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In-column preparation of a brush-type chiral stationary phase using click chemistry and a silica monolith

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Abstract

Brush-type chiral stationary phases have been prepared both from a silica monolith and, separately, from 10 μm porous silica beads via a process of *in-column* modification including attachment of the chiral selector via copper catalyzed azide-alkyne cycloaddition. Azide functionalities were first introduced on the pore surface of each type of support by reaction with 3-(azidopropyl) trimethoxysilane, followed by immobilization of a proline-derived chiral selector containing an alkyne moiety. This functionalization reaction was carried out in dimethylformamide in the presence of catalytic amounts of copper(I) iodide. The separation performance of these triazole linked stationary phases was demonstrated in enantioseparations of four model analytes, which afforded separation factors as high as 11.4.

Keywords

Chiral stationary phase; Click chemistry; Enantioseparation; Monolithic column; Silica

1 Introduction

Monolithic stationary phases for chromatography emerged in the early 1990s as attractive formats of separation media from membranes [1] to columns [2–4]. Monolithic separation columns provide access to reduced back-pressure and rapid mass transfer kinetics, which enables a reduction in analysis time with the associated benefits of higher sample throughput, a feature that is particularly important for on-line process monitoring. In addition to the “classical” reversed phase separation mode [5–9], monolithic separation media have also been found useful for chiral separations.

The first chiral separations using monoliths were demonstrated with molecularly imprinted synthetic polymers as early as 1993 [10–13]. For example, Matsui *et al.* used an acrylate polymer monolith imprinted with a single enantiomer of phenylalanine anilide to achieve the separation of the racemate of the templating compound in HPLC mode [10]. Enantioselective imprinted monoliths have also been used widely in CEC mode [14–17].

Chiral stationary phases (CSPs) based on porous polymer monoliths have been prepared by incorporating a chiral co-monomer and then used for the separation of amino acid derivatives and benzodiazepines using HPLC in normal phase mode [18]; organic polymer monoliths with

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immobilized human serum albumin have been applied for the separation of the enantiomers of warfarin and tryptophan [19].

Silica monoliths for chiral affinity chromatography have also been prepared by attachment of penicillin G acylase to epoxy modified silica [20]. Chen *et al.* synthesized chiral silica monoliths for ligand-exchange μ -HPLC of amino acid derivatives [21]. Lubda *et al.* attached β -cyclodextrin to silica monoliths and achieved fast reverse phase separations of pharmaceuticals [22] while Bayer *et al.* prepared monolithic CSPs using derivatized β -cyclodextrins [23]. In a series of papers, Chankvetadze *et al.* have described the immobilization of 3,5-dimethylcarbamate derivatives of cellulose and amylose on silica monoliths and the subsequent use of these CSPs for the separation of a variety of chiral compounds in normal phase mode [24–28]. An interesting study conducted by Mallik *et al.* compared separations of racemic warfarin using three different stationary phase formats: a glycidyl methacrylate-co-ethylene dimethacrylate monolith, a Chromolith silica monolith, and a stationary phase consisting of packed 7 μ m spherical silica particles, all modified with α_1 -acid glycoprotein for chiral affinity chromatography. The silica monolith outperformed the other formats in terms of both resolution and separation factor [29].

Brush-type CSPs for the HPLC separations of enantiomers featuring small molecules as the selectors attached to porous silica beads were introduced by Pirkle in the late 1970s [30–32]. Interestingly, much less is known about the preparation and use of their monolithic counterparts. To our knowledge, only one previous example is known in which Lubda and Lindner immobilized tert-butylcarbamoylquinine on a silica monolith and separated N-functionalized amino acids and Suprofen [33].

A key aspect of the preparation of brush-type CSPs is the reaction used to anchor the low molecular weight chiral selector onto the sorbent since the particular reaction affects the properties of the linker, which then also impacts the chiral recognition process. The choice of the selector itself is also dictated by the immobilization reaction since its attachment should be free of side-reactions. The most common approach is that originally reported by Pirkle [30–32] involving substitution reactions using a nucleophile bound to the stationary phase. However, other routes have also been explored. For example, the chiral ligands have been attached to the support through alkene moieties via free radical addition of surface-bound thiols [34,35]. Hydrosilylation using hydride-silica and platinum or rhodium catalysts has also been demonstrated [36–38].

Although the azide-alkyne cycloaddition was first described in 1967 [39], the more recently developed version of this reaction, copper-catalyzed azidealkyne cycloaddition (CuAAC) [40,41], has garnered much attention in the various fields of synthetic chemistry owing to its mild reaction conditions and virtually orthogonal reactivity compared to more traditional methods of organic synthesis [42–45]. This reaction has also been used to afford stationary phases for chromatographic separations. For example, we have developed two complementary approaches to porous polymer beads containing alkyne or azide functionalities and used them to prepare stationary phases for reversed phase and affinity HPLC of peptides and proteins [46]. Finn and coworkers have prepared an azide derivative of agarose beads and used the CuAAC reaction to immobilize an alkyne-modified biotin and a hexapeptide. Alternatively, they also used a complementary alkyne derivative of agarose to prepare a sorbent for the selective separation of a specific antibody [47]. Lindner and coworkers have applied this reaction to attach alkyne-containing derivatives of cinchona alkaloids to azide functionalized silica beads and used these materials for very selective enantioseparations of amino acid derivatives in reverse phase HPLC [35]. Guo *et al.* modified silica particles with azidopropyl (triethoxy)silane and reacted these beads with alkyne containing oligo(ethylene glycol) [48], benzyl propargyl ether, octyne, dodecyne, and propargyl alcohol [49] to obtain

chromatographic stationary phases. The same group then used this approach to immobilize β -cyclodextrin for enantioseparations involving drug racemates and other chiral compounds realizing modest selectivity factors varying from 1.05 to 1.47 [50].

This report demonstrates for the first time the suitability of the versatile copper-catalyzed azide-alkyne cycloaddition for the *in-column* preparation of enantioselective brush-type stationary phases based on monolithic silica and compares the chromatographic performance of the resulting chiral monolithic column with that of a column packed with porous silica particles modified using the same procedure.

2 Experimental

2.1 Materials and Methods

HPLC grade solvents from Fisher Scientific (Pittsburgh, PA) and reagents from Aldrich were used as obtained. Luna spherical porous silica 10 μm particles and the Onyx monolithic silica column in poly(ether-ether-ketone) (PEEK) enclosure were obtained from Phenomenex (Torrance, CA). The specific surface area of the Luna particles was 410 m^2/g as measured in our lab, while product literature quotes the specific surface area of the Onyx monolith to be 300 m^2/g .

Reactions in the synthesis of amino acid derivatives were monitored for completion by TLC on fluorescent silica plates visualized with UV and/or phosphomolybdic acid stain. Elemental analyses were performed at the UC Berkeley Microanalysis Facility.

Isothermal nitrogen adsorption/desorption was carried out using an ASAP 2020 sorptometer from Micromeritics (Norcross, GA). The specific surface area was calculated using the BET model, while mesopores were described using the BJH model. IR spectra were recorded on a 3100 FT-IR spectrometer (Varian, Palo Alto, CA) using thin films between KBr disks or as dispersions in a fused KBr pellet. Gas chromatography was performed with a Hewlett-Packard HP 6890 GC (Hewlett-Packard, Palo Alto, CA) equipped with a 30.0 $\text{m} \times 250 \mu\text{m} \times 0.25 \mu\text{m}$ J&W DB-XLB column and HP 5973 mass selective detector. NMR spectra were obtained with a Bruker Avance 500 instrument (Billerica, MA) using solutions in chloroform-*d* unless noted otherwise. NMR chemical shifts are reported as δ in ppm relative to TMS (δ 0.00), and coupling constants are given as *J* values in Hz; peak multiplicity is described using the following abbreviations: s, singlet; d, doublet; t, triplet; quar, quartet; quin, quintet; dd, doublet of doublets; dt, doublet of triplets; td, triplet of doublets; m, multiplet; br, broad signal. DEPT experiments were used to assign the ^{13}C NMR resonances as CH_3 , CH_2 , CH, or C.

2.2 Chromatography

The 10 μm porous silica particles were packed into 4.6 mm i.d. $\times$ 10 cm long stainless steel columns using conventional slurry packing techniques [51,52]. Silica particles (1.5 g) were suspended in 22 mL of 1:1 toluene/cyclohexanol by manual agitation followed by sonication in an ultrasonic bath for 15 min. The column was filled with the slurry solvent and attached to the reservoir which was then filled with the prepared slurry. The assembly was attached to the Haskel pump (Burbank, CA) and pressurized to 31 MPa using methanol as the pushing solvent. Once 125 mL of solvent had been collected, the gas supply valve to the pump was closed and the pressure was allowed to fall to ambient. The particle bed was then settled by a series of three rapid re-pressurizations from ambient to the packing pressure of 31 MPa. The column was then removed from the apparatus and quickly sealed.

Separations were carried out using a Waters system (Medford, MA) comprised of a 2695 separations module and 996 photodiode array detector controlled using Empower software. UV data extracted at 210 nm were analyzed using the PeakFit v4.12 program from Systat

Software (Richmond, CA). All chromatograms were baseline corrected using PeakFit's non-parametric correction algorithm and peaks were fit to an exponentially-modified Gaussian function. Plate numbers were calculated from peak widths at half-height.

Separation factors k were calculated using equation $k = (t_r - t_o)/t_o$ where t_r is the retention time of the analyte enantiomer and t_o is the elution time of the unretained void volume marker 1,3,5-tri-*t*-butylbenzene. Selectivity factors α were calculated using equation $\alpha = k_2/k_1$ where subscripts 1 and 2 refer to the less and more retained enantiomer, respectively. Asymmetry values were calculated as the ratio of the back half-width of the peak to the front-half-width at 10% peak height.

2.3 Syntheses

Racemic analytes N-(3,5-dinitrobenzoyl)leucine diallyl amide **3** and diethyl amide **4** as well as N-(3,5-dinitrobenzoyl)alanine diallyl amide **5** and diethyl amide **6** were synthesized from the parent free amino acids as described elsewhere [53,54].

To prepare (3-azidopropyl)trimethoxysilane **1**, sodium azide (120 mmol, 7.80 g) and sodium iodide (18.0 mmol, 2.70 g) were stirred together in 200 mL anhydrous dimethylsulfoxide (DMSO) and (3-chloropropyl)trimethoxysilane (60.0 mmol, 11.9 g) was added in one portion. The reaction was followed by withdrawing a sample, diluting it with an equal volume of CDCl₃, filtering through a 0.2 μ m PTFE syringe filter to removed precipitated salts, and analyzing by ¹H NMR. The chemical shift of the triplet corresponding to the CH₂ group at the 3 position decreases from δ 3.5 to δ 3.2 upon substitution of chloride by azide. Complete conversion was observed after heating at 60 °C for 6 h and was accompanied by formation of a fine, white precipitate. After cooling the vessel to room temperature, 200 mL hexanes were added and the mixture was stirred vigorously for 1 h to extract the DMSO layer. After the two layers had separated, the hexanes phase was mostly removed to a clean flask via cannula under nitrogen pressure and concentrated *in vacuo* to obtain a clear, colorless liquid. The extraction procedure was repeated 4 more times to obtain a combined total of 9.7 g of crude product. ¹H NMR analysis indicates that the crude product contains 6 % w/w DMSO which was not expected to interfere with subsequent use and the crude material was used as such with all quantities being corrected for the purity of the reagent. Yield 9.7 g crude = 9.1 g pure compound, 44 mmol, 74 %. ¹H NMR (500 MHz): δ 0.603 (t, 2, J = 8), 1.616 (m, 2, J₁ = 7, J₂ = 8), 3.177 (t, 2, J = 7), 3.485 (s, 9). ¹³C NMR (125 MHz): δ 6.34 (CH₂), 22.49 (CH₂), 50.58 (CH₃), 53.74 (CH₂). ²⁹Si NMR (99 MHz): δ -42.87. ν_{max} (cm⁻¹): 2944, 2842, 2098, 1683, 1464, 1192, 1090, 821.

N-prolinoyl-5-aminoindan was prepared as described previously [55]. ¹H NMR (500 MHz): δ 1.714 (m, 2), 2.037 (m, 3), 2.156 (m, 1), 2.494 (s, 1), 2.848 (dt, 4, J = 7.5, 11.5), 2.935 (m, 1), 3.019 (m, 1), 3.805 (dd, 1, J = 5, 9), 7.124 (d, 1, J = 8), 7.263 (dd, 1, J = 1.75, 8), 7.545 (s, 1), 9.692 (s, 1). ¹³C NMR (125 MHz): δ 25.61 (CH₂), 26.23 (CH₂), 30.77 (CH₂), 32.31 (CH₂), 32.98 (CH₂), 47.27 (CH₂), 60.98 (CH), 115.58 (CH), 117.34 (CH), 124.33 (CH), 136.06 (C), 139.67 (C), 144.97 (C), 173.16 (C).

N-(N-(5-hexynoyl)prolinoyl)-5-aminoindan **2**, was prepared from a mixture of N-prolinoyl-5-aminoindan (2.5 mmol, 576 mg), 5-hexynoic acid (3.75 mmol, 420 mg), 4-dimethylaminopyridine (0.25 mmol, 31 mg), and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (5 mmol, 959 mg) that was dissolved under stirring in 20 mL dry dichloromethane (DCM). After 3 days, the clear yellow solution was diluted with 10 mL DCM, poured into a separatory funnel, and washed sequentially with 15 mL each of 5 % acetic acid, water, 10 % aqueous sodium hydrogen carbonate, and brine. The DCM solution was dried over magnesium sulfate, filtered and concentrated *in vacuo* giving 822 mg of viscous, amber oil. This oil was purified on a silica column eluting with an ethyl acetate gradient in hexanes

to obtain 650 mg of clear, colorless oil (2.00 mmol, 80 % yield). ^1H NMR (500 MHz): δ 1.81 (m, 2), 1.89 (quin, 2, $J = 7$), 1.95 (t, 1, $J = 2.8$), 2.01, (quin, 2, $J = 7.5$), 2.15 (m, 1), 2.28 (td, 2, $J = 2.8, 6.8$), 2.48 (td, 2, $J = 3, 7.3$), 2.53 (dd, 1, $J = 7, 11.5$), 2.81 (quar, 4, $J = 7.4$), 3.47 (quar, 1, $J = 7$), 3.57 (td, 1, $J = 2.3, 9.1$), 4.75 (d, 1, $J = 7.5$), 7.07 (d, 1, $J = 8$), 7.16 (d, 1, $J = 8$), 7.42 (s, 1), 9.52 (br s, 1). ^{13}C NMR (125 MHz): δ 18.02 (CH_2), 23.47 (CH_2), 25.21 (CH_2), 25.77 (CH_2), 26.84 (CH_2), 32.49 (CH_2), 33.10 (CH_2), 33.13 (CH_2), 47.81 (CH_2), 60.67 (CH), 69.42 (CH), 83.70 (C), 116.17 (CH), 117.93 (CH), 124.47 (CH), 136.62 (C), 139.89 (C), 145.11 (C), 168.98 (C), 173.49 (C). ν_{max} (cm^{-1}) 3284, 2949, 1686, 1673, 1619, 1547, 1491, 1426, 1325, 1282, 1198, 818. HRMS (FAB): m/z found $[\text{M}^+]$ 384.183420, $\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2$ requires 324.183778.

2.4 In-column modification

The apparatus used for *in-column* modifications of silica-containing columns comprised the Waters 515 HPLC pump connected to an empty 250×4.6 mm i.d. HPLC column tube serving as the reagent reservoir. The solvent from the pump was delivered to the top of this reservoir and its outlet was connected to the silica-containing column located in a chromatographic oven enabling precise control of temperature during modification reactions. All connections were made with stainless steel capillary tubing.

For silanization with **1**, the silica-containing column was kept in the oven at a desired temperature, flushed with 100 mL anhydrous toluene and then the reservoir was opened, emptied, and re-filled with a 30 % w/v solution of **1** in anhydrous toluene. The pump was run at a flow rate of 0.5 mL/min for 1 minute to displace approximately 1 void volume of the column, and then the flow rate was reduced to 0.1 mL/min for 1 h. The oven was then switched off and the column flushed with 100 mL dry toluene at ambient temperature.

The procedure and conditions for end-capping of stationary phases with 1,1,1,3,3,3-hexamethyldisilazane (HMDS) were identical to those used for silanization with **1**, except for using a 15 % w/v solution of HMDS in toluene instead of **1**.

To modify the azidopropyl functionalities attached to both types of silica phases using the copper-catalyzed azide-alkyne cycloaddition, the column was flushed with 100 mL anhydrous dimethylformamide (DMF) and then the reservoir was filled with a solution of **2** (436 mg, 1.34 mmol), *N,N*-diisopropylethylamine (DIPEA) (520 mg, 4.03 mmol), and copper iodide (13.3 mg, 69.8 μmol) in 2.3 mL DMF. The reservoir was pressurized with nitrogen to minimize dilution of the reagent and the effluent was collected in a 10 mL graduated cylinder. When 3 mL of effluent had been collected, the column was removed, sealed with end plugs and kept at ambient temperature for 2 days. At this time the column was reconnected and flushed with 100 mL DMF. The solvent in the system was changed to water and the reservoir was then filled with 25 mM disodium salt of EDTA in water. This was pumped through at 0.5 mL/min until 100 mL H_2O had been collected. The column was then washed with 100 mL of acetonitrile and 50 mL 2-propanol before conditioning with mobile phase until a stable baseline was achieved.

3 Results and discussion

3.1 Preparation of CSPs

In-column modification of HPLC stationary phases is a useful technique due to the ease with which various bonded phases may be produced independent of parameters used to pack the sorbent [56,57]. While particulate media may be modified in batch mode before packing the column, functionalization using flow-through techniques is required for silica monoliths encased in polymer or prepared in capillaries in order to obtain the desired stationary phase displaying homogenous coverage of the support [22]. To carry out the in-column modification

of our HPLC columns, we assembled an apparatus from standard HPLC hardware with an empty column tube placed between the pump and the column containing the silica support, which enables the use of a variety of reagents without the risk of damaging the pump. For example, the CuAAC reaction is sometimes accompanied by deposition of metallic copper due to disproportionation of the copper(I) catalyst which is highly undesirable to occur inside the pump.

To estimate the effects of mixing due to the incoming solvent on the reagent concentration in the reservoir, we filled the reservoir with a solution of aniline in toluene, then toluene was pumped in for a fixed period of time. The reservoir was then opened, its contents were removed and subjected to analysis by gas chromatography. This showed that after one reservoir volume of toluene had been pumped through the system, the amount of aniline in the reservoir had fallen to about 10% of its original concentration. Although this result is likely to vary slightly depending on flow rate and solvent viscosity, it is a useful estimate of the rate of reagent delivery in the modified column.

The first step in the preparation of a CSP using the CuAAC reaction involves incorporation of one of the coupling partners onto the surface of the silica stationary phase material. Surface modification with azide groups is commonly used due to the commercial availability of functional haloalkylsilanes, which may be converted to azidoalkylsilanes by simple treatment with sodium azide. In contrast, alkyne modified silica has only been reported once previously [50]. Starting from 3-(chloropropyl)trimethoxysilane we prepared 3-(azidopropyl)trimethoxysilane and proceeded to surface modify the silica as shown in Figure 1. An initial “batch mode” reaction carried out by treatment of silica particles with an excess of silane **1** as a 10 % w/v solution in refluxing toluene for 1 h affords a loading of 0.31 mmol/g azide functionalities and may be considered the maximum extent of functionalization with this particular reaction system. Modification with **1** is accompanied by the appearance of a peak at 2100 cm^{-1} in the IR spectrum characteristic of the azide moiety. The *in-column* reaction of 10 μm porous beads packed in a stainless steel column was run at $110\text{ }^{\circ}\text{C}$ using a 20 % w/v toluene solution of **1** over the course of 1 h affording beads containing 0.98 % N, corresponding to 0.23 mmol/g azide functionalities and a ligand density of $0.58\text{ }\mu\text{mol/m}^2$.

Preliminary screening experiments were carried out to determine the optimal conditions for CuAAC modification of silica containing azidopropyl functionalities. Three sets of reaction conditions were tested in batch mode using phenylacetylene as the alkyne component and 5 mol% copper catalyst in each case. First, we tested the system previously demonstrated by our group for azide-alkyne modification of polymeric supports involving the use of copper iodide and the accelerating ligand tris-(1-benzyl-[1.2.3]-triazol-4-ylmethyl)amine in acetonitrile [46]. We also used the “click chemistry” conditions described by Vsevolod *et al.* with copper (II) sulfate and sodium ascorbate in aqueous methanol [40]. Finally, we tested the approach reported by Tornøe *et al.* [41] using copper(I) iodide and DIPEA in DMF. Evaluation of the various reactions via elemental analysis for carbon content in the products showed that the latter reaction conditions led to the highest incorporation of organic ligands and to the most pronounced decrease in intensity of the IR peak at 2100 cm^{-1} .

These conditions were subsequently used to modify an azidopropylated particulate silica column with the alkyne containing chiral selector **2** to produce **CSP-1** as shown in Fig. 1. The reaction was initially attempted using a flow-through procedure at $60\text{ }^{\circ}\text{C}$ to accelerate the reaction as the azide-alkyne cycloaddition reactions are known to be slow. Back pressure in the system slowly increased during the reaction until it reached the pressure limit tolerated by the pump. Upon disassembly of the system, the inlet frit of the packed column was found to be blocked with a teal blue solid. The reaction was then repeated at room temperature and this problem was not observed. In order to accommodate the slow kinetics of the reaction a stopped-

flow method was applied in which the packed column was filled with the reaction mixture, the flow stopped, and the reaction then left to proceed. Once the modification reaction was completed, the column was treated with EDTA and thoroughly flushed with water and acetonitrile to remove copper salts.

The two-step modification procedure described above was then applied to a monolithic silica column. The product literature supplied with the Onyx column listed a maximum operating temperature of 45 °C, possibly due to differences in coefficients of thermal expansion of the silica and the PEEK enclosure. Therefore the silanization reaction was carried out at this temperature to avoid compromising the integrity of the monolithic column. To help compensate for the lower reaction temperature used in this case, the concentration of **1** was increased to 40% w/v. Subsequent immobilization of selector **2** via CuAAC proceeded smoothly at room temperature to afford monolithic **CSP-M1**.

In order to establish that the CuAAC reaction is in fact responsible for selector immobilization a negative control experiment was carried out. A packed bed column was carried through the set of transformations described above with the exception that CuI was omitted from the second step. The analyte with the largest α value **3** (see below) was injected on this column in order to give the analysis the greatest sensitivity to the presence of the chiral selector. Only a single peak was observed with a retention factor of 0.15 allowing us to conclude that no selector immobilization takes place in the absence of the copper salt. The low capacity factor highlights an important advantage of using the CuAAC reaction for preparing CSPs. The azidopropyl groups do not create substantial nonspecific retention in the normal phase mode, therefore no end-capping of the residual, unreacted coupling partner is required after immobilization of the chiral selector.

3.2 Separation of racemates

Both triazole-linked CSPs were tested for chiral recognition in normal phase mode using 10% 2-propanol in hexanes as the mobile phase. Fig. 2 shows the structures of four racemic analytes known to be enantioseparated using CSPs containing our selector. Separation factors found for **CSP-1** and **CSP-M1** followed a trend similar to that reported previously for this selector/analyte system [55,58]. Specifically, Table 1 shows that higher α values were observed for derivatives of leucine than for those of alanine, and for diallylamides rather than diethylamides. Chromatograms showing the separations of analyte **3** on packed bed and monolithic columns are presented in Fig. 3.

Both Fig. 3 and Table 1 indicate that the performance of monolithic **CSP-M1** with heavy peak tailing and much lower α values lags behind that of **CSP-1** prepared from silica beads. The likely cause of this poor behavior is the low surface coverage achieved during silanization as a result of the low reaction temperature used for modification of the monolith, thus leaving a large amount of unreacted silanol groups. These cause extensive non-specific interactions, which “dilutes” the effects of chiral discrimination and explains the low separation factors, while the polar interactions between the silanols and the Lewis-basic amide groups of the analytes accounts for the observed tailing.

In an attempt to improve the performance of **CSP-M1**, the remaining silanol functionalities of the monolith were end-capped with trimethylsilyl groups by *in-column* treatment with HMDS in toluene at 45 °C to afford **CSP-M2**. Table 1 shows that the end-capping dramatically improves the performance of the column. The separation factors measured for monolithic **CSP-M2** are close to those measured for its packed bed counterpart **CSP-1**. The retention factors of the less hydrophobic alanine derivatives **5** and **6** decrease for both enantiomers. In contrast, the retention factors of more retained enantiomer of the leucine derivatives **3** and **4** increase, while the retention factor of the less retained enantiomers decrease. The increase in retention

factor for the more retained enantiomer, which is observed despite a reduction in nonspecific interactions, can be attributed to elimination of “phase collapse” where the brush strands lay over on the surface of the stationary phase and the chiral selector interacts with free silanol groups [59]. Elimination of free silanols by end-capping thus increases the availability of the selector strands to interact with the analytes. Fig. 4 shows chromatograms of analyte **3** before and after end-capping of the monolithic CSP.

Asymmetry values were found to be higher in the monolithic CSPs compared to **CSP-1**. The reason for the tailing in the monolith is likely due to two factors. First the tailing is more significant for the longer-retained, more polar compounds, and may result from interactions with residual silanols. This assumption is supported by the observation that end-capping led to a significant improvement in peak asymmetry. However, even for the very weakly retained analyte **3** the asymmetry values are slightly higher than for **CSP-1**. This effect has been noted by other groups comparing monolithic silica and packed bed columns and is ascribed to both structural heterogeneity and mass-transfer kinetic effects [60].

3.3 Effect of flow rate

The effect of mobile phase velocity on column efficiency expressed as plate height was investigated using analyte **3**, which exhibits the highest α value. For a given volumetric flow rate, the efficiency of the monolith is higher than that of the packed bed column. The observed increase in efficiency in the monolithic CSP may be due, at least in part, to the lower linear velocities. Although the two columns exhibit similar back pressure for a given flow rate, the linear flow velocity in the monolithic column is about 75% of that in the packed bed column with spherical 10 μm particles since the monolith has a higher total porosity and thus a larger cross sectional area through which the mobile phase can flow. Additionally, the approximately 1 μm skeleton size of the monolith provides a shorter diffusion path in the mesopores as compared to the 10 μm particles, a feature that also contributes to the higher efficiency in the monolithic column. The van Deemter plots for both enantiomers of analyte **3** are shown in Fig. 5.

The minimum plate heights at a flow rate of 0.5 mL/min for **3** in **CSP-M2** were 20 and 17 μm for the first and second eluting enantiomers, respectively. The efficiency in the monolithic column was always higher for the later eluting enantiomer. The van Deemter curve for monolithic columns is usually reported to remain relatively flat even at high linear velocities. However, in our experiments the loss of efficiency at higher flow rates is evident. A similar behavior was also observed for some compounds by Gerber *et al.* who compared monolithic and packed columns and found a relationship between the decrease in efficiency and molecular size of the analyte [61]. They suggest that this effect results from different mass transfer kinetics within the mesoporous skeletons forming the monolith. Larger molecules have smaller diffusion coefficients and thus do not efficiently penetrate the 13 nm mesopores. The loss of efficiency we observed for **3** (MW = 404) is similar to the losses they observed for a pharmaceutical compound (MW = 811), lending support to our assumption. Other factors that contribute to increased plate height with increasing velocity may also be involved, such as binding-site heterogeneity or slow kinetics of diastereomeric complex formation due to the multi-point nature of the chiral interaction.

4. Concluding Remarks

We have demonstrated the utility of the copper-catalyzed azide-alkyne cycloaddition reaction for *in-column* preparation of a brush-type chiral stationary phase. The surface of silica monoliths or packed beads of 10 μm particles were readily modified with azide groups by treatment with 3-(azidopropyl) trimethoxysilane and then coupled to an alkyne-containing chiral selector. The CuAAC reaction occurs under mild conditions and the residual azide

functionality does not seem to contribute to non-specific retention making this reaction particularly useful for preparation of CSPs. Excellent enantioselectivity towards π -acidic amino acid amide derivatives was observed with both monoliths and beads. Although our *in-column* approach to CSPs is demonstrated with a single selector, it is reasonable to expect that it can also be used for the attachment of other selectors that contain an alkyne functionality.

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Abbreviations

CSP, chiral stationary phase; CuAAC, copper catalyzed azidealkyne cycloaddition; DIPEA, N,N-diisopropylethylamine; HMDS, 1,1,1,3,3,3-hexamethyldisilazane; PEEK, poly(ether-ether-ketone).

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Figure 1.

Copper-catalyzed azide-alkyne cycloaddition immobilization of a chiral selector on azidopropyl silica. Conditions (a) **1**, toluene, Δ ; (b) **2**, CuI, DIPEA, DMF.

R	R'	
	Allyl	Ethyl
Isobutyl	<u>3</u>	<u>4</u>
Methyl	<u>5</u>	<u>6</u>

Figure 2.
Structures of N-(3,5-Dinitrobenzoyl)amino acid dialkyl amide analytes.

Figure 3. Separation of **3** on triazole-linked stationary phases (a) **CSPM1** and (b) **CSP1**. Conditions as in Table 1.

Figure 4. Separation of **3** on monolithic CSPs (a) before and (b) after end-capping. Conditions as in Table 1.

Figure 5. Plate height vs. linear velocity for (a) the first and (b) second eluting enantiomers of **3** on CSP-M2 (●) and CSP1 (■). Conditions: Mobile phase 10% 2-propanol in hexanes, ambient temperature, 1 μL injections of ~1 mg/mL analyte solutions.

Retention and separation factors of amino acid amides on triazole-linked CSPs. Conditions: 10% 2-propanol in hexanes, 0.5 mL/min, ambient temperature, 1 µL injections of 1 mg/mL analyte solutions.

Table 1

Racemat	CSP1 ^a		CSP-M1 ^b		CSP-M2 ^c	
	k ₁	k ₂	α	k ₁	k ₂	α
<u>3</u>	0.77	8.74	11.4	0.11	0.35	3.2
<u>4</u>	0.99	8.49	8.6	0.24	0.52	2.1
<u>5</u>	1.87	14.2	7.6	0.54	1.00	1.9
<u>6</u>	3.64	19.2	5.3	1.70	2.54	1.5
				0.20	0.81	4.1

^aColumn packed with 10 µm silica particles;

^bMonolithic silica column before end-capping;

^cMonolithic silica column after end-capping with trimethylsilyl groups.

Table 2
Peak asymmetry values of amino acid amides on triazole-linked CSPs. Conditions as in Table 1.

Analyte	CSP1		CSP-M1		CSP-M2	
	A _{sl}	A _{s2}	A _{sl}	A _{s2}	A _{sl}	A _{s2}
<u>3</u>	1.00	0.86	2.29	2.41	2.09	1.87
<u>4</u>	0.82	1.11	2.53	2.79	2.17	2.06
<u>5</u>	1.36	1.18	4.05	4.60	2.34	3.08
<u>6</u>	2.42	1.88	6.01	7.29	2.60	4.1