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Itaconic Acid-Based Organic-Polymer Monolithic Column for Hydrophilic Capillary Electrochromatography and Its Application in Pharmaceutical Analysis

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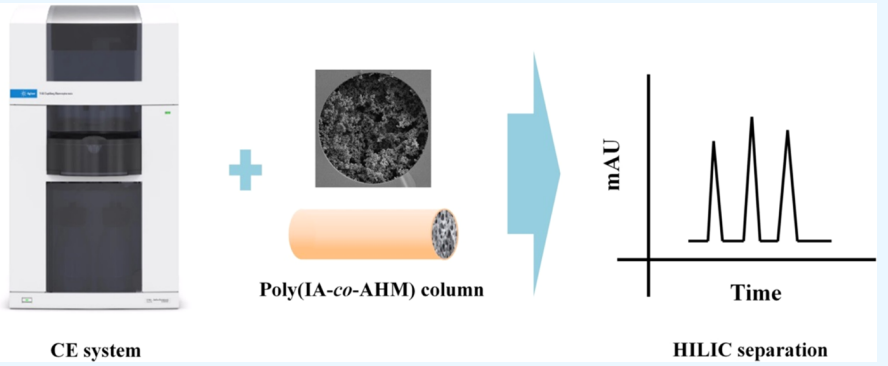

ABSTRACT: Itaconic acid is an excellent hydrophilic monomer owing to the dicarboxylic group possessing strong polarity. This study reports on the preparation of a new organic-polymer monolithic column poly(itaconic acid-*co*-3-(acryloyloxy)-2-hydroxypropyl methacrylate) (poly(IA-*co*-AHM)) featuring excellent hydrophilic chromatography ability and its application in pharmaceutical analysis. The monolithic column was successfully synthesized by using the monomer itaconic acid and the cross-linker AHM through an in situ copolymerization method. Optical microscopy, scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR) were employed for the characterization of the poly(IA-*co*-AHM) monolithic column, and all of these demonstrated that the prepared itaconic acid-based monolithic column exhibited satisfactory permeability and a homogeneous porous structure. Owing to the carboxylic groups of itaconic acid, a cathodic electroosmotic flow (EOF) was generated on the itaconic acid-based monolithic column among the pH ranges of the mobile phase from 4.0 to 9.0. Depending on the powerful hydrophilic interactions, different kinds of polar substances, including thioureas, nucleoside drugs, sulfonamides, and polypeptides, were separated efficiently by the itaconic acid-based monoliths poly(IA-*co*-AHM). The separations of polar compounds were successfully realized, even at a lower level of 50% acetonitrile content on this monolithic column. The highest column efficiencies corresponding to *N*,*N*′-dimethylthiourea and idoxuridine were 102 720 and 124 267 N/m, respectively. The poly(IA-*co*-AHM) monolithic column displayed excellent repeatability, whose relative standard deviations (RSDs) of the retention time and peak area were both lower than 5.0%. All experimental results demonstrated that the new itaconic acid-functionalized monolithic column was greatly appropriate to separate the polar compounds under the HILIC mode.

1. INTRODUCTION

Capillary electrochromatography (CEC), a hybrid derived from liquid chromatography (LC) and capillary electrophoresis (CE), possesses a number of desirable features such as rapid analysis, high efficiency, high selectivity, small usage of sample, good economic operation, and extraordinary versatility in the matter of stationary phases and detection.[1−3] CEC is still a valuable research field that has garnered wide attention from the academia owing to its wide applications, e.g., in the biological analysis of amino acids, polypeptides, and proteins,[4−6] enantioseparations,[7−9] as well as pharmaceutical analysis.[10−12] The development of the technology of new chromatographic columns is the core of CEC, which includes three main column types, namely, monolithic, packed, and open-tubular columns.[2,13,14] Through the interactions between the solutes and stationary phases or the effective combination of these interactions and the intrinsic electrophoretic mobility

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of the solutes, the charged and uncharged substances can be separated well under the chromatographic modes comprising reversed-phase,[15,16] hydrophilic interaction chromatography (HILIC),[17] ion-exchange,[18,19] and ligand affinity behavior.[5,20]

HILIC is a complementary method to reversed-phase liquid chromatography, and it was defined by Alpert for the first time.[21] The organic-rich mobile phase employed in HILIC makes the column backpressure lower, allowing fast analysis and the compatibility of HILIC coupling with MS detection. HILIC is a prioritized choice to separate polar and hydrophilic substances. However, the lack of commercial chromatography columns with strong hydrophilicity and specific separation selectivity is the bottleneck that restricts the development of hydrophilic interaction capillary electrochromatography (HI-CEC).

To develop novel chromatographic columns in HI-CEC, monoliths are fascinating and promising due to their advantages of excellent permeability, fast mass transfer, ease of fabrication with macro- or micropores, versatility in surface chemistries, and powerful separation ability.[22,23] Organic polymer-modified and silica-modified polar monoliths have been synthesized for HI-CEC. Compared to silica-modified monoliths, stronger pH tolerance makes organic polymer-modified monoliths more popular in HI-CEC. Rassi et al.[24] synthesized a hydrophilic organic-polymer stationary phase poly(carboxyethyl acrylate-*co*-ethylene glycol dimethacrylate) postfunctionalized with Tris, whereon many different kinds of polar substances such as nucleotides, hydroxybenzoic acids, dansyl amino acids, and phenoxy acid herbicides were separated successfully by HI-CEC. Li et al.[19] fabricated a novel zwitterionic monolith with the monomer 3-dimethyl-(3-(*N*-methacrylamido) propyl) ammonium propane sulfonate (DMMPPS) and the cross-linker pentaerythritol triacrylate (PETA) through thermal-initiated copolymerization for HI-CEC. The prepared monolithic column poly(DMMPPS-*co*-PETA) exhibited a typically hydrophilic retention behavior with the acetonitrile content higher than 80% so that the polar compounds (e.g., amides, nucleotides, and nucleosides) were excellently separated. In the meantime, the column efficiency was up to 93 000 N/m, and the RSDs of retention times were lower than 4.5%. Chen et al.[25] synthesized a new mixed-mode poly(VBP-*co*-EDMA-*co*-VBTA) monolith featuring reversed-phase/hydrophilic (RP/HILIC) performance for CEC, and the separation mode switched from RP to HILIC as the mobile phase comprised around 80% acetonitrile. Under the HILIC mode, baseline separations of polar thioureas and alkaline substances were achieved successfully, and the RSDs of the retention times were less than 5.0%.

Nevertheless, for HILIC separations, a high content of organic solvent miscible with water (e.g., acetonitrile or methanol) in the mobile phase is normally necessary. In the HILIC field, these problems cannot be ignored because of the following reasons: (1) the narrow source of the mobile phase results in a lower degree of freedom for optimizing the mobile phase components, and (2) the poor solubility of polar compounds in an organic-rich mobile phase (generally, >85% organic solvents).[26]

In order to tackle the problems above, a prior strategy is the development of strongly hydrophilic stationary phases that can enhance the retention for polar compounds, with mobile phases comprising a higher water content. Itaconic acid (IA) ($\log P$ −0.301) is a promising functional monomer for the fabrication of hydrophilic stationary phases owing to the carboxyl and vinyl groups in its structure. 3-(Acryloyloxy)-2-hydroxypropyl methacrylate (AHM) ($\log P$ 1.121), a novel cross-linker possessing a typically hydrophilic feature, has a stronger polarity compared to the frequently used cross-linker PETA ($\log P$ 1.212). IA and AHM were good functional monomers for the preparation of hydrophilic monoliths. Moreover, there were no reports about the fabrication of monolithic stationary phases by using IA and AHM.

In this research, IA and AHM were first applied to fabricate a new hydrophilic itaconic acid-functionalized monolith poly(IA-*co*-AHM) employing PEG400 and H_2O as binary porogens through in situ copolymerization. The dosage of the monomer, cross-linker, and porogens was optimized in detail. Optical microscopy, SEM spectroscopy, and FTIR were implemented to evaluate the successful preparation of the poly(IA-*co*-AHM) monolithic column. The resulting itaconic acid-based monolithic column was employed in CEC to separate the various kinds of polar substances including thioureas, nucleoside drugs, sulfonamides, as well as polypeptides. The polar compounds could be baseline-separated perfectly by CEC even with a lower content of 50% acetonitrile in the mobile phase solvent. The proposed monolithic column had advantages of rapid separation, high resolution, high column efficiency, and excellent reproducibility, which indicated its enormous application potential.

2. EXPERIMENTAL SECTION

2.1. Materials and Chemicals. Fused-silica capillary with I.D. 100 μm and O.D. 365 μm was purchased from Yongnian Ruifeng Chromatographic Devices (Hebei, China). Syringes were obtained from Yu'an (Henan) Holding Co., Ltd. (China). IA was obtained from Shanghai Macklin Biochemical Co., Ltd. (China). AHM was obtained from TCI Company (Shanghai, China). Toluene, Ala−Tyr, cytarabine, Leu−Leu, azacitidine, Leu−Gly, cyclocytidine, methanol, and acetonitrile were purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. (China). 3-(Triethoxysilyl)propyl methacrylate (γ-MAPS) was obtained from J&K Scientific Co., Ltd. (Guangzhou, China). Hydrochloric acid (HCl), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), sodium persulfate ($Na_2S_2O_8$), PEG400, *N*-methylthiourea, sodium hydroxide (NaOH), *N*,*N*′-dimethylthiourea, and phosphoric acid (H_3PO_4) were obtained from Shanghai Aladdin Reagent Factory (China). Thiourea, sulfamethoxazole, sulfamethazine, and sulfadiazine were obtained from Alfa-Aesar (Tianjin, China). Sulfamerazine, idoxuridine, and 5-methyluridine were obtained from Sigma-Aldrich (Beijing, China). All of the reagents used in the experiment were of analytical grade.

2.2. Instrumentation. FTIR was performed on a Thermo Nexus 470 FTIR system (MA, USA) to obtain the infrared spectra data. The cross-sectional morphology of the prepared monolithic columns was examined by a Carl Zeiss Ultra Plus field emission scanning electron microscope (FESEM, Carl Zeiss, Germany) with an accelerating voltage of 5.0 kV. Ultrapure water used in the experiment was produced through a Milli-Q CLX system (Merck, Germany). The backpressure of the obtained itaconic acid-based monolithic columns was measured on an LC-20AD NANO pump (Shimadzu, Japan). All HI-CEC separations were implemented on an Agilent 7100 CE system with a diode array detector (Waldbronn, Germany). Data collection and processing were carried out using a chromatographic workstation (Chemistry Station).

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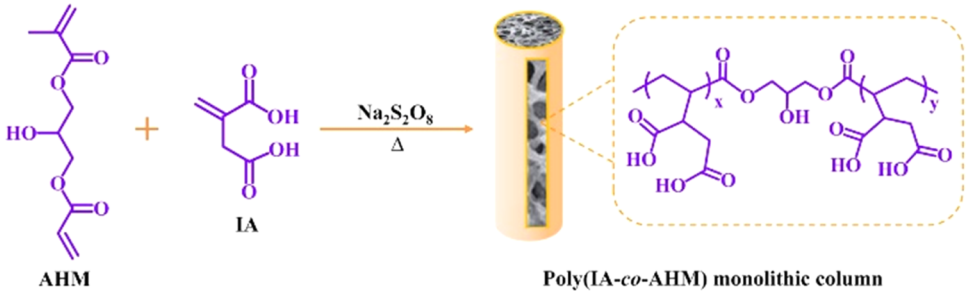

Figure 1. Scheme for the preparation of a poly(IA-*co*-AHM) monolithic column by in situ copolymerization.

Table 1. Composition of the Copolymerization Mixtures of the Hydrophilic Poly(IA-*co*-AHM) Monolithic Column[a]

	monomers	cross-linker	porogens			
column	IA (wt %)	AHM (wt %)	H_2O (wt %)	PEG400 (wt %)	backpressure (MPa)	permeability (10^{-13} m^2)
1	8	12	44	36	>20	–
2	8	12	32	48	1.3	1.61
3	8	12	20	60	0.3	6.97
4	6	14	32	48	0.4	5.23
5	10	10	32	48	4.9	0.43

[a]Backpressure was obtained with methanol as the mobile phase at a flow rate of 0.5 μL min^{-1}. "–" represents no detection.

2.3. Preconditioning of Capillary. Before the monolithic column preparation, the preconditioning of the fused-silica capillary was performed according to the steps described next. First, the bare capillary was flushed with NaOH, H_2O, HCl, and methanol sequentially, and the specific operation was according to the literature.[6] Then, the capillary was dried by nitrogen and filled with a mixing solution consisting of γ-MAPS and methanol (50/50, v/v) to vinyl-functionalize the inner wall of the capillary through a 45 °C water bath overnight.[27] After that, methanol was used to rinse γ-MAPS-functionalized capillaries to get rid of the unreacted silane reagents, and nitrogen was used to dry the capillary. The capillary was stored in a dry environment for further use.

2.4. Synthesis of Poly(IA-*co*-AHM) Monolithic Column. Based on our previous work, the poly(IA-*co*-AHM) monolithic column was synthesized successfully.[6] At first, the prepolymerizable mixture to prepare the monolithic column was composed of 8% (w/w) IA, 12% (w/w) AHM, $Na_2S_2O_8$ (1.5% (w/w) of the total amount of functional monomers and cross-linkers), 48% (w/w) PEG400, and 32% (w/w) H_2O. The ingredients were placed into a 2 mL Eppendorf tube and sufficiently mixed through ultrasonic vibration to form a homogeneous solution. After that, the solution was injected into the γ-MAPS-functionalized capillary by a syringe. The two ends of the capillary should be plugged using a silicone stopper. In order to synthesize the hydrophilic itaconic acid-based monolithic column, the capillary above should be kept in a water bath at 65 °C for 18 h. Finally, methanol was applied to rinse the itaconic acid-based monolithic column so that the unreacted monomers and cross-linkers, as well as residual porogenic solvents, were cleaned up.

2.5. Sample and Buffer Solution Preparation. Sample solutions: Standard solutions (2.0 mg/mL) of *N*,*N*′-dimethylthiourea, thiourea, and *N*-methylthiourea were obtained with methanol as the solvent. Standard solutions (5.0 mg/mL) of idoxuridine, 5-methyluridine, cytarabine, azacitidine, and cyclocytidine were prepared in turn through dissolving them in a mixing solution comprising H_2O and methanol (v/v, 50/50). Standard solutions (1.0 mg/mL) of sulfonamides were prepared by individually dissolving them in ultrapure water. A standard solution (2.0 mg/mL) of toluene was made with methanol as the solvent.

Buffer solutions: The mobile phase was prepared with phosphate-buffered solutions with different pH values and acetonitrile. The phosphate-buffered solutions were prepared using $Na_2HPO_4{\cdot}12H_2O$, and their pH was regulated using a solution of phosphoric acid (pH 4.0–9.0, H_3PO_4–Na_2HPO_4). All solutions were stored in a 4.0 °C refrigerator for further use.

2.6. CEC Experiments. Before the CEC experiments, a detection window (around 3.0 mm) was made on the monolithic column. Through burning the polyimide coating on the outer surface of the silica capillary, the detection window was made after the continuous monolithic stationary phases. For the monolithic column used in the CEC experiments, its effective length and total length were 25.0 and 33.5 cm, respectively.

Then, the itaconic acid-based monolithic columns used in the experiment were first conditioned with the mobile phase for 0.5 h at a flow velocity of 0.05 mL/h by a mechanical pump. After that, a CE system (Agilent 7100) was used to equilibrate the monolithic column for 0.5 h at a voltage of 20 kV. All sample solutions and the mobile phase were filtrated by the 0.22 μm nylon membranes.

2.7. Equations. In this study, the formula $k = (t_r - t_0)/t_0$ was employed to compute the retention factor (k) of the analytes, where t_r and t_0, respectively, represent the retention times of analytes and the unretained compound.

The formula $K = (F \times \eta \times L)/(\Delta P \times \pi \times r^2)$ was applied to calculate the permeability (K) of the prepared itaconic acid-based monolithic columns using methanol as the mobile phase, where F represents the velocity of volume flow, η represents the viscosity coefficient of the mobile phase, L represents the total length of the monolithic column, and ΔP represents the backpressure value of the monolithic column. In particular, the dynamic viscosity of methanol is 0.580×10^{-3} kg m^{-1} s^{-1} at 25 °C.[28]

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The formula $v_{EOF} = \varepsilon_0\varepsilon_r\zeta E/\eta$ was employed to calculate the electroosmotic flow (v_{EOF}), where ε_0 represents the dielectric constant of a vacuum, ε_r represents the permittivity of the eluent, ζ represents the zeta potential, E represents the intensity of the applied electric field, and η represents the dynamic viscosity of the eluent.[25]

3. RESULTS AND DISCUSSION

3.1. Optimization of the Preparation of Itaconic Acid-Based Monolithic Columns. The fabrication process of the itaconic acid-based monolithic column poly(IA-*co*-AHM) with the HILIC mode is depicted in Figure 1. Varying amounts of monomers, cross-linkers, and porogens as well as a range of itaconic acid-based monolithic columns were synthesized (Table 1). The binary porogens made by PEG400 and H_2O had a significant effect on the permeability of the itaconic acid-based monolithic column poly(IA-*co*-AHM). The influence of the proportion of H_2O to PEG400 in the prepolymerizable mixture on the formation of poly(IA-*co*-AHM) monoliths was investigated. In the case of Column 1, Column 2, and Column 3, the permeability increased obviously from "Blocked" to 6.97 $\times$ 10^{-13} m^2 by changing the ratio of H_2O to PEG400 from 44:36 to 20:60. The resulting optical microscope images of Columns 1, 2, and 3 corresponding to the ratio of H_2O to PEG400 at 44:36, 32:48, and 20:60, respectively, are displayed in Figure 2. It could be observed that the higher proportion

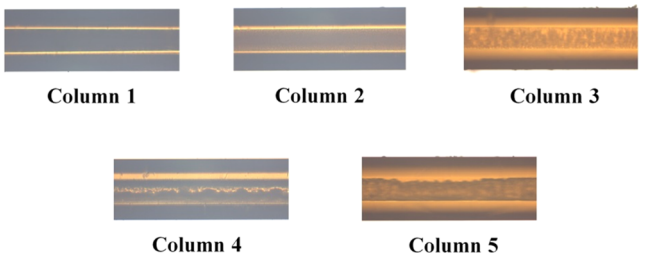

Figure 2. Optical microscope images of the monolithic column, 400×.

(44%) of H_2O in the copolymerization solution, e.g., Column 1, would result in a nontransparent monolithic bed whose permeability was blocked; the middle proportion (32%) of H_2O in the copolymerization solution, e.g., Column 2, would result in a homogeneous and semitransparent monolithic bed whose permeability was 1.61 $\times$ 10^{-13} m^2; and the lower proportion (20%) of H_2O in the copolymerization solution, e.g., Column 3, would result in a slack monolithic bed whose permeability was 6.97 $\times$ 10^{-13} m^2. The reason might be that H_2O was a poor porogen, which was adverse to the production of the porous skeleton structure. Therefore, the ratio of H_2O to PEG400 at 32:48 was selected.

The proportion between the monomer IA and the cross-linker AHM cannot be ignored for the permeability of the poly(IA-*co*-AHM) monoliths. As the ratio of IA to AHM changes from 6:14 to 10:10, the permeability corresponding to Column 4, Column 2, and Column 5 fell gradually from 5.23 $\times$ 10^{-13} to 0.43 $\times$ 10^{-13} m^2. As seen from Figure 2, when the ratio of IA to AHM was 6:14 (Column 4), the monolithic bed was cracked, although Column 4 had the best permeability, it could not generate a stable current, which was necessary for CEC; when the ratio of IA to AHM was 8:12 (Column 2), the morphology and permeability of Column 2 were excellent and met the requirements of CEC; and when the ratio of IA to AHM was 10:10 (Column 5), the monolithic bed was inhomogeneous and partially broke away from the inwall of the capillary, which always formed bubbles under the high voltage of CEC. Depending on the results above, the optimal ratio of IA to AHM was 8:12.

According to the permeability and the optical microscope images of these monolithic columns above, Columns 2 and 3 could be used in the CEC experiments, and their hydrophilic performance was further compared. The chromatogram is shown in Figure 3. It could be seen that test analyte thioureas

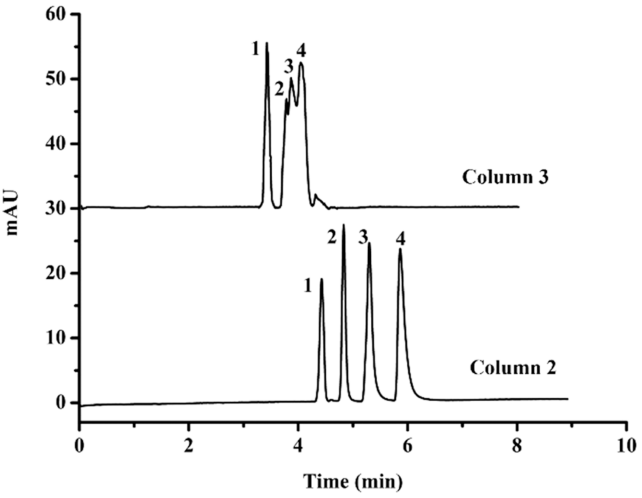

Figure 3. Separation of thioureas on the poly(IA-*co*-AHM) monolithic column (Columns 2 and Column 3). Experimental conditions: mobile phase, 60% acetonitrile in 10 mM, pH 7.0 phosphate buffer; applied voltage: 20 kV; electrokinetic injection, 5 kV × 5 s; detection wavelength, 214 nm. Peaks: 1, toluene; 2, *N*,*N*′-dimethylthiourea; 3, *N*′-dimethylthiourea; 4, thiourea.

were separated completely using Column 2, but the peaks overlapped using Column 3. The experimental results indicated that Column 2 had a better separation performance than Column 3.

In conclusion, comprehensively considering the permeability, morphology, and separation performance of the itaconic acid-based monolithic columns, the optimal hydrophilic monolithic column was Column 2.

3.2. SEM. The morphology of the cross section of Column 2 was investigated with SEM. The monolithic column was cut into about 2.0 mm segments, and then, these segments were immobilized onto the side of a circular sample holder. After gold was sprayed on the segments for 1.0 min, the sample holder with the segments was put into FESEM to obtain the SEM images. In Figure 4, the SEM micrographs of Column 2 are displayed. It could be observed that the monolithic column poly(IA-*co*-AHM) had a satisfactory morphology. In Figure 4A, the polymer stationary phases of Column 2 were continuous and complete, bonding tightly to the inwall of the capillary and possessing generous and well-distributed pore structures that were beneficial to the increase of the permeability. In Figure 4B, the polymers showed visible spheres, which were close together to form large grape-like clusters. In Figure 4C, it could be seen that the polymer spheres possessed a honeycomb-like structure, which was good for the enlargement of the specific surface area of the polymers.

3.3. FTIR Analysis. The FTIR experiment was carried out to characterize the successful fabrication of the itaconic acid-

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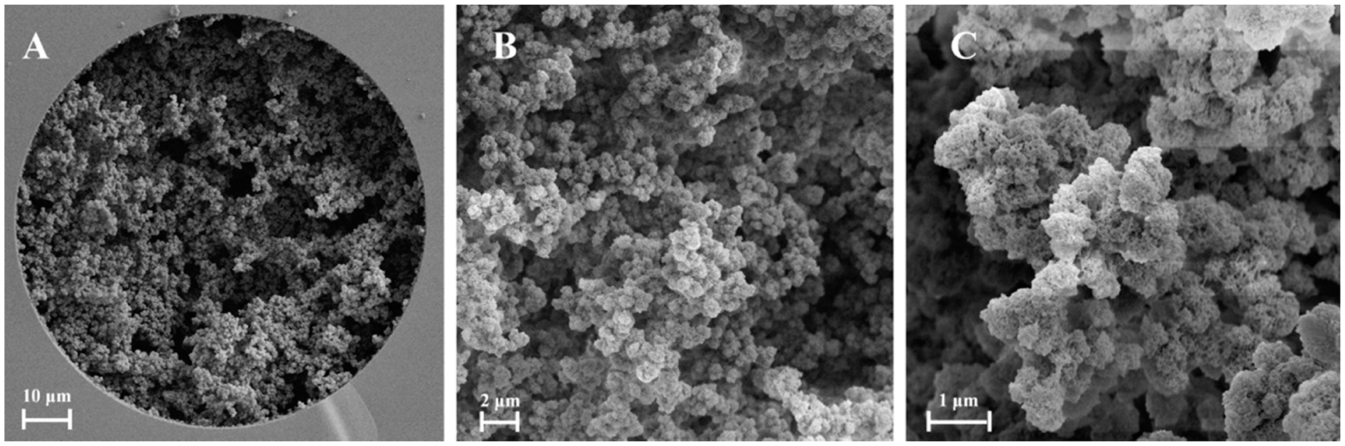

Figure 4. SEM images of the stationary phases of Column 2 (A, 750×; B, 3000×; C, 10 000×).

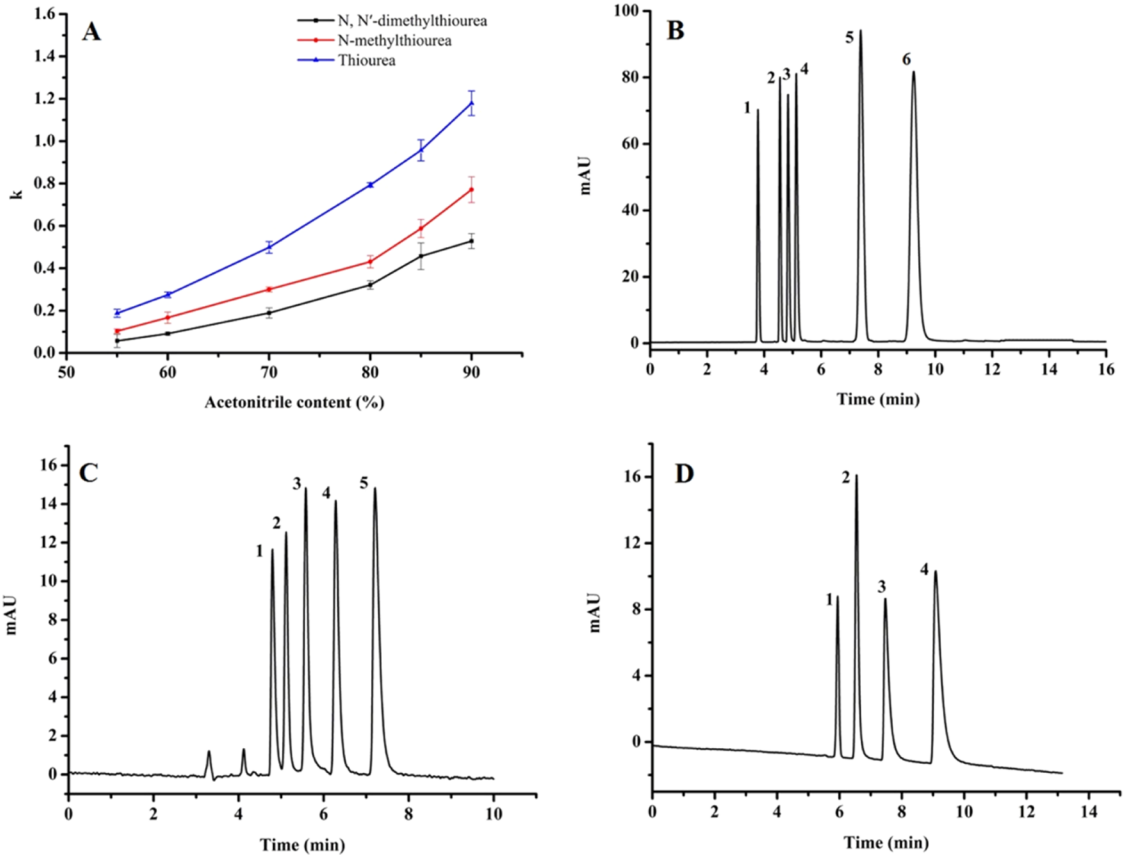

Figure 5. (A) Relationship between the retention factor (k) and acetonitrile content on the poly(IA-*co*-AHM) monolithic column to separate thioureas with toluene as an unretained marker. Experimental conditions: mobile phase, different acetonitrile contents in 10 mM, pH 7.0 phosphate buffer; other conditions are the same as in Figure 2. (B) Separation of nucleoside drugs. Experimental conditions: mobile phase, 60% acetonitrile in 10 mM, pH 8.0 phosphate buffer; applied voltage, 25 kV; electrokinetic injection, 10 kV × 5 s; detection wavelength, 214 nm. Peaks: 1, toluene; 2, idoxuridine; 3, 5-methyluridine; 4, cytarabine; 5, azacitidine; 6, cyclocytidine. (C) Separation of sulfonamides. Experimental conditions: mobile phase, 50% acetonitrile in 15 mM, pH 8.0 phosphate buffer; applied voltage, 20 kV; electrokinetic injection, 5 kV × 5 s; detection wavelength, 256 nm. Peaks: 1, toluene; 2, sulfamethoxazole; 3, sulfamethazine; 4, sulfamerazine; 5, sulfadiazine. (D) Separation of polypeptides. Experimental conditions: mobile phase, 50% acetonitrile in 20 mM, pH 6.0 phosphate buffer; applied voltage, 20 kV; electrokinetic injection, 5 kV × 10 s; detection wavelength, 214 nm. Peaks: 1, toluene; 2, Leu−Leu; 3, Leu−Gly; 4, Ala−Tyr.

based monoliths (Column 2). First, the outer coating of the capillary was burned and removed. After that, a capillary without coating was applied to fabricate the poly(IA-*co*-AHM) monolithic column. Finally, a poly(IA-*co*-AHM) monolithic column was cut out into small pieces and then crushed into powder for FTIR.

The FTIR spectrum of functional groups of stationary phases is displayed in Figure S1. The peak of the −COOH stretching vibration of itaconic acid was observed at 1727 cm^{-1}. The peaks at 2958 and 1176 cm^{-1} individually corresponded to the $-CH_3$ stretching vibration and the C−O−C stretching vibration in the cross-linker AHM.[29] The peak

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Table 2. RSDs of Retention Time and Peak Area of the Poly(IA-*co*-AHM) Monolithic Column (Column 2)[a]2

	retention time (RSD%)			peak area (RSD%)		
analytes	intraday (n = 5)	interday (n = 5)	column-to-column (n = 3)	intraday (n = 5)	interday (n = 5)	column-to-column (n = 3)
N,*N*′-dimethylthiourea	0.65	0.86	1.78	1.26	1.81	2.68
N-methylthiourea	0.93	1.76	2.33	2.06	3.03	3.92
thiourea	1.57	2.35	3.17	2.87	4.31	4.86

[a]The experimental conditions were the same as in Figure 2.

of the −OH bending bands of AHM was at 987 cm^{-1}.[30] In the FTIR spectrum of the poly(IA-*co*-AHM) monolithic column, all characteristic peaks described above suggested that the hydrophilic monolith was prepared successfully via itaconic acid and AHM.

3.4. Retention Performance and HILIC. The retention performance of the itaconic acid-based monolithic column (Column 2) was evaluated. *N*-Methylthiourea (log *P* −0.700), *N*,*N*′-dimethylthiourea (log *P* −0.285), and thiourea (log *P* −1.020) were used as the test compounds, while toluene (log *P* 2.720) was used as the unretained marker. The relationship between the retention factors of the thioureas and the acetonitrile proportion in the mobile phase was investigated. As shown in Figure 5A, with the increase of the acetonitrile proportion from 55 to 90%, the retention factor k of three thioureas increased evidently. In Figure 2, three kinds of thioureas and toluene were separated well according to the peak order toluene < *N*,*N*′-dimethylthiourea < *N*-methylthiourea < thiourea, which was in good agreement with the polarity of the test compounds from weak to strong. The symmetry factors of the peak of *N*,*N*′-dimethylthiourea, *N*-methylthiourea, and thiourea were 0.81, 0.71, and 0.41, respectively. All of these above demonstrated that a typical HILIC retention behavior existed in Column 2.[31] The separation mechanism might be attributed to the hydrophilic interactions between carboxyl groups. In addition, the chromatogram (Figure 3) showed that Column 2 was fit for the excellent baseline separation of thioureas. The resolution was more than 2.82. The column efficiencies for *N*,*N*′-dimethylthiourea, *N*-methylthiourea, and were 102720, 57740, and 44184 N/m, respectively.

3.5. EOF Study. Owing to the carboxyl groups from the monomer itaconic acid (pKa_1 = 3.85, pKa_2 = 5.44), a strong cathodic EOF used to drive the running buffer passing the monolithic column in CEC experiments was generated by the itaconic acid-based monolithic column. For EOF, the pH value, the buffer concentration, and the content of organic solvent in the mobile phase are meaningful factors. The influence of these factors on the EOF was studied in detail using toluene as the unretained marker in HI-CEC.

Experimental results of the EOF study are shown in Figure S2. As shown in Figure S2A, the cathodic v_{EOF} enhanced obviously as the pH values were increased from 4.0 to 9.0. It was attributed to the increase of ζ resulting from the enhancement of the thickness of the electric double layer corresponding to the enlarged ionization of carboxyl groups immobilized on the surface of the monoliths. On increasing the pH to more than 7.0, the carboxyl groups were almost absolutely ionized so that v_{EOF} remained nearly the same. As shown in Figure S2B, as the buffer concentration was increased from 5 to 25 mM, the v_{EOF} decreased to some extent. Due to the increase in buffer concentration, the thickness of the electric double layer decreased, which resulted in the reduction of ζ. As shown in Figure S2C, when the acetonitrile content (v/v) increased from 55 to 90%, the v_{EOF} showed an uptrend. The reason is that the ε_r/η ratio of acetonitrile is more than the ε_r/η ratio of water; when the acetonitrile content increases, the ε_r/η ratio of the mobile phase increases, which is better for the enhancement of v_{EOF}.[32]

3.6. Column Efficiency. In separation science, the column efficiency corresponds to the theoretical plates. As shown in Figure S3, the relationship between the plate height and the flow velocity was investigated with *N*,*N*′-dimethylthiourea as the test analyte in HILIC. The chromatographic condition was 60% acetonitrile at pH 7.0, 10 mM phosphate buffer, and the voltage used in CEC was altered from 12.5 to 25 kV. In Figure S4, a U-like trend for the plate height is observed with the change in flow velocity. At 20 kV, the lowest plate height of 9.73 μm relating to the column efficiency of 102 720 N/m was obtained.

3.7. Repeatability. Repeatability is an important parameter for evaluating the separation ability of a novel chromatographic column. The repeatability of the itaconic acid-based monolith (Column 2) was assessed by determining RSDs of the retention time and the peak area of three thioureas (Table 2). The repeatability values of intraday (n = 5) and interday (n = 5) were assessed on the same itaconic acid-based monolithic column, while the repeatability of column-to-column (n = 3) was assessed on the same day. All of the RSDs of the retention time and peak area of thioureas were lower than 5.0%. In addition, there was no obvious change in the separation ability of the itaconic acid-based monolithic column after 130 runs. It could be seen that the itaconic acid-based monolithic column had outstanding repeatability.

3.8. Applications. *3.8.1. Separation of Nucleoside Drugs.* The resulting itaconic acid-based monolithic column (Column 2) was used to separate five nucleoside drugs including idoxuridine (log *P*, −0.593), cytarabine (log *P*, −1.808), 5-methyluridine (log *P*, −1.166), azacitidine (log *P*, −2.191), and cyclocytidine (log *P*, −2.35). Toluene was the unretained marker. In Figure 5B, it could be seen that a mixture of nucleoside drugs was separated successfully within 10 min using the mobile phase consisting of 60% (v/v) acetonitrile in phosphate buffer (10 mM, pH 8.0). The peak order was toluene < idoxuridine < 5-methyluridine < cytarabine < azacitidine < cyclocytidine following the polarity from high to low. The resolution among nucleoside drugs was larger than 2.33. The column efficiencies of idoxuridine, 5-methyluridine, cytarabine, azacytidine, and cyclocytidine were 124 267, 108 404, 101 341, 66 223, and 25 986 N/m, respectively. The symmetry factors of the peak of idoxuridine, 5-methyluridine, cytarabine, azacytidine, and cyclocytidine were 0.70, 0.59, 0.54, 0.66, and 0.79, respectively. All of these experimental results indicated that the itaconic acid-based monolithic column had a powerful hydrophilic chromatographic behavior, which is very suitable for separating nucleoside drugs.

3.8.2. Separation of Sulfonamides. Many of the sulfonamides, including sulfamethazine (log *P* 0.296, p*K*a

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7.89), sulfadiazine (log *P* −0.074, p*Ka* 6.81), sulfamerazine (log *P* 0.107, p*Ka* 7.35), and sulfamethoxazole (log *P* 0.659, p*Ka* 5.81), were separated using the hydrophilic monolithic column poly(IA-*co*-AHM) (Column 2). In Figure 5C, the sulfonamides were separated perfectly by employing 75% acetonitrile in phosphate buffer (15 mM, pH 8.0) as the running buffer. Under this chromatographic condition, the sulfonamides were eluted in the order of toluene < sulfamethoxazole < sulfamethazine < sulfamerazine < sulfadiazine, following the polarity from weak to strong. The powerful hydrophilic interactions were beneficial for the separation of sulfonamides. The resolution among sulfonamides was greater than 2.07. The column efficiencies of sulfamethoxazole, sulfamethazine, sulfamerazine, and sulfadiazine were 73 952, 72 744, 62 480, and 48 996 N/m, respectively. The symmetry factors of the peak of sulfamethoxazole, sulfamethazine, sulfamerazine, and sulfadiazine were 0.62, 0.54, 0.50, and 0.40, respectively.

3.8.3. Separation of Polypeptides. The chromatogram of polypeptides is shown in Figure 5D. The polypeptides including Ala−Tyr (log *P* −4.27; pKa_1 3.03, pKa_2 8.66), Leu−Leu (log *P* −1.53; pKa_1 3.16, pKa_2 8.60), and Leu−Gly (log *P* −3.24; pKa_1 3.15, pKa_2 8.34) were separated excellently using a poly(IA-*co*-AHM) monolithic column under the optimal chromatographic condition of 50% acetonitrile in phosphate buffer (10 mM, pH 6.0). At the chromatographic condition, the polypeptides were all almost neutral. For polypeptides, the resolution was more than 3.78. The peak order is toluene < Leu−Leu < Leu−Gly < Ala−Tyr. For Leu−Leu, Leu−Gly, and Ala−Tyr, the symmetry factors were 0.62, 0.35, and 0.32 and the column efficiencies were 90 056, 48 316, and 32631 N/m, respectively. All of the results demonstrated that the HILIC mode was very useful for the separation of polypeptides.

4. CONCLUSIONS

An innovative itaconic acid-based monolithic column poly(IA-*co*-AHM) featuring powerful hydrophilicity was first synthesized for HI-CEC separations. Itaconic acid is an outstanding hydrophilic monomer owing to its abundant carboxyl groups. The outstanding separation ability from hydrophilic interactions was useful for the separation of thioureas, nucleoside drugs, sulfonamides, as well as polypeptides. All separations could be achieved successfully within 10 min, possessing a resolution of more than 2.07. The advantages of high column efficiency, high resolution, good repeatability, and rapid separation made the itaconic acid-based monolithic column poly(IA-*co*-AHM) have a huge application potential. The glittering combination of itaconic acid and AHM was a preferred choice for the preparation of new hydrophilic organic-polymer monolithic columns.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08031.

FT-IR spectra of the stationary phases of Column 2; relationship between the plate height and linear velocity of *N*,*N*′-dimethylthiourea; experimental conditions: mobile phase, 60% acetonitrile in 10 mM, pH 7.0 phosphate buffer; applied voltage, from 12.5 to 25 kV; electrokinetic injection, 5 kV × 5 s; detection wavelength, 214 nm (PDF)

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Notes

The authors declare no competing financial interest.

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