and 100% B from 15-30 min; flow rate: 1 mL/min; temperature: ambient; detection: UV@214 nm (0.100 AUFS). (Adapted from Ref. 44 with permission).

porphyrin-silica stationary phases has not, as of yet, been matched with high efficiency. Peak shapes are routinely poor with a high degree of peak tailing. Experimental evidence appears to implicate a number of chemical sources for the low efficiencies observed, including: a) heterogeneity in the porphyrin structure and surface topology; b) incomplete silica surface deactivation; c) multiple modes of solute retention; d) and slow dissociation kinetics for solute interactions.

First, in the preparation of porphyrin derivatives, approximately 2-5% of the product is the corresponding chlorin (a reduced form of porphyrin with 2 additional hydrogens on one of the pyrrole rings). To date, no attempt has been made to synthesize stationary phases free of chlorin contamination. Although the porphyrin and chlorin are very similar in structure, even a slight deviation in stationary phase structure is likely to lead to different interaction energies with solutes.

Second, heterogeneity in the surface coverage has not been examined in detail. Because the surface coverage is much lower than a monolayer it is both conceivable and likely that large variations in porphyrin density occur over the silica surface. This is supported by the knowledge that porphyrins tend to aggregate into stacks[75] and this phenomenon is likely to occur during the immobilization procedure where high concentrations of the activated porphyrin derivatives are employed.

This stacking behavior would result in a highly varied surface structures with regions of densely stacked porphyrins as well as regions of highly spaced immobilized porphyrins. The resulting stationary phase can exhibit at least two, and likely more, distinct interaction behaviors.

Third, because of the comparatively low porphyrin coverages, compared to conventional columns such as ODS, there exist a large number of exposed silanols and amine sites. Thus, secondary solute interactions with the silica surface can be a significant source of solute retention.

A fourth contributor to band broadening in ligand exchange applications (peptide separations and anion exchange) relates to the high association constants for metal ion ligation reactions, which are generally much greater than those typically found in dynamic chromatographic systems (e.g. ODS, amine, phenyl). Hence, the slow kinetics of ligand dissociation can contribute significantly to band broadening in such systems. If dynamic chromatographic behavior is desired it appears that metal ligation interactions must be mitigated either through the addition of a competing complexation agents, judicious choice of mobile phase pH, or alteration of the porphyrin structure to sterically hinder solute ligation.

Further research into the relationship between each of these factors and peak tailing is in progress. As the contribution of each is understood, their adverse effect on solute efficiency can hopefully be reduced or eliminated, making the porphyrin-silica phases more useful as versatile supports for a variety of modern HPLC applications.

ACKNOWLEDGEMENTS

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