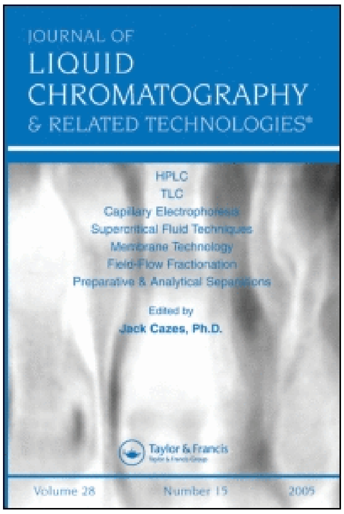

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Porous Hybrid Organic-Inorganic Particles in Reversed-Phase Liquid Chromatography

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Abstract: Reversed-phase chromatographic media have recently become available that are based on porous hybrid organic-inorganic particles. The present paper reviews hybrid particles that are made from organosilanes (organic moiety) and tetraalkoxysilanes (inorganic moiety). The hybrid particles are defined and classified within the context of a broader definition of hybrid materials. First syntheses and chromatographic evaluations are discussed for this class of hybrid packing materials. Publications are then described, which characterize two distinguishing chemical properties of hybrid particles vs. silica gel: 1) less acidic silanols, and 2) markedly longer lifetimes in alkaline mobile phases. These properties are achieved without sacrificing mechanical strength, as is found for fully organic particles, i.e., polymers, with the same chemical features. Literature reports are then reviewed that employ hybrid based reversed-phase column packings for HPLC. Topics covered include fundamental retention mechanism studies, methods development studies, and applications made possible with the hybrid based products. Further review is presented on the use of theses hybrid particles for UPLC. The hybrid particles afford good mechanical strength without sacrificing retention and loading capacity, as is found for non-porous particles. Applications employing hybrid based particles in the UPLC mode are then reported.

Keywords: Hybrid organic-inorganic, Reversed-phase, HPLC applications, UPLC applications

INTRODUCTION

Liquid chromatography (LC) based separations continue to grow in popularity for innumerable classes of compounds. The increasing utility of LC for

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today's end user has been driven by advances in all of the different components of an LC system that must ultimately come together to offer a separations solution. These components can simplistically be broken out into software, hardware, and the column or device which contains a chromatographic media, all of which have benefited from advances in a myriad of scientific and engineering disciplines. The column is often referred to as the "heart" of a separations system because the device is responsible for the actual separation of a mixture into its subcomponents. A column material's chemical and physical properties determine which mechanisms (e.g., reversed-phase, ion-exchange, size-exclusion, etc.) are at its disposal to affect the separation.

As for many technologies, separations materials have traditionally been either an inorganic based material (e.g., silica gel, alumina) or an organic based material (e.g,. poly-divinylbenzene). Significant improvements have been achieved for both classes of materials in terms of providing better separations. For example, reversed-phase (RP) high performance liquid chromatography (HPLC) silica gel packings have gone from irregular to spherical, and from 10 μm to 3 μm in diameter, to afford improved peak efficiency for the end user. At the same time, the LC end user continues to require higher performance from his separations material to analyze increasingly complex and challenging mixtures. To meet that demand, scientists working on new LC materials have recently turned their attention to hybrid materials. Hybrid material approaches have already found success in a number of diverse technology arenas, and the study of hybrid materials chemistry is a rapidly growing sub-discipline of the many classical scientific disciplines.[1]

Succinctly defining the term "hybrid" is a difficult task. To quote Gomez-Romero and Sanchez: "When it comes to formal classifications, hybrid materials tend to resist rigid categorization. Their grouping is too large–and growing–to allow systematic grouping criterion".[2] This difficulty is, thus, born partly from the vast combinations of organic and inorganic moieties, which defy simple classification. It is also born partly from perspective of the individual. For example, a simple C_{18} bonded phase on silica gel would be considered by some to be a hybrid phase, since an organic component (C_{18} ligand) and an inorganic component (SiO_2) are coupled together by a chemical bond. At the same time, others would classify polystyrene coated onto silica gel as a hybrid, despite the organic component (polystyrene) having no chemical bond to the inorganic component (SiO_2). No deliberate attempt to classify what does or doesn't constitute a "hybrid material" will be made in the present review. However, hybrid materials covered in this review will be of the particulate sol-gel type and involve simultaneous formation of organic (organosiloxane) and inorganic (silicon dioxide) building blocks or repeat units. The initiated reader is directed to Novak and also Wen and Wilkes for presentation of two classification systems for sol-gel hybrid materials.[3,4] Many diverse combinations of synthetic variables are available to make new materials by this route.[5]

Porous hybrid particles used in RPLC will be reviewed with categorization broken out into high and ultra performance LC modes. The hybrid material's chemical and physical properties will be described, and their utility for the end user will also be demonstrated through citation of published RPLC applications. Our objective is not to be exhaustive but is rather to introduce the chromatographic scientist to the subject matter and provide a starting point for further literary investigation. It is very important to note that hybrid approaches to other LC separation modes and formats are very active areas of investigation but will not be covered here. The use of hybrid materials in electrochromatography is arguably quite advanced, and a number of reviews in this area have been published.[6–8] More recently, hybrid sol-gel monolithic materials have been reported.[9–11] Theses hybrid monoliths are expected to provide for new applications beyond those found for silica based and polymer based monoliths.[12]

Hybrid Particles in HPLC

Although reports of hybrid materials for HPLC may seem to have appeared only in the last ten years or so, the design and use of hybrid materials is in fact 30 years old. In a 1974 patent application and 1976 publication, Unger and coworkers made what would today be called hybrid packing materials, and described their potential to solve problems found in RPHPLC materials that existed then (and some would argue still exist now).[13,14] Unger sought to introduce the stationary phase directly onto the surface and into the skeletal structure by the simultaneous hydrolytic condensation of a monomeric organosilicon compound [e.g., R'sSi(OR)$_4$] with a tetraalkoxysilane [e.g., Si(OR)$_4$] or a polyalkoxysiloxane. Unger rationalized that this approach would provide better control of the particle's pore structure properties, lead to more stable packings, and obviate the need for subsequent surface bonding steps that would be required to impart a chromatographic selectivity. Despite the envisioned promise of this hybrid approach, little chromatographic data followed these first reports. A review of the data suggests the particles suffered from poor morphology (i.e., non-spherical) and microporosity which had a deleterious impact on efficiency. At the same time this hybrid approach was reported, nominal 5 μm spherical silica particles were being introduced into the field, affording improved packed bed stability and efficiency. This advance in silica gel performance may have pushed column material technology to move forward along classical inorganic and organic lines, leaving this first hybrid approach behind.

As research activity in the hybrid materials sciences began to increase in the late 1980's, new reports soon followed for their use in liquid chromatography applications. In 1991, Loy and Shea prepared a phenyl-bridged polysilsesquioxane xerogel and compared it to a commercial silica for the separation of aromatic hydrocarbons under normal phase conditions.[15]

Despite the large (25–38 μm) and irregularly shaped particles, the hybrid xerogel showed good efficiency, as well as a different selectivity vs. silica gel. More recently, an ethenyl-bridged xerogel was prepared with a decaoctahedron particle shape and 6–8 μm particle size.[16] Unlike most LC materials which are amorphous, the materials were highly ordered as determined by XRD and TEM analysis. Large surface areas of 700–800 m^2/g and small pore diameters of 28–40 Å were obtained. In chromatographic analyses of nitroaniline isomers and polyaromatic hydrocarbons, the hybrid materials did not have as good efficiency as a commercial, 60 Å amorphous silica gel. The authors attributed the poor efficiency to poor packed bed quality caused by the decaoctahedron particle shape, as well as slow mass transfer caused by the narrow pores.

A common theme in the above reports of hybrid materials for HPLC is a dual proficiency for the investigators in both materials chemistry and chromatography. This limited progress in the use of hybrid materials as separation media. Arguably, the first porous hybrid material to become commercially available to chromatographers without synthesis proficiency was a methylsiloxane/silicon dioxide support introduced by Fisk, Jiang, and other scientists from Waters Corporation.[17] Starting with the concept first introduced by Unger (*vide supra*), the Waters group was able to develop a hybrid particle with spherical morphology, good porosity, and good mechanical strength.[18] A schematic illustration of the synthesis and structure of the hybrid organic-inorganic particle is shown in Figure 1. The HPLC particle contained a methyl group that was incorporated into the internal framework and surface of the material, where about every third silicon atom is substituted with a methyl group, i.e., empirical formula $SiO_2(CH_3SiO_{1.5})_{0.5}$. Interestingly, the particles were found to have 7% carbon before any surface modification, attributed to the incorporated methyl groups. C_8 and C_{18} surface concentrations were limited to about 2.5 μmol/m^2. These values were lower than those found for silica (3.2–3.4 μmol/m^2) and were ascribed to the presence of the methyl groups on the surface instead of silanols.[19] Subsequent investigations found that approximately 10–15% of the particles' methyl groups were located on the chromatographically accessible surface.[20]

Once bonded with C_{18}, C_8, and embedded polar group ligands, the hybrid based column packings were reported to have improved peak shape for basic analytes and the ability to operate in alkaline mobile phases not obtainable with analogous silica based column packings.

This approach to a porous hybrid packing was recently extended to a particle with the empirical formula $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$, prepared from the co-condensation of 1,2-bis(triethoxysilyl)ethane with tetraethoxysilane.[21] These particles were found to have about 6% carbon before any surface modification, attributed to the incorporated ethyl-bridged groups. Unlike the methyl formulation, the majority of the ethyl-bridged groups were found to reside within the skeletal structure with a minority of groups on the chromatographically accessible surface. This depiction was

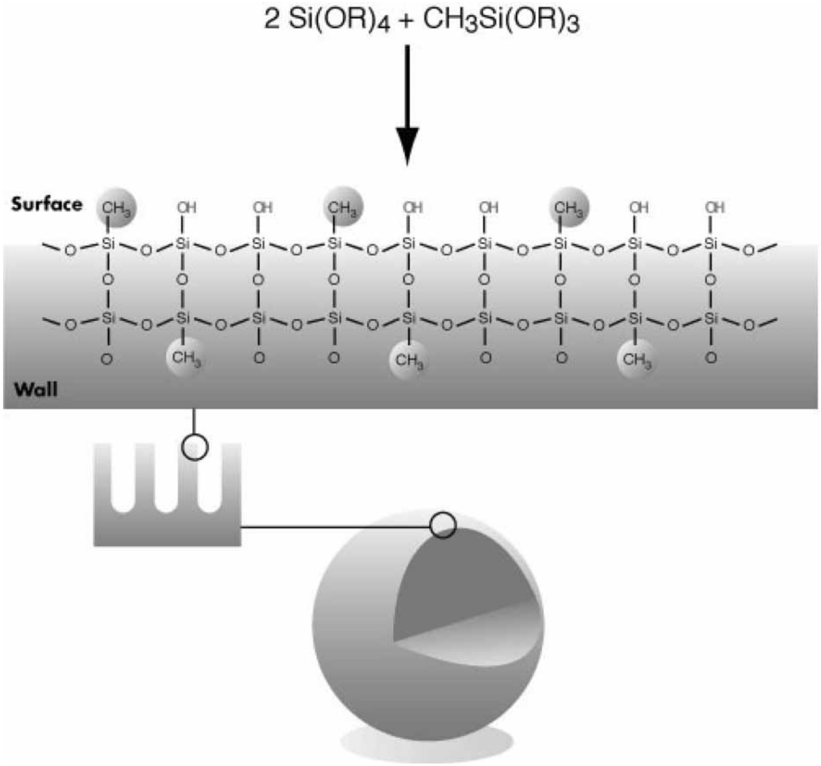

Figure 1. Schematic illustration of the synthesis and structure of $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid organic–inorganic particles. Views of the particle structure are shown on three size scales: at the bottom an entire 5 μm particle with a cutaway to indicate that the particle is solid, in the middle a segment showing idealized 125-Å pores, and on the top a schematic illustrating the idealized chemical structure. Reprinted from reference,[18] Copyright 2000, with permission from Waters Corporation.

supported by C_{18} surface concentrations being in the ranges found for silica packings (3.2–3.4 μmol/m^2). Substitution of methyl groups with ethyl-bridged groups led to a marked increase in alkaline chemical stability, as measured by an accelerated lifetime test run at pH 10 and 50°C. As shown in Figure 2, the C_{18} bonded hybrid (H2-C_{18}) had about a six fold increase in lifetime over a similarly bonded C_{18} silica (S-C_{18}) column. Interestingly, the unbonded hybrid particle (H2) had almost the same chemical lifetime as the C_{18} hybrid particle, leading the authors to conclude the substantial lifetime improvements were due to the bridging ethyl groups.

In another report of a porous hybrid particle, Ding and coworkers prepared a column packing with the empirical formula $SiO_2(HOCH_2CH_2CH_2\text{-}SiO_{1.5})_{0.25}$.[22] For this material, the authors concluded that the propanol group was located primarily at the surface of the particle. The unbonded hybrid

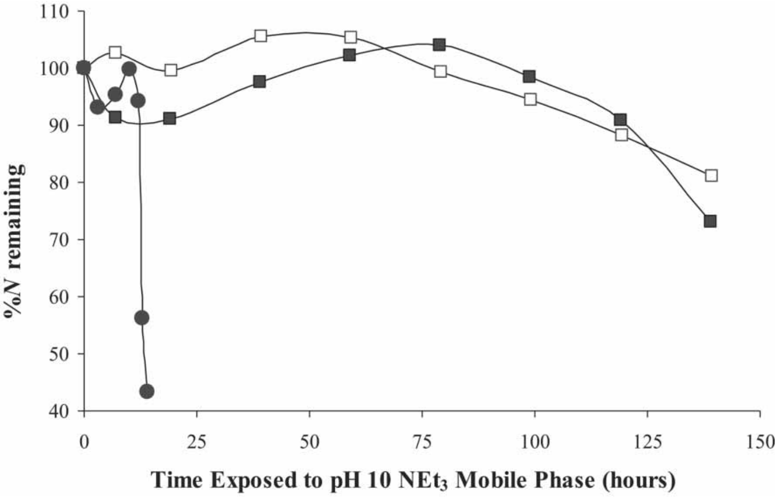

Figure 2. Loss of efficiency (5σ method) for acenaphthene on H2-C_{18} (closed squares) and S-C_{18} (closed circles) columns exposed to 50 mM triethylamine solution at 50 C. For unbonded H2 (open squares), the efficiency loss of the void marker, uracil, was reported. See experimental section of reference for detailed test conditions. Reprinted with permission from reference.[21] Copyright 2003 American Chemical Society.

column packing showed a ten fold improvement in chemical stability vs. silica gel, as measured by an accelerated lifetime test run at pH 10 and 50°C. The alcohol group was also converted to a cyanopropyl group in a two step process. When compared to a trifunctionally bonded cyanopropyl bonded phase on silica gel, the hybrid version showed a moderate improvement in acidic chemical stability, as measured by an accelerated lifetime test run using a 1% TFA solution at 80°C.

Analytical/separation scientists have conducted studies to quantitatively characterize the intrinsic and unique properties of hybrid materials. Other investigators have utilized these unique features of low silanol activity and alkaline stability to address fundamental retention mechanism questions and develop new separations strategies. In one study, Rosés and coworkers measured the silanol acidity of a hybrid material with the empirical formula $SiO_2(CH_3SiO_{1.5})_{0.5}$ vs. a modern high purity and an older low purity silica gel.[23] The study measured the retention of $LiNO_3$ with a methanol/water mobile phase buffered to different pH values. Both unbonded and C_{18} bonded materials were tested. As shown in Figure 3, the retention of Li^+ showed little dependence on pH up to a pH of 8 for the hybrid material (XTerra), whereas the low purity (Resolve) and the high purity silica gel (Symmetry) showed changes at pH 3 and about pH 5 respectively. When the data was used to calculate pK_a values for the three materials, the hybrid

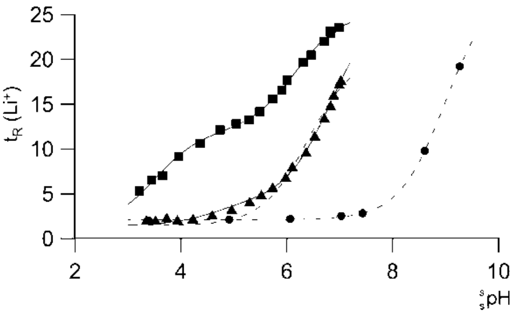

Figure 3. Dependence of the retention of Li^+ in silica columns, (■) Resolve silica, (▲) Symmetry silica, (●) underivatized XTerra, with the pH of the methanol–(0.001 M NaAc + HCl) (60:40) mobile phase detected by conductometry. See reference for calculation of fitting lines. Reprinted from reference,[23] Copyright 2003, with permission from Elsevier.

material was found to have a single value of about 9, while the low purity and high purity silica gels had two values of about 3.5/6.2 and 4.6/6.8, respectively. Similar trends were found for the C_{18} bonded phases. Original reports on the $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid material attributed its good performance for bases (peak shape) to the surface methyl hybrid groups, which acted as an efficient pre-endcap, but the Rosés paper suggests that the methyl group lowers the silanol pK_a to achieve the peak shape improvement.

Similar, although perhaps less quantitative results to the above study were achieved by Neue and coworkers by studying bretylium tosylate retention on the same materials.[24] The paper further successfully mapped out the retention patterns of a broad range of acids, bases, and polyfunctional analytes over a pH range of 2 to 11, deconvoluting the contributions of ion exchange and ion exclusion to "reversed-phase" retention of ionizable analytes. From these studies, they showed that retention of singly charged acid and base analytes can decrease by a factor of 10 to 30 in going from the non ionized to the ionized form. The extended pH range and low silanol acidity of the $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid material was more recently used to create a more refined understanding of how silanols on a C_{18} modified surface interact with positively charged analytes. In this paper, the authors proposed a multiplicative reversed-phase ion exchange mechanism as a universal and dominant mode of interaction between a charged basic analyte with a reversed-phase packing.[25]

Gritti and Guichon have used the low silanol activity of a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid material as part of studies to understand retention mechanism between a solute and an adsorbent. Three papers have studied the retention of propranolol under a variety of mobile phase conditions (e.g., pH, buffer type and ionic strength, etc.).[26–28] A fourth paper reports on adsorption data for caffeine and phenol. In that paper, the authors conclude that observed high energy adsorption sites were not explained by an interaction with the bare surface of the adsorbents, but rather was due to the existence of spots inside the C_{18} bonded layer where molecules could become embedded.[29] In a more recent paper, the surface heterogeneity of a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid was characterized and compared against that of a non endcapped C_{18} silica.[30] The authors used to the data to address reported inconsistencies between different groups regarding saturation capacities for stationary phases. A more detailed discussion of their results is beyond the scope of the present review's objectives, but clearly the hybrid materials' properties have found utility in advancing the understanding of retention mechanism.

The alkaline stability of C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column packings has recently been used by Kaliszan and coworkers to develop a pH gradient separation method.[31–33] Recognizing that the retention of ionogenic analytes in reversed-phase HPLC is strongly dependent on the pH of the eluent, the authors were able to develop a predictive method to elute acids and bases by changing the pH of the mobile phase. Development of the method required the use of aqueous buffers at pH 10.5. In addition to the hybrid material, a purely polymeric packing was also used because of its alkaline stability. The authors reported that the pH gradient method may be particularly suitable when separating bioanalytes that are sensitive to high concentrations of organic mobile phase. They also reported that the pH gradient method proceeds two to three times faster than a conventional organic gradient giving narrow, symmetrical peaks. More recently, the authors applied the pH gradient technique to determine the pK_a values of pharmaceutical compounds.[34] The LC method gave close but not identical pK_a values as those found in the literature, but at the same time the method was relatively quick and did not require highly purified nor large quantities of sample. In another report, Kaliszan and coworkers described the development of a pH and organic solvent double gradient RPHPLC method, attempting to combine the advantages of both gradient separations.[35] A pH 11.5 aqueous buffer was used in this method with a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column packing. The practical utility of the double gradient method was recently demonstrated in the analysis of opipramol from a biological matrix.[36]

The chemical stability of hybrid based bonded phases has led to a variety of reports describing how to use these materials to address many practical separations challenges. In a paper discussing the challenges of analyzing strong bases, McCalley called out the advantages of running at high pH but also warned the user about long term column stability.[37] Comparison of $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid and silica based embedded polar group carbamate bonded phases found the hybrid bonded phase to have generally lower efficiencies in acetonitrile pH 3 and pH 7 phosphate buffer mobile phases. In the same report, reduced base asymmetry factors on the methyl hybrid packing were also achieved using pH 7 mobile phases and elevated temperature. In a later report, McCalley found the reduced asymmetry factors resulted primarily from a reduction in the pK_a of the basic analytes that was caused by the elevated temperature of the mobile phase.[38]

In the area of developing methods for good resolution of analytes, Neue and coworkers described an efficient method development strategy that uses both acidic and alkaline mobile phases (e.g., pH 3, pH 9) to quickly develop good separations.[39] They reported pH provides larger selectivity differences in ionizable analytes than changes in mobile phase organic modifier. C_{18}, embedded polar groups, and phenyl bonded phases on a $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid packing material were employed for their chemical stability, and to further adjust selectivity via the bonded phase structure. Capote and coworkers employed the low silanol activity and stability of a methyl hybrid packing to develop a gradient elution method for the separation of tetracycline, its fermentation impurities, and it degradation products.[40] In that report, they scouted mobile phase pH and temperature values as high as pH 8.0 and 55°C. The authors found that an increase in temperature improved selectivity between critical pair tetracycline/2-acetyl-2-decarboxamidotetracycline, and settled on conditions of pH 7.5 and 40°C to balance separation and column lifetime. Similar mobile phase conditions were employed to achieve a robust method for the separation of oxytetracycline and its impurities.[41] In another method development report, Faux and coworkers employed a 3-(cyclohexylamino)-1-propane-sulfonic acid (CAPS) buffer at pH 10.5 to separate six human immunodeficiency virus protease inhibitors and two non nucleoside reverse transciptase inhibitors in a single run.[42] The authors reported more than three hundred assays were performed on a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid packing material without any chromatographic criteria modification and without any major change in system back pressure. Hybrid based bonded phases have also been used successfully at intermediate pH with potassium phosphate buffers, which are known to degrade many columns fairly quickly. Abuga developed an isocratic method on an embedded polar group RP_{18} bonded hybrid column packing for the impurity profiling of clarithromycin bulk manufacturing samples. The stability indicating method mobile phase consisted of acetonitrile–0.2 M potassium phosphate buffer pH 6.80–water (40:3.5:56.5). The optimum column temperature was reported to be between 50–60°C.[43] Another intermediate pH method on a hybrid based packing was reported for the analysis of azithromycin, its synthesis intermediates, and its degradation product in bulk samples.[44] In this case, the mobile phase consisted of acetonitrile–0.2 M K_2HPO_4 pH 6.5–water (35:10:55) run at a column temperature of 70°C. An embedded polar group, RP_{18} bonded hybrid column packing was used in a similar manner as above for the separation of erythromycin, its known related substances, and its degradation products.[45] The mobile phase consisted of acetonitrile–0.2 M potassium phosphate buffer pH 7.0–water (35:5:60), and the column temperature was reported to be between 60–70°C. When compared against a method run at pH 9 on a polymeric column material, the hybrid separation afforded improved selectivity and efficiency. The hybrid column was stable for about 3 months under these conditions. As expected, the polymeric column showed no chemical degradation in the same time period.

In the area of preparative HPLC, Neue and coworkers have shown that the preparative loadability of basic analytes can be increased up to twenty fold by employing alkaline mobile phases.[46] The authors employed a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column packing to run preparative scale separations at pH 10. Figure 4 shows the significantly improved loading of three bases, diphenhydramine, oxybutynin, and terfanadine, achieved at pH 10 vs. pH 3. A theoretical account was also presented for why the effect is observed.

Hybrid based column packings have also found utility in several reports that employed MS detection systems. Kim and coworkers recently reported

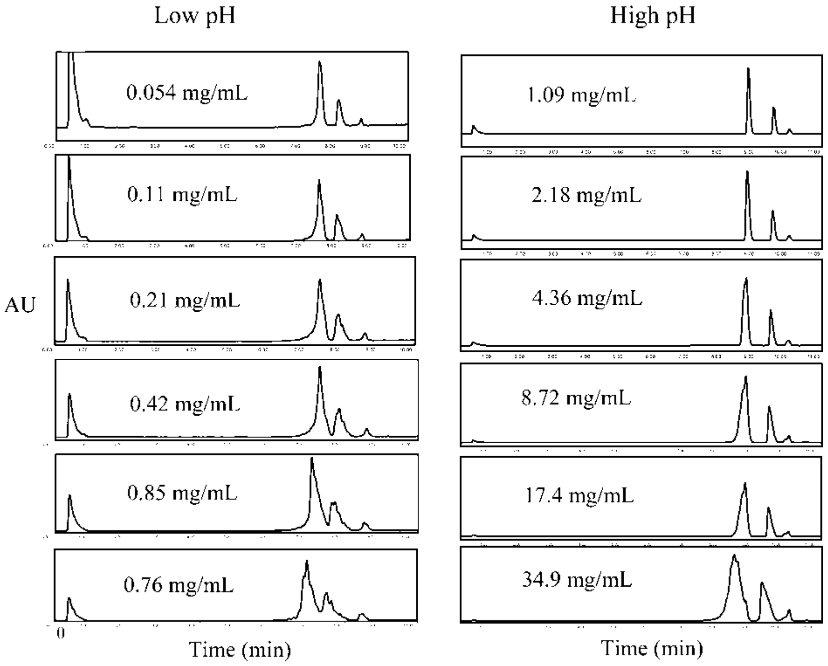

Figure 4. Loading studies for diphenhydramine, oxybutynin and terfenadine at acidic and basic pH. Column: XTerraPrep MS C_{18}, 50 mm × 19 mm, 5 μm. Conditions: gradient from 5 to 95% acetonitrile over 5 min with a 7 min pre-equilibration in 5% acetonitrile. Buffers: 10 mM ammonium formate at pH 3.75 and 10 mM ammonium hydrogen carbonate at pH 10.0. The samples were dissolved in DMSO. Flow rate: 30 mL/min (=3.2 column volumes per minute). Detection: UV, 254 nm. Elution order: diphenhydramine, oxybutynin, terfenadine. Reprinted from reference,[46] Copyright 2003, with permission from Elsevier.

a LC-MS/MS method to measure ambroxol in human plasma down to a quantification limit of 0.2 ng/mL.[47] The MS system was run in positive ion mode. Plasma samples were extracted with diethyl ether under basic conditions, and centrifuged. The supernatant was evaporated, reconstituted, and then injected onto C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column packing using an acetonitrile: 20 mM ammonium acetate pH 8.8 (10:90) mobile phase. The authors reported that a 3.5 μm particle size column used in this way lasted for at least 600 injections. In another MS application, Mroczek and coworkers reported an on-line characterization of a series of novel pyrrolozidine alkaloids and their N-oxides from two plant species.[48] An ion trap MS method was employed with an APCI interface. A 15 mM ammonia in acetonitrile mobile phase was used. The authors reported that the alkaline mobile phase under RP mode gave improved peak symmetry and resolution over a previously used ion pair HPLC method or a RPHPLC method run in an acidic mobile phase. The chemical stability of the hybrid packing was also advantageously used to analyze an acidic compound using MS detection. Cheng and coworkers found that the MRM negative ion response for ibuprofen went up about a factor of 40 by going from a pH 2.7 to pH 8 when run on a methyl hybrid column packing.[49] The authors attributed this gain to increased deprotonation of the acidic analyte.

Hybrid based materials in HPLC have also been used successfully with super heated water and deuterium oxide (D_2O) mobile phases. In one report, Saha and coworkers separated a methanolic extract of ginger powder on an embedded polar group RP_{18} bonded hybrid column packing using super heated D_2O.[50] A temperature gradient method (50 to 130°C at 4°C/min afforded a good separation, and the separated components were then analyzed by an on-line NMR spectrometer. Attempts to separate the extract on a polybutadiene coated zirconia phase failed due to low efficiency. In another report, Louden and coworkers analyzed crude extracts of ecdysteroid containing plants using super heated D_2O as a mobile phase on a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column.[51] A column temperature of 160°C was employed to elute the ecdysteroids into an on line set of UV, IR, MS, and NMR spectrometers for full characterization. An earlier report employed similar chromatographic conditions to separate and characterize on line a set of model pharmaceutical compounds including acetaminophen, antipyrine, 4-aminoantipyrine, norantipyrine, caffeine, phenacetin, p-aminobenzoic acid, propranolol, sufacetamide, and sulfanilamide.[52] In a most recent report, a C_{18} bonded $SiO_2(O_{1.5}SiCH_2\text{-}CH_2SiO_{1.5})_{0.25}$ hybrid column packing was evaluated using pure high temperature water as a mobile phase at temperatures up to 200°C.[53] The authors observed linear van't Hoff plots for alkyl benzenes and aromatic alcohols from 150 to 200°C, but non linear behavior for toluene from 30 to 200°C when 30% acetonitrile was added to the mobile phase. The authors attributed the non linear behavior to a conformational change in the stationary phase at about 97°C. In a separate stability study with a pure water mobile phase, retention time for butylbenzene was found to degrade by 10% after a 1 month period of continuous running at 200°C.

Hybrid based packings have also been used advantageously in the separation of biomolecules. Gilar and co workers reported an ion pair RPHPLC method for the separation of synthetic oligonucleotides on a 2.5 μm C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column packing.[54] The use of small particle size, elevated column temperatures, and relatively slow flow rates afforded an efficient separation of up to 30mer oligonucleotides on a 50 mm length column. The method separated "trityl off" oligonucleotides, and about 1000 analyses could be run in 0.1 M triethylammonium acetate–acetonitrile mobile phases at 60°C. As part of the study, a mathematical model was developed to predict oligonucleotide retention from its sequence and length. The authors extended this technology to the separation and purification of dye labeled oligonucleotides.[55] MS compatible mobile phase conditions were developed, and purification of a 0.1 μmole oligonucleotide synthesis in a single injection was demonstrated. In another report, highly efficient separations were developed for native and chemically modified antisense oligonucleotides from their metabolites or failure synthetic products.[56] Taylor and coworkers used a C_{18} bonded $SiO_2(CH_3SiO_{1.5})_{0.5}$ hybrid column to separate out nine peptides with insecticide properties from the extracts of protein enriched flour of commercial field peas.[57] The peptides were resolved using a gradient method of 10 mM aqueous ammonium hydroxide and acetonitrile with a column temperature of 50°C. The peptides' molecular masses were then measured directly with ESI mass spectroscopy. By comparison, the authors found that a C_{18} silica column only resolved three peptides under acidic mobile phase conditions.

Hybrid Particles in UPLC

Particle based LC separations have benefited in terms of speed and resolution with the development of small particle (<2 μm) packed microbore (1–2 mm ID) and capillary columns (10–150 μm ID).[58–60] The increase in efficiency due to small particle packed columns results from improved mass transfer, reduced band broadening, and diminished eddy diffusion. For all the theoretical benefits, wide spread adoption of small particle technologies has been limited for practical reasons.[61,62] LC instruments did not have pumps able to routinely operate at the pressures created by columns packed with <2 μm particles. The instruments also failed to have the low band spread and more sensitive detectors required to realize the benefits of such columns. For the column itself, <2 μm packed columns were only available to those who had expertise in particle synthesis and column packing.

Several academic groups have addressed the aforementioned instrument issues. LC pumping systems have been designed and built that can achieve the necessary pressures (3,450–6,800 bar or 50,000–100,000 psi) to deliver mobile phases at the required linear velocities for longer length capillary columns (30–50 cm).[61,63–66] Commercially available systems have been recently introduced that can achieve the necessary pressures (1,050 bar or 15,000 psi) required for microbore columns (2–15 cm).[62]

Until recently, $<2\,\mu m$ particle technology was limited to nonporous materials. Highly spherical monodispersed silica particles were typically prepared from the homogeneous Stöber type sol-gel process.[67–69] Alternately, a heterogeneous growth condensation reaction could be employed using tetraalkoxysilanes.[70,71] The synthesis of uniform nonporous hybrid organic/inorganic particles were first reported in the mid 80's using growth processes, as well as emulsion condensation reactions similar to those used for silica.[72–77] In 1997, Unger and coworkers reported the use of a mixed condensation of tetraethoxysilane with octadecyltrimethoxysilane to grow monodisperse hybrid particles between 0.1–2 μm.[78,79] In a subsequent reaction step, the C_{18} group was removed by calcination as a means to introduce porosity. More recently, Colón and coworkers reported the use of a similar condensation growth process of tetraethoxysilane and octyltriethoxysilane to prepare 0.1–0.7 μm hybrid nonporous particles.[80] By eliminating the calcination step they were able to prepare unique hydrophobic nonporous particles for CEC and UHPLC applications that did not require an additional step to make the bonded phase. The use of C_{18} nonporous hybrid particles, packed into 50 μm capillaries, showed good efficiencies, short run times, and great chemical stability after equilibration in an acetonitrile/water mobile phase at pH <1 (35 h or 90 column volumes) and pH 11.7 (10 h or 285 column volumes).[65]

The major drawback to the use of nonporous particles (both silica and hybrid) as packing materials has been the low specific surface areas (SSA, typically $<3\,m^2/g$) of these materials.[63,81] The lower retentivity and loading capacity of separations performed using nonporous particles vs. separations performed with porous particles (SSA 150–300 m^2/g and SPV 0.7–0.9 mL/g) can be understood by comparing the phase ratio (β) of these materials. Phase ratio is a comparison of the surface area of the packed chromatographic bed to volume of mobile phase in the column.[82] The phase ratio determined for most nonporous particles (1–2 μm) is normally $<10\,m^2/mL$. In contrast, porous particles can have phase ratios ranging from 80–200 m^2/mL. An 8-20-fold increase is expected in loading capacity and retentivity when porous particles are employed instead of non-porous partcles.[81] Thus, readily available, porous reversed-phase packing materials have been needed for UPLC to reach its true and practical potential.

Commercial suppliers have recently begun to offer $<2\,\mu m$ reversed-phase silica microbore columns, but these columns have been found to suffer from low efficiency caused by overly broad particle size distributions.[66] Regardless of the column's efficiency, a silica based column would still suffer from high silanol activity and poor chemical stability in alkaline mobile phases (vs. a hybrid based packing). Waters recently introduced a commercially available porous <2 μm hybrid reversed-phase column. The nominal 1.7 μm particle had the empirical formula $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ and was prepared from the cocondensation of 1,2-bis(triethoxysilyl)ethane with tetraethoxysilane, and these hybrid based column packings were found to have low silanol activity and very good lifetime in alkaline mobile phases as described in the HPLC section above.

The combined availability of both <2 μm porous hybrid packings and UPLC instrumentation has initiated further chromatography mechanism studies and application reports from the broader population of chromatography end users. In 2005, Kele and coworkers compared the performance of 1.7 μm C_{18} bonded $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ hybrid column packings with other commercially available silica based <2 μm columns.[83,84] The hybrid column showed increased column efficiency, mechanical stability, and chemical stability when used with low (pH 2) or high (pH 11.3) pH mobile phases at elevated temperature (60°C). Thermal properties of the 1.7 μm C_{18} bonded $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ hybrid packed columns were studied under isothermal, non-isothermal, and adiabatic conditions, and were not found to severely limit the use of <2 μm particles for UPLC.[85]

Different 1.7 μm bonded phases on $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ hybrid packings have been evaluated in microbore columns for rapid method development, showing distinct selectivity differences between different surface bondings (e.g., C_{18}, C_8, embedded polar group RP_{18}, and phenyl) and improved gradient peak capacities.[86–88] Metabonomic LC-MS profiling of rat and other biological matrices using microbore columns has shown improved chromatographic resolution and a dramatic reduction in analysis time.[89–91] In another report, an UPLC system with a 1.7 μm C_{18} hybrid column was compared against a HPLC system with a 3.5 μm C_{18} silica column used in the analysis of *in vitro* metabolized dextromethrophan.[92] The authors reported significant gains in sensitivity, chromatographic resolution, and speed of analysis for the UPLC method.

The use of even smaller 1.5 μm C_{18} bonded $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ hybrid particles packed in capillaries (30 μm ID, 15–50 cm in length) has been explored by Jorgenson and coworkers. Their initial studies showed that hybrid columns had similar performance and mechanical stability to 1.0 μm nonporous silica columns at ultra-high pressures (4,500 bar or 65,000 psi). Increased loading capacities (35 fold) and retentivity were obtained on the C_{18} hybrid columns when compared to nonporous silica.[81] The authors established this increased loading by injecting increasing concentrations of ascorbic acid, hydroquinone, and 4-methyl-catechol onto columns packed with the porous hybrid and non porous silica materials. Using electrochemical detection, peak areas were ascribed to the total amount of analyte injected into the column, and overloading was then taken to be indicated by an increase in the 4σ peak width as a function of peak area. Figure 5 illustrates these findings. An exponential function was fit to the data to calculate the point at which peak widths increased by more

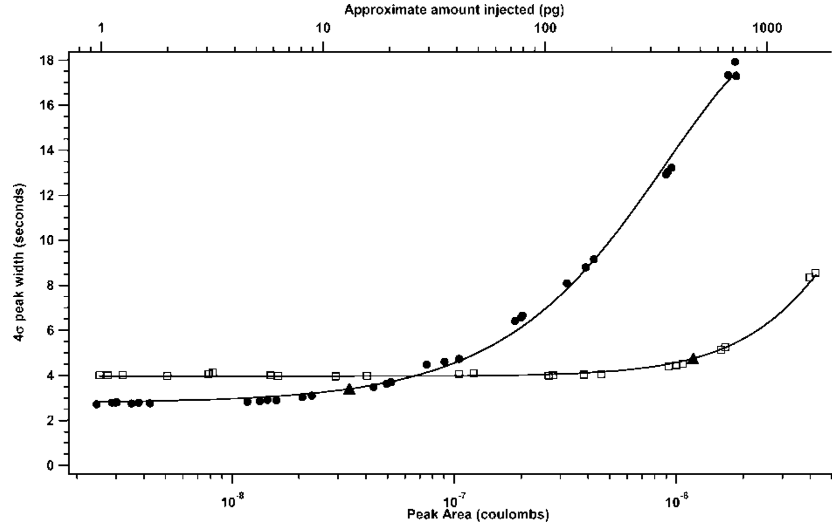

Figure 5. Overloading of 4-methylcatechol on nonporous silica (●) and porous ethyl-bridged hybrid (γ) columns. Overloading is indicated by an increase in the peak width versus area. Traces through the markers are exponential fit functions used to calculate the peak areas at which the peak widths increased by 20% over their initial values (▲). Reprinted with permission from reference,[81] Copyright 2004 American Chemical Society.

than 20% over their initial values, and this calculated value was defined to be the onset of overloading for each column (closed triangles in Figure 5).

Improved gradient loading and peak capacities vs. nonporous silica capillary columns were also observed by Smith and coworkers for tryptic digests separations.[63] Jorgenson and coworkers also reported the use of C_{18} bonded $SiO_2(O_{1.5}SiCH_2CH_2SiO_{1.5})_{0.25}$ hybrid packings in capillary columns for intact proteins separations, showing improved MS signal-to-noise and dramatically increased protein recovery with respect to nonporous silica columns at pressures >1,500 bar.[93] Certainly, new reports are expected as porous hybrid organic-inorganic column packing materials and UPLC capable instrumentation become more available to the separation science community.

CONCLUSIONS

Hybrid materials chemistry represents an emerging and increasingly vast technology that continues to provide advantages over many traditional inorganic or organic based materials in a multitude of products, including liquid chromatography media. Porous sol-gel type hybrid particles described here involve simultaneous formation of the organic (organosiloxane) and inorganic (silicon dioxide) building blocks. The RP column packings built on these materials are characterized by low silanol acidity, excellent stability towards alkaline mobile phases, and good mechanical strength. From fundamental retention studies to new methods, HPLC and UPLC separation science has clearly benefited from the attributes of hybrid materials. More reports are expected that will utilize the unique properties of hybrid materials. At the same time, a vast number of organic-inorganic combinations remain unexplored at the molecular building block level. This fact hints (if not exclaims) that hybrid technology will continue to contribute to the advancement of the separation sciences.

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