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"Click Chemistry" in the Preparation of Porous Polymer-Based Particulate Stationary Phases for μ-HPLC Separation of Peptides and Proteins

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With the use of the copper(I)-catalyzed (3 + 2) azide−alkyne cycloaddition, an element of "click chemistry," stationary phases carrying long alkyl chains or soybean trypsin inhibitor have been prepared for use in HPLC separations in the reversed-phase and affinity modes, respectively. The ligands were attached via a triazole ring to size monodisperse porous beads containing either alkyne or azide pendant functionalities. Alkyne-containing beads prepared by direct copolymerization of propargyl acrylate with ethylene dimethacrylate were allowed to react with azidooctadecane to give a reversed-phase sorbent. Azide-functionalized beads were prepared by chemical modification of glycidyl methacrylate particles. Subsequent reaction with a terminal aliphatic alkyne produced a reversed-phase sorbent similar to that obtained from the alkyne beads. Soybean trypsin inhibitor was functionalized with *N*-(4-pentynoyloxy)succinimide to carry alkyne groups and then allowed to react with the azide-containing beads to produce an affinity sorbent for trypsin. The performance of these stationary phases was demonstrated with the HPLC separations of a variety of peptides and proteins.

There are many types of columns available for HPLC, both commercial products and those developed in academic laboratories. Most of these use packings based on only two types of porous beads made either of silica or of synthetic polymers. Polymeric stationary phases may be prepared with a broad range of functionalities in a single step by the copolymerization of functional monomers. Although simple, this approach requires reoptimization of the entire preparation process to control the ultimate properties of the beads each time a new monomer is used. Silica-based stationary phases are typically obtained in a two-step process: first, the silica beads themselves are prepared, and then their surface is modified in a separate functionalization step. A similar two-step approach is also used with polymers; a classical example of this process is the preparation of ion-exchange resins by chemical modification of poly(styrene-*co*-divinylbenzene) beads.

The two-step approach is particularly attractive as it enables the formation of numerous stationary phases from a single type of material with optimized size and porous properties. For example, glycidyl methacrylate beads have proven to be rather versatile and have been used to prepare a wide range of stationary phases for HPLC in a variety of separation modes.[1−6] The beads are typically modified using standard electrophile−nucleophile displacement chemistry, or via ring-opening of a pendant reactive moiety such as an epoxide or an azlactone. Although these reactions work well, they limit the type of functionality that can be presented on the surface of the beads as some sensitive functional groups may not survive the modification reaction intact. For example, immobilization of a protein ligand carried out in aqueous solution using beads with azlactone or epoxide groups to form stationary phases for affinity chromatography is accompanied with a spontaneous hydrolysis of the reactive functionalities of the support.

With emerging targets in fields as diverse as environmental studies, proteomics, metabolomics, and biotechnology, high-performance liquid chromatography faces new challenges. Most of these new areas require highly efficient separations of very complex mixtures for which currently available columns may not perform adequately. Therefore, the development of new, versatile separation media is desirable. One approach to achieve this goal is the development of stationary phases with the desired chemistry attached in a controlled manner via the selective reaction of complementary functionalities orthogonal to those that may be found on the ligand to be attached to the bead. The copper(I)-catalyzed (3 + 2) azide−alkyne cycloaddition, an element of the "click chemistry" popularized by Sharpless' group,[7] is a very efficient coupling reaction that provides an ideal reactivity profile

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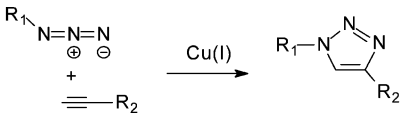
for this purpose. Equation 1 shows a general scheme of the Cu(I)-catalyzed reaction of a terminal alkyne with a terminal azide affording a 1,4-disubstituted triazole ring:



(1)

This reaction has already been widely used for the synthesis of substituted triazoles in solution.[8−12] Because azide and alkyne functionalities do not interfere with common biological processes, the copper-catalyzed azide−alkyne cycloaddition has been used for profiling enzyme activity[13−17] and to incorporate new functionalities in proteins,[18] cells,[19] and viruses.[20] This "click" reaction was also applied for the preparation and functionalization of dendrimers[21,22] as well as for the synthesis[23] and modification[24] of linear polymers. In addition, the utility of this reaction has also been demonstrated in chemical modifications performed on solid polymer supports. For example, it has been integrated with solid-phase peptide synthesis to produce peptidotriazoles[25,26] and for the installation, via a "click linker", of sensitive functionalities on Merrifield resin for solid-phase organic synthesis.[27,28] Finally, Puna et al. have recently prepared an azide derivative of diaminodipropylamine agarose beads and used this click reaction to immobilize alkyne-containing versions of biotin and a hexapeptide.[29] They also used a complementary alkyne derivative of agarose to attach 4-azido-*N*-(4-(4-oxobutoxy)phenyl)butyramide; this aldehyde-containing ligand was then used for the selective separation of a specific antibody.

The objective of this communication is to demonstrate for the first time the suitability of the versatile copper-catalyzed azide−alkyne (3 + 2) cycloaddition for the preparation of efficient stationary phases for the separation of peptides and proteins in HPLC mode.

EXPERIMENTAL SECTION

Materials and Methods. HPLC grade water, acetonitrile (ACN), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF) were purchased from Fisher Scientific (Pittsburgh, PA). All proteins and peptides including ribonuclease A, lysozyme, cytochrome *c*, myoglobin, albumin, trypsin, Phe-Gly-Phe-Gly, bradykinin, angiotensin II, angiotensin I, insulin, and Kunitz-type soybean trypsin inhibitor (SBTI) were obtained from Sigma (St. Louis, MO). Octadecyne was purchased from GFS Chemicals. All other reagents were purchased from Aldrich. Monomers, glycidyl methacrylate, propargyl acrylate, and ethylene dimethacrylate, were distilled under vacuum prior to use; other reagents were used as provided. Polystyrene seed particles were obtained from Bangs Laboratories (Fishers, IN). Polymer beads were physically characterized by isothermal nitrogen adsorption/desorption using an ASAP 2010 sorptometer from Micromeritics (Norcross, GA); surface area was calculated using the BET model, while mesopore information was derived using the BJH model. Reactions were monitored for completion by TLC on fluorescent silica plates, visualizing with UV and/or phosphomolybdic acid stain. Organic extracts were dried over $MgSO_4$ and solvents were removed in vacuo using a rotary evaporator. Elemental analyses were performed at the UC Berkeley Microanalysis Facility. IR spectra were recorded on a Mattson Genesis II FT-IR as thin films between NaCl disks or as dispersions in KBr. NMR spectra were recorded on Bruker AV-400 or DRX-500 instruments as solutions in chloroform-*d*. 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; DEPT experiments were used to assign the ^{13}C NMR resonances as CH_3, CH_2, CH, or C. Teflon-coated 251 μm i.d. capillaries were purchased from Polymicro Technologies (Phoenix, AZ).

Care in Handling Azides. Azides must be handled with care, and all work must be performed behind a safety shield. Avoid high temperatures and reactions that would lead to products containing a high weight percent of the azide moiety in the final isolated product.

1-Azidooctadecane (1). This was prepared by a modification of a procedure described by Alvarez and Alvarez.[30] Sodium azide (7.15 g, 110 mmol) and tetrabutylammonium iodide (20 mg, 54 μmol) were dissolved in DMSO (220 mL); 1-bromooctadecane (33.3 g, 100 mmol) was added, and the biphasic mixture was heated at 80 °C for 16 h. Upon cooling, the mixture was poured into water (200 mL) and then extracted with hexanes (3 × 200 mL); the combined organic extracts were washed with water (3 × 200 mL) and brine (300 mL) and worked up as usual to afford a straw-colored oil. This was purified by flash chromatography on 150 g of silica gel, eluting with petroleum ether; the product **1** was obtained as a clear, colorless oil (28.66 g, 97.0 mmol, 97%

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yield). ^{1}H NMR (400 MHz): δ 3.24 (t, 2H), 1.59 (quin, 2H), 1.26 (br m, 30 H), 0.88 (t, 3H). ^{13}C NMR (125 MHz): δ 14.0 (CH_3), 22.7 (CH_2), 26.7 (CH_2), 28.8 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.50 (CH_2), 29.56 (CH_2), 29.64 (CH_2), 29.67 (CH_2), 29.69 (CH_2), 29.706 (CH_2), 29.714 (CH_2), 29.72 (CH_2), 31.9 (CH_2), 51.4 (CH_2); the peaks were not sufficiently dispersed to identify each C, even when resolution-enhancing processing was used. Elemental Anal. Calcd for $C_{18}H_{37}N_3$: C, 73.2%; H, 12.6%; N, 14.2%. Found: C, 73.6%; H, 12.8%; N, 14.4%.

***N*-(4-Pentynoyloxy)succinimide (2).** 4-Pentynoic acid (1.00 g, 10.2 mmol), *N*-hydroxysuccinimide (2.35 g, 20.4 mmol), *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (2.15 g, 11.2 mmol), and 4-(dimethylamino)pyridine (415 mg, 3.40 mmol) were combined under an Ar atmosphere, and dry DCM (20 mL) was introduced. After stirring for 3 days, the mixture was poured into water containing 5% v/v acetic acid. The layers were separated, and the aqueous phase was extracted with DCM (2 × 25 mL). The combined organic extracts were washed with brine (60 mL) and worked up as usual giving a clear, colorless oil. This was purified using chromatography on 21 g of silica gel, eluting with hexanes with an ethyl acetate gradient (0−100%); the product was obtained as a white solid (0.99 g, 5.1 mmol, 50% yield). Mp: 70−72 °C. ^{1}H NMR (400 MHz): δ 2.08 (t, 1H, $J = 2.5$), 2.61 (td, 2H, $J = 2.5$, $J = 6$), 2.84 (s, 4H), 2.89 (t, 2H, $J = 6$). ^{13}C NMR (100 MHz): δ 13.4 (CH_2), 25.1 (CH_2), 29.6 (CH_2), 69.8 (C), 80.8 (CH), 166.8 (C), 169.2 (C). ν_{max}/cm^{-1}: 2255, 1816, 1790, 1743, 1431, 1373. HRMS (EI): m/z found [MH+] 196.0603, $C_9H_{10}NO_4$ requires 196.0610; found [M+] 195.0532, $C_9H_9NO_4$ requires 195.0532. Elemental Anal. Calcd for $C_9H_9NO_4$: C, 55.4%; H, 4.65%; N, 7.18%. Found: C, 55.1%; H, 4.69%; N, 7.12%.

Monodisperse Porous Polymer Beads. Poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (GMA−EDMA) beads **3** were prepared via a templated suspension polymerization described previously.[31] Poly(propargyl acrylate-*co*-ethylene dimethacrylate) (PA−EDMA) beads **4** were prepared in an analogous manner as follows: polystyrene seed particles with a diameter of 560 nm (0.05 mL total bead volume) were first activated by swelling in an emulsion prepared by sonicating dibutyl phthalate (0.33 mL) in 15 mL of an aqueous solution of SDS (37.5 mg, 0.25% w/v). After primary swelling leading to particles with a diameter of 1 μm was complete, they were added to an emulsion consisting of the porogenic solvents cyclohexanol (18.8 mL) and 1-dodecanol (2.1 mL), monomers EDMA (8.4 mL) and propargyl acrylate (5.6 mL), and initiator AIBN (290 mg) in 80 mL of an aqueous solution of SDS (200 mg, 0.25% w/v). The secondary swelling was allowed to proceed until the calculated bead diameter of 5 μm was obtained. Sufficient 4% w/v poly(vinyl alcohol) (PVA, M_n 106 000−110 000, 100% hydrolyzed) solution was added to the mixture to afford a final concentration of 1% w/v PVA in the polymerization mixture, and $NaNO_2$ (16 mg) was added to suppress polymerization in the aqueous phase. The mixture was sparged with N_2 for 20 min and then polymerized in an orbiting shaker bath at 70 °C for 16 h. The beads were washed by repeated decantation with water, methanol, and THF and then isolated by filtration.

C_{18} RP Stationary Phase from PA−EDMA Beads (5). 1-Azidooctadecane (1.06 g, 3.6 mmol), CuI (23 mg, 120 μmol), and MeCN (5 mL) were combined and sonicated briefly to produce an emulsion. PA−EDMA beads were added, and the mixture was rotated using a rotary evaporator motor at ambient temperature for 18 h; phase separation occurred during this time. The beads were isolated by filtration on a fine glass frit and washed with DCM (20 mL), MeCN (40 mL), 10 mmol/L HCl in 75% MeOH (20 mL), 75% MeOH (20 mL), 5 mmol/L NaOH in 75% MeOH (20 mL), 75% MeOH (20 mL), MeOH (40 mL), THF (40 mL), and Et_2O (40 mL). The beads were air-dried and then placed in a vacuum oven at 40 °C for 14 h. An amount of 237 mg of beads **5** were obtained as a white powder. Elemental analysis of nitrogen indicated that the beads contained 0.54 mmol/g triazole units: C, 63.0%; H, 7.75%; N, 2.29%. ν_{max}/cm^{-1}: 3276, 2977, 2924, 1714(br), 1521, 1475, 1429, 1389; the intensity of the band due to the alkyne C−H stretch (3276) is smaller relative to that of other bands as compared to the spectrum of the starting material.

Azide-Modified GMA−EDMA Beads (6). GMA−EDMA beads **3** (2.00 g, ca. 5 mmol epoxide groups based on monomer ratios) and sodium azide (1.95 g, 30 mmol) were added to a flask, and water (7 mL) and DMF (14 mL) were added. The flask was rotated in an oil bath at 50 °C for 20 h using a rotary evaporator motor. The beads **6** with 3-azido-2-hydroxypropyl functionalities were isolated by filtration on a fine glass frit and washed with DMF (50 mL), water (100 mL), methanol (60 mL), THF (60 mL), and Et_2O (30 mL) before drying in a vacuum oven at 40 °C for 12 h. Incorporation of azide groups was determined by elemental analysis for nitrogen: C, 52.4%; H, 7.73%; N, 6.65%, corresponding to 1.6 mmol/g azide groups. ν_{max} (cm^{-1}): 3324 (v br), 2947, 2892, 2103, 1718 (br), 1475, 1456, 1388.

C_{18} RP Sorbent from Azide-Containing Beads (7). CuI (7.6 mg, 40 μmol) and 1-octadecyne (250 mg) were mixed in MeCN (5 mL) and sonicated briefly to aid in mixing; the emulsion took on a pale yellow hue. Azide-modified beads **6** (250 mg, ∼0.4 mmol azide) were added, and the mixture was rotated at ambient temperature for 2 days. The beads **7** were isolated by filtration on a fine glass frit and washed with $CHCl_3$ (30 mL), MeOH (30 mL), 3 mmol/L disodium EDTA in 10% MeOH (30 mL), H_2O (30 mL), MeOH (30 mL), THF (45 mL), and Et_2O (30 mL), then dried in a vacuum oven at 40 °C for 13 h. Elemental analysis: C, 53.8%; H, 7.62%; N, 6.46%.

Alkyne-Modified Soybean Trypsin Inhibitor (8). SBTI (478 mg) was added to a 50 mL polypropylene vial followed by 25 mL of 10 mmol/L sodium phosphate buffer (pH 7.0); the pH of the resulting solution was 5.6, and some material remained undissolved. NaOH (0.5 mol/L) was added until all of the protein dissolved, and then the pH was adjusted to 7 by dropwise addition of 0.5 mol/L HCl. *N*-(4-Pentynoyloxy)succinimide **9** was dissolved with mild heating in 5.0 mL ethylene glycol, and the solution was added at once to the protein solution. The resulting suspension was rotated for 15 h at ambient temperature; all of the solids dissolved after about 10 h. The pH of the solution fell to 5.3 during the course of the reaction, and some protein precipitated near the end of the reaction time; prior to workup, the pH was raised to 7 using 0.5 mol/L NaOH. The crude protein solution was purified by gel filtration on 50 g of Sephadex G-25 in H_2O. The resulting solution was analyzed by MALDI-TOF MS using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+] m/z 20 500. The observed mass increase relative to that of unmodified SBTI

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([M+] m/z 20 000) indicates acylation of ca. 6 of 10 possible amine sites on the protein.

Attachment of Alkyne-Modified SBTI to Azide-Containing Beads (10). CuI (190 μg, 1.0 μmol) was added as 17.2 μL of a 11.1 mg/mL suspension in H_2O to tris-(1-benzyl-[1,2,3]-triazol-4-ylmethyl)amine[32] (1.1 mg, 2 μmol) in 0.5 mL MeOH. The mixture was sonicated until homogeneous, and then the methanol was evaporated under a stream of argon. The resulting white solid was dissolved in 10 mL of 70 μmol/L alkyne-modified SBTI (as obtained from the gel filtration step above), and azide beads (50 mg, 80 μmol azide) were added. The mixture was slowly agitated for 16 h at ambient temperature. The beads **10** were isolated by centrifugation (60 rpm, 5 min) and then washed by resuspending the solids in water (15 mL), agitating for a few minutes, and centrifuging; this wash step was repeated seven times. The final supernatant was decanted, and the wet beads were packed in a capillary as described below. The absorbance of the supernatant at 280 nm was measured and compared to the value before the reaction; on the basis of this analysis the protein loading of the beads was estimated to be ca. 2 μmol/g. Elemental analysis: C, 52.2%; H, 7.49%; N, 7.61%; S, 0.48%.

Chromatography. Stationary phases for reversed-phase chromatography were packed into the capillary from 6 mg/mL slurry in an isodensity solvent mixture consisting of a 25:75 (v/v) mixture of THF and DCM at a constant pressure of 13.8 MPa. The packed capillary was attached to an HPLC system consisting of an Ultra Plus II pump and pump controller (Micro-Tech Scientific, Vista, CA) and pressurized to 27.6 MPa using 50:50 (v/v) ACN−water mixture until no further compression of sorbent within the capillary was observed. Typically, a compression amounting to 3−5 mm was observed for a 400 mm long packed segment. The packed capillary was cut into 150 mm columns that were then fitted with inlet and outlet fittings. A similar procedure was used for packing columns with sorbent containing immobilized soybean trypsin inhibitor except that water was used instead of organic solvents. The column length was 100 mm.

The HPLC system consisted of the Ultra Plus II pump and pump controller and a UVIS 205 detector (Linear, Thermo Electron, Waltham, MA). The column temperature was held constant at 35 °C using a Polaratherm series 9000 oven (Selerity Technologies Inc., Salt Lake City, UT), unless stated otherwise. The data were processed by Chrom Perfect Spirit (Justice Laboratory, Denville, NJ). Baseline was subtracted in chromatograms of proteins and peptides to eliminate the effects of refractive index variations resulting from the use of the gradient. Typically, the injected sample volume was 0.6 μL. Mixtures of proteins and peptides were prepared by mixing 1 mg/mL stock solutions of individual components. Retention factors k for alkyl benzenes were calculated using equation

$$k = (V_a - V_o)/V_o$$

where V_o and V_a are the retention volumes of acetone and analyte, respectively.

Column Stability. The capillary column packed with sorbent **5** was flushed for almost 400 h representing about 3300 column volumes of 1% trifluoroacetic acid (TFA) at a temperature of 80 °C and flow rate 1 μL/min. At selected times, the temperature in the thermostat was decreased to 35 °C and the column cleaned with a blank gradient of 0−100% ACN in water in 15 min before the test substances were injected. Separations of acetone and benzene were carried out using 50% aqueous ACN. The separations of proteins were achieved using a linear gradient of from 5% to 100% ACN in 0.1% aqueous TFA in 15 min. All these separations were performed at a flow rate of 6 μL/min.

Table 1. Properties of Azide- and Alkyne-Containing Porous Polymer Beads

beads	specific surface area, m^2/g	pore volume mL/g	pore size nm
4	243	0.83	10
6	76	0.35	16

RESULTS AND DISCUSSION

Preparation of Stationary Phases. The triazole forming "click" reaction we used to modify the stationary phases is derived from the original 1,3-dipolar cycloaddition of organic azides to carbon−carbon multiple bonds discovered by Huisgen et al.[33] and later enhanced and popularized by Sharpless.[7] Since both azide and alkyne functional groups are relatively stable to many synthetic procedures, either group can be incorporated on the stationary phase as desired. Thus, the solid support can include alkyne functionality to later react with a ligand bearing an azide group or vice versa. To demonstrate this versatility, we prepared both types of porous polymer beads: azide beads and alkyne beads. First, using a procedure developed earlier in our laboratory,[34] we prepared uniformly sized particles **3** containing reactive epoxide functionalities by the staged, templated polymerization of glycidyl methacrylate. A simple, single-step reaction of these beads with sodium azide in water−DMF afforded beads **6** with the desired azide groups present in 2-hydroxy-3-azidopropyl moieties. Using an analogous approach, we also prepared beads **4** featuring pendant alkyne groups by copolymerization of propargyl acrylate and ethylene dimethacrylate. The properties of these beads are summarized in Table 1.

Both types of beads were then reacted with the complementary reagent, 1-octadecyne and 1-azidooctadecane, respectively, to afford the two analogous hydrophobic stationary phases suitable for reversed-phase HPLC. In both cases the alkyl chains are attached to the polymer support via a triazole ring (see Schemes 1 and 2).

Hydrophobicity and Methylene Selectivity. The effect of surface chemistry on separation properties is easily followed by determination of methylene selectivity. This value is a measure of the ability of the stationary phase to separate alkyl-substituted homologues of benzene. Plots of retention factors for columns packed with the original beads and their alkylated counterparts as recorded for a homologous series of alkylbenzenes, including benzene and its methyl, ethyl, propyl, butyl, and pentyl derivatives, are shown in Figure 1. Clearly, the original propargyl acrylate-

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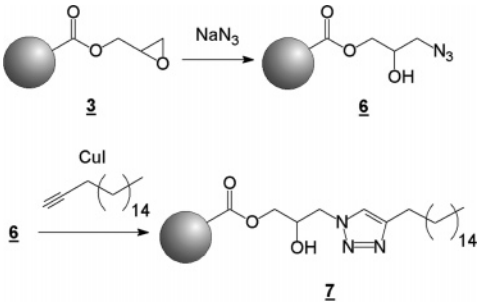
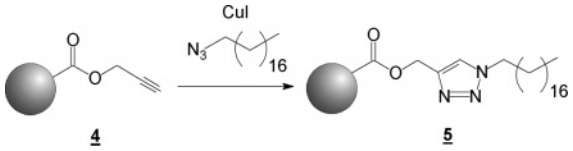
Scheme 1



Scheme 2



based stationary phase itself exhibits significant retentivity, and its alkylation leads to only a small increase in methylene selectivity as demonstrated by the increase in slope of the solid straight lines from 0.12 to 0.18. In contrast, the stationary phase with 2-hydroxy-3-azidopropyl functionalities is rather hydrophilic. Therefore, it shows very little retention toward alkylbenzenes and exhibits a marginal selectivity as indicated by the slope value of only 0.03. This value increases considerably after modification by click chemistry to a slope of 0.17, which is almost identical to that of the alkylated propargyl acrylate-based stationary phase. This increase clearly confirms that the chemical modification using click chemistry affords a retentive hydrophobic surface. On the basis of the results of the elemental analysis of nitrogen, as shown in the Experimental Section, the surface coverage with C16 alkyl chains is 2.2 μmol/m^2. This value is on par with typical monomeric stationary phases for reversed-phase HPLC that contain 2−3.5 μmol/m^2.[35] It is worth noting that in contrast to silica-based phases, the alkyl chains of the polymer backbone also contribute to the overall hydrophobicity of the packing which may then appear higher than expected if only the C16 chains are taken into account.

Typical C18 silica columns exhibit methylene selectivity in the range from 1.3 to 1.6 calculated as the ratio of retention factors for toluene and benzene.[36−38] The values for our stationary phases are 1.44 for **4**, 1.58 for **5**, 0.96 for **6**, and 1.51 for **7**. These high selectivities show that the high hydrophobicities of alkylated packings **5** and **7** exceed those of many ODS columns.

Separation of Proteins and Peptides. Both alkylated stationary phases **5** and **7** were used for the reversed-phase separation of peptides and proteins in the gradient elution mode, and their separation abilities were compared to those of the nonalkylated beads **4** and **6**. As expected from the methylene selectivity data indicating similar properties for **4** and **5**, separations using columns packed with these stationary phases are similar, as shown in Figures 2 and 3. However, the slightly higher hydrophobicity

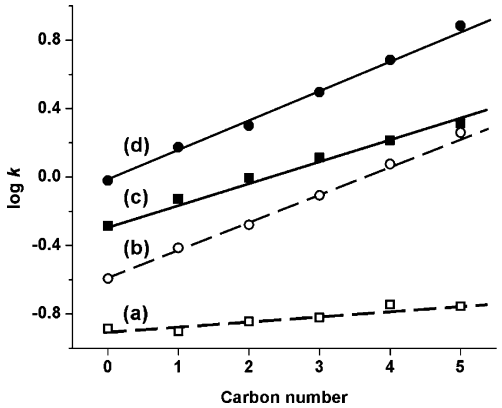


Figure 1. Effect of the number of carbon atoms in the alkyl group of substituted benzene homologues on their retention in columns packed with 2-hydroxy-3-azidopropyl methacrylate (a), alkylated 2-hydroxy-3-azidopropyl methacrylate (b), propargyl acrylate (c), and alkylated propargyl acrylate beads (d). Conditions: column 150 mm × 0.251 mm i.d.; mobile phase 50:50 (v/v) acetonitrile−water; flow rate 6 μL/min; column temperature 35 °C.

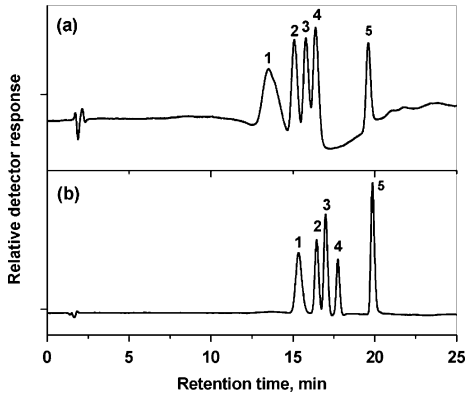


Figure 2. Gradient elution of peptides using columns packed with the original propargyl acrylate beads **4** (a) or the alkylated propargyl acrylate beads **5** (b). Conditions: column 150 mm × 0.251 mm i.d.; mobile phase A, 0.1% aqueous TFA; mobile phase B, acetonitrile; gradient 0−65% B in A in 15 min; flow rate 6 μL/min; column temperature 35 °C. Peaks: Phe-Gly-Phe-Gly (1), bradykinin (2), angiotensin II (3), angiotensin I (4), insulin (5).

of **5** leads to somewhat longer retention times for both peptides and proteins. In addition, the performance of this column is better in terms of peak shape and resolution.

The situation is different for the other pair of stationary phases derived from glycidyl methacrylate beads. As shown in Figure 4, sorbent **6** based on 2-hydroxy-3-azidopropyl methacrylate beads is hydrophilic due to the presence of hydroxyl groups, and thus it does not retain small peptides. The only retained peak is insulin. Similarly, as shown in Figure 5, the separation of proteins using this stationary phase is also poor. As expected, after alkylation of the beads the retention of peptides is significantly enhanced, and very good separation of angiotensin I and insulin can easily be achieved (Figure 4b). The unusual elution pattern with broad tailing peaks of bradykinin and angiotensin II results from the large volume of the gradient mixer and capillary connecting it with the injector. As a result, the gradient of the mobile phase is delayed by about 14 min. Therefore, these two slightly retained peptides elute in the isocratic mode as poor peaks. Since the Phe-Gly-Phe-Gly peptide is not retained and elutes close to the column dead volume, its peak is sharp. However, this stationary phase

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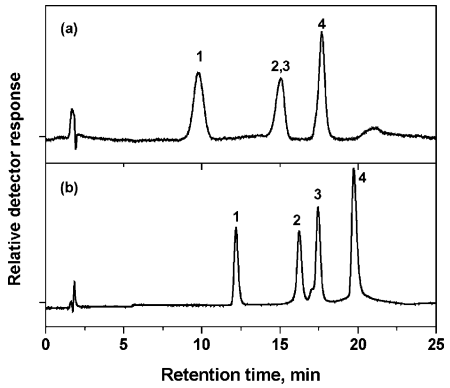


Figure 3. Gradient elution of proteins using columns packed with the original propargyl acrylate beads **4** (a) or with alkylated propargyl acrylate beads **5** (b). Conditions: column 150 mm × 0.251 mm i.d.; mobile phase A, 0.1% aqueous TFA; mobile phase B, acetonitrile; gradient 5−100% B in A in 15 min; flow rate 6 μL/min; column temperature 35 °C. Peaks: ribonuclease A (1), cytochrome *c* (2), lysozyme (3), myoglobin (4).

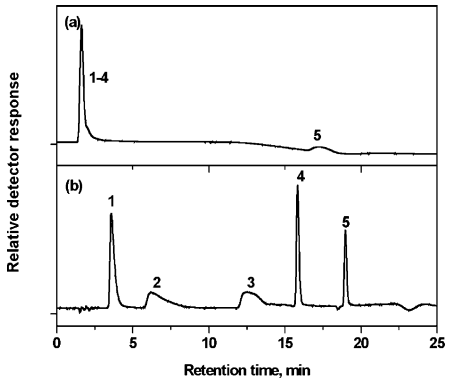


Figure 4. Gradient elution of peptides using columns packed with 2-hydroxy-3-azidopropyl methacrylate beads **6** (a) or with alkylated 2-hydroxy-3-azidopropyl methacrylate beads **7** (b). Conditions: column 150 mm × 0.251 mm i.d.; mobile phase A, 0.1% aqueous TFA; mobile phase B, acetonitrile; gradient 0−65% B in A in 15 min; flow rate 6 μL/min; column temperature 35 °C. Peaks: Phe-Gly-Phe-Gly (1), bradykinin (2), angiotensin II (3), angiotensin I (4), insulin (5).

enables an excellent baseline separation of all five proteins as shown in Figure 5b since all of them are sufficiently retained.

Protein Immobilization and Affinity Chromatography. As discussed above, the chromatographic experiments with 2-hydroxy-3-azidopropyl methacrylate beads clearly demonstrated their hydrophilicity. This feature is particularly advantageous in the design of separation systems for affinity chromatography where nonspecific hydrophobic interactions should be minimized. Accordingly, we prepared a stationary phase for the affinity-based separation of trypsin through the immobilization of soybean trypsin inhibitor (SBTI) onto the hydrophilic beads. As shown in Scheme 3, the SBTI was modified using a mild acylating agent—the *N*-hydroxysuccinimide ester of 4-pentynoic acid, **2**—to provide the necessary complementary alkyne functionality. This modified protein was then attached to the hydrophilic azide beads using a ligand-accelerated version of the copper mediated coupling reaction.

Figure 6 shows that a column packed with beads **10** containing this affinity ligand binds a significant amount of trypsin at neutral pH. Trypsin release is achieved only after the pH is lowered to 2.

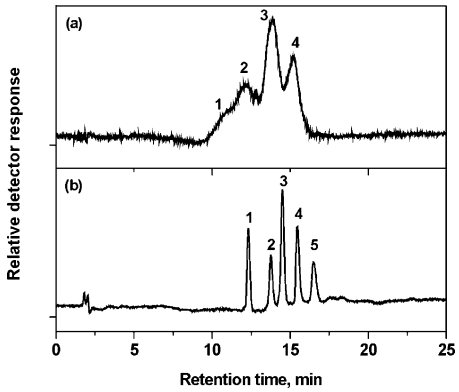
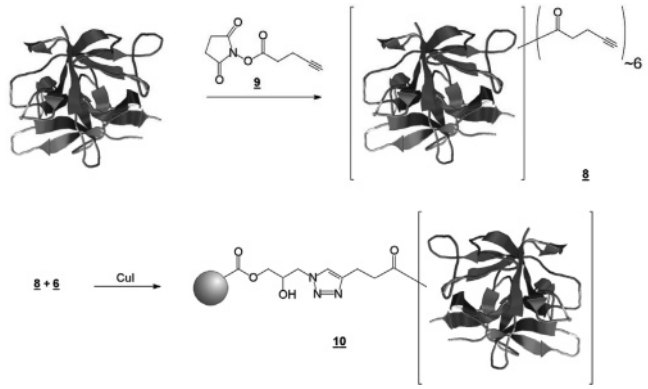


Figure 5. Gradient elution of proteins using columns packed with 2-hydroxy-3-azidopropyl methacrylate beads **6** (a) or with alkylated 2-hydroxy-3-azidopropyl methacrylate beads **7** (b). Conditions: column 150 mm × 0.251 mm i.d.; mobile phase A, 0.1% aqueous TFA; mobile phase B, acetonitrile; gradient 5−100% B in A in 15 min; flow rate 6 μL/min; column temperature 35 °C. Peaks: ribonuclease A (1), cytochrome *c* (2), lysozyme (3), myoglobin (4), albumin (5).

Scheme 3



This behavior confirms the expected formation of a reversible complex between trypsin and SBTI.

Column Stability. We also assessed the stability of the stationary phases containing long alkyl chains attached via the triazole ring under extremely harsh conditions. The capillary column packed with sorbent **5** was flushed with 1% trifluoroacetic acid (TFA) for over 380 h, representing about 3300 column volumes, at a high temperature of 80 °C. Despite these treatment conditions, repeated measurements of the retention of both

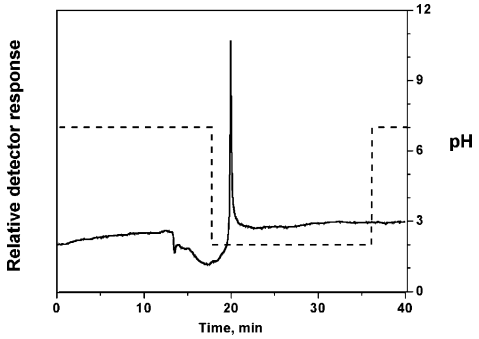


Figure 6. Affinity chromatography of trypsin using a column packed with 2-hydroxy-3-azidopropyl methacrylate beads containing immobilized soybean trypsin inhibitor **10**. Conditions: injection of 1.0 μg of trypsin in an aqueous solution of pH 7; desorption at pH 2 (0.1% aqueous TFA); flow rate 6 μL/min; column temperature 23 °C.

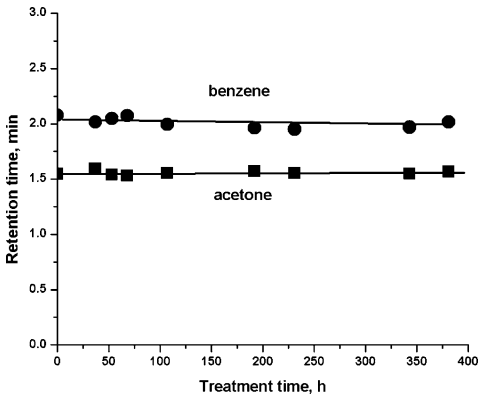


Figure 7. Effect of treatment of the column using 1.0% aqueous trifluoroacetic acid at 80 °C on the retention of acetone and benzene. Conditions: column 150 mm × 0.251 mm i.d.; mobile phase 50:50 (v/v) acetonitrile−water; flow rate 6 μL/min; column temperature 35 °C.

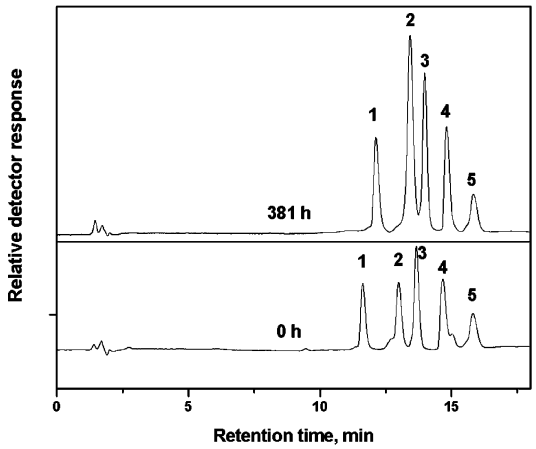


Figure 8. Separation of standard proteins using a column packed with beads **7** immediately after its preparation and after 381 h of treatment with 1.0% aqueous trifluoroacetic acid at 80 °C. Conditions: column 150 mm × 0.251 mm i.d.; mobile phase A, 0.1% aqueous TFA; mobile phase B, acetonitrile; gradient 5−100% B in A in 15 min; flow rate 6 μL/min; column temperature 35 °C. Peaks: ribonuclease A (1), cytochrome *c* (2), lysozyme (3), myoglobin (4), albumin (5).

acetone and benzene shown in Figure 7 do not exhibit any appreciable variation. Similarly, separations of proteins presented in Figure 8 vary very little. Interestingly, the retention of the less retained proteins slightly increases suggesting an increase in hydrophobicity of the stationary phase. These observations clearly confirm the excellent stability of the support−ligand linkage.

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CONCLUSION

In this short communication we have presented the application of "click chemistry" in HPLC and demonstrated that this approach can afford stationary phases with well-defined chromatographic properties. Two approaches were used in this work; the reaction of alkyne-carrying beads with azide-containing small molecules gave similar end results as the inverse arrangement of functional groups, as good surface coverage is obtained in each case. However, the azide-functionalized support proved to be more versatile as its hydrophilic nature can be exploited in systems where the undesirable nonspecific interactions of the supporting matrix with biological polymers such as proteins should be minimized. A closer look at the attachment chemistry shown in Schemes 1 and 2 reveals its similarity with columns featuring polar groups such as amide, carbamate, urea, sulfonamide, and others embedded between the hydrophobic alkyl chain and the support surface.[39−41] These stationary phases are gaining popularity since they enable excellent separations of basic compounds, change selectivity, and increase wettability, a particularly valuable property in separations requiring mobile phases with a large proportion of water. Although this aspect is not targeted in this report, it will certainly be interesting to use this functionalization approach to prepare stationary phases for small molecules using silica-based supports and study this effect.

While this report has only shown the power of "click chemistry" to prepare stationary phases for the separation of peptides and proteins in both reversed-phase and affinity chromatography modes, many other types of functionalization are possible. For example, click chemistry using functional azides or alkynes could be used to prepare packings for other chromatographic modes such as normal phase, ion-exchange, or hydrophobic interaction. Because of its gentle nature, click chemistry seems especially well suited for the immobilization of proteins, a feature that can be exploited for the immobilization of enzymes and the creation of enzymatic reactors. We see a major opportunity for using these reactions in conjunction with the photografting techniques that we have recently developed[42] in the field of microfluidics. The orthogonal character of the click reactions with respect to many other functionalization techniques enables the attachment of a variety of ligands without compromising the native functionalities of the biopolymer. Experiments in this direction are currently in progress.

ACKNOWLEDGMENT

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