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"Click dipeptide": A novel stationary phase applied in two-dimensional liquid chromatography

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ABSTRACT

2D-HPLC is an important technique for the separation of complex samples. Developing new types of stationary phases is of great interest to construct 2D-LC systems with high orthogonality. In this study, a novel stationary phase-Click dipeptide (L-phenylglycine dipeptide) was prepared by immobilization of α-azido L-phenylglycine dipeptide on alkyne-silica via click chemistry. In the preparation of this new material, an efficient, inexpensive and shelf-stable diazo transfer reagent (imidazole-1-sulfonyl azide hydrochloride) was utilized to transfer the amino group of L-phenylglycine to corresponding azido group under mild conditions. The Click dipeptide thus prepared was confirmed by FT-IR, solid state CP/MAS ^{13}C NMR and elemental analysis. The Click dipeptide packing showed high orthogonality with C18, which reached 63.5%. An off-line 2D-RP/RPLC system was developed to analyze a traditional Chinese medicine (TCM)-*Rheum Palmatum* L. The results showed high orthogonality between Click dipeptide and C18 as well as great separating power in the practical separation of complex samples.

1. Introduction

Reversed phase liquid chromatography (RPLC) is the most popular analytical tool in separation science, and has dominated the analysis field for more than 30 years [1]. However, with the increasing demand for the separation and analysis of complex samples in the fields of proteomics, metabolomics, and natural products [2], the traditional RPLC could not satisfy some of these requirements. In an attempt to overcome these limitations, two-dimensional liquid chromatography (2D-LC) was gradually developed [3]. Up to now, great progress in 2D-LC has been achieved in methodology, technique and application [4], and many different 2D-LC modes have been constructed, such as SEC-RP [5], IEX-RP [6], NP-RP [7], HILIC-RP [8], and so on. Even so, there are still many problems in practical application of 2D-LC, such as orthogonality, solvent compatibility and low peak capacity [9,10].

2D-LC systems based on RP/RP should be the very useful combination due to its high separation efficiency, great peak capacity and the completely miscible mobile phases used in each dimension [4,9]. But 2D-RP/RPLC system is not completely perfect because of the limited orthogonality between the two dimensions [11]. This problem can be tackled partially by varying the mobile phases in the two dimensions, such as changing the gradient conditions in both dimensions [12], taking a different mobile phase composition (i.e., methanol, acetonitrile, tetrahydrofuran) into account [13], or utilizing mobile phases with different pH values in both dimensions [14]. However, the results are not satisfactory enough, because the improvement of orthogonality is always limited. Another alternative is to combine different kinds of stationary phases with totally different physicochemical surfaces. In other words, there is still a demand to develop independent retention mechanisms in each dimension, such as Click OEG-C18 [4,15]. Nevertheless, the types of stationary phases which can be combined with C18 for the purpose of high orthogonality in RP-RP mode are still rare. In this case, it is extremely important to develop new types of stationary phases with different selectivity from that of C18 column.

As far as we know, few peptide-bonded silica stationary phases have been reported [16,17]. Short peptide composed of hydrophobic amino acid residue is obviously different from long-chain alkane on the structures. Thus, it may bring about high orthogonality for 2D-RP/RPLC separation by combining this kind of peptide bonded silica with C18 stationary phase. The immobilization of peptide onto silica is usually accomplished by *N*,*N*′-dicyclohexylcarbodiimide (DCC) coupling method [18–20]. However, this bonding method is not efficient enough for heterogeneous reaction, thus, the resultant surface coverage is usually low. Moreover, long reaction time and anhydrous solvent are required.

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Recently, sharpless's "click chemistry" [21] has been widely used in preparation of covalently bonded silica separation materials [15,22–26]. This strategy showed powerful ability for immobilization of different stationary phases. In 2007, an efficient, safe and conveniently prepared diazotransfer reagent was reported to transfer amino group to corresponding azido group with high selectivity [27].

In this paper, we describe an easy route for preparation of a novel Click dipeptide stationary phase by introducing azido group into dipeptide artfully. The results of orthogonality evaluation and off-line 2D-LC separation of complex samples show that Click dipeptide and C18 stationary phases are highly orthogonal.

2. Experiment

2.1. Chemicals and reagents

Spherical silica (5 μm particle size; 10 nm pore size; 300 $m^2 g^{-1}$ surface area) was purchased from Fuji Silysia Chemical Ltd. (Japan). Copper iodide was purchased from Acros (USA). HPLC-grade acetonitrile and formic acid were purchased from Tedia (USA) and Acros (USA), respectively. Water was purified on a Milli-Q system (USA). 3-Isocyanatopropyl-triethoxysilane and propargylamine were domestic reagents and purified by distillation before use. L-phenylglycine was purchased from Shanghai Kayon Biological Technology Co., Ltd. (Shanghai, China). *N*,*N*'-dicyclohexylcarbodiimide and 4-dimethylaminopyridine (DMAP) were purchased from Shanghai Medpep Co., Ltd. (Shanghai, China). The test solutes (listed in Table 1) used for orthogonality evaluation were commercially available and used without further purification.

2.2. Sample preparation

The test probes (uracil, phenylamine, phenol, toluene, phenylethane), used to evaluate the performance of Click dipeptide column, were dissolved in acetonitrile/water (20/80, v/v) to afford 1 mg mL^{-1} concentration.

The solutes (listed in Table 1) used for orthogonality evaluation were dissolved in acetonitrile to form about 1 mg mL^{-1} concentration.

Table 1
List of test solutes used for orthogonality evaluation.

No.	Aromatic compounds
1	Benzene
2	Toluene
3	Chlorobenzene
4	Bromobenzene
5	Nitrobenzene
6	Anisole
7	Ethyl benzoate
8	2-Chloroacetophenone
9	4-Nitroacetophenone
10	2-Chlorobenzaldehyde
11	Cinnamaldehyde
12	3-Methylcinnamaldehyde
13	2-Chlorocinnamaldehyde
14	Indole
15	5-Methoxyindole
16	6-Methylindole
17	2-Chloronitrobenzene
18	4-Chloronitrobenzene
19	4-Chlorophenol
20	2-Nitrophenol
21	3-Nitrophenol
22	1-Naphthol
23	2-Naphthol
24	2-Methylaniline
25	4-Ethylaniline

Rheum palmatum L. was used to further investigate the orthogonality of the 2D-RP/RPLC system. The sample was prepared as follows: 100 g *R. palmatum* L. was ground into powder and decocted in 1 L water at 100 °C for 2 h. The supernatant liquid was filtered and the residue was re-decocted in another 1 L water at 100 °C for 1.5 h. The combined solutions were condensed to fine powder (about 1.7 g), which was re-dissolved in 30 mL of methanol/water (75:25, v/v), then the mixture was kept in a refrigerator overnight. The extract was filtered through a 0.2 μm regenerated cellulose membrane prior to injection.

2.3. Preparation of Click dipeptide stationary phase and column packing

The diazotransfer reagent imidazole-1-sulfonyl azide hydrochloride **1** was prepared according to the reference [27].

Condensation of 3-isocyanatopropyl-triethoxysilane with propargylamine in anhydrous DMF afforded terminal alkynyl triethoxysilane **2**, which was then directly polymerized with silica beads to afford the terminal alkyne-silica beads **3** [26].

The amino group in L-phenylglycine was smoothly transferred to corresponding azido L-phenylglycine by using an efficient, inexpensive and shelf-stable diazo transfer reagent **1** [27]. Subsequent condensation of the azido L-phenylglycine **4** with L-phenylglycine methyl ester **5** afforded the dipeptide azide **6**. Then "clicking" between the dipeptide azide and the terminal alkyne-silica afforded Click dipeptide silica **7**. The general synthesis scheme is shown in Fig. 1.

2.3.1. Imidazole-1-sulfonyl azide hydrochloride **1** [27]

A suspension of NaN_3 (3.2 g, 50 mmol) in anhydrous MeCN (50 mL) in 100 mL bottom round flask was cooled in an ice bath, then sulfuryl chloride (4.0 mL, 50 mmol) was added drop-wise to this flask. Then the mixture was stirred overnight at room temperature. At this stage, imidazole (6.8 g, 100 mmol) was added in one portion to the ice-cooled mixture. After stirring for 3 h at room temperature, the mixture was diluted with EtOAc, the organic layer was in sequence washed with H_2O and saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4 and filtered. A solution of HCl in EtOH which was prepared by the drop-wise addition of AcCl (5.4 mL, 76 mmol) to ice-cooled dry ethanol (19 mL), was added slowly to the filtrate while stirring in an ice bath. Then the suspension was filtered and the filter cake was washed with EtOAc to get imidazole-1-sulfonyl azide hydrochloride **1** (7.66 g, 73%) as white crystal. ^1H NMR (D_2O) δ: 9.32 (dd, 1H, J = 1.3, 1.6 Hz, H-2), 8.02 (dd, 1H, J = 1.6, 2.2 Hz, H-5), 7.58 (dd, 1H, J = 1.3, 2.2 Hz, H-4).

2.3.2. α-Azido L-phenylglycine **4** [27]

To the mixture of L-phenylglycine (3.02 g, 20 mmol), K_2CO_3 (6.92 g, 25 mmol) and $CuSO_4{\cdot}5H_2O$ (0.05 g, 0.5 mmol) in MeOH (100 mL), imidazole-1-sulfonyl azide hydrochloride **1** (5.04 g, 24 mmol) was added, and the reaction system was stirred at room temperature overnight. Removal of the solvents left a residue, which was diluted with H_2O and acidified with conc. HCl until pH to 2, the mixture was extracted with ethyl acetate (100 mL × 3). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. Flash chromatography gave the α-azido L-phenylglycine **4** (2.14 g, 60%) as light yellow solid. ^1H NMR ($CDCl_3$) δ: 9.84 (br, 1H, CO_2H), 7.43 (m, 5H, Ar–H), 5.05 (s, 1H, N_3–CH).

L-Phenylglycine methyl ester **5** was synthesized according to a method described in the literature [28]. AcCl (12.0 mL, 169 mmol) was added drop-wise to ice-cooled MeOH (75 mL) and then L-phenylglycine (8.61 g, 57 mmol) was added. The mixture was heated under reflux for 3 h. The solvent was evaporated to get L-phenylglycine methyl ester hydrochloride as white solid. Then the