Preparation of Benzyl Quinine-modified Monolithic Column for Reversed-phase Capillary Electrochromatography

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N-Benzylquininium chloride is a versatile functional monomer with quinoline and benzyl groups, which is beneficial for reversed-phase chromatography. In this study, a novel monolithic column with reversed-phase mode was synthesized using N-benzylquininium chloride as the monomer and 3-(acryloyloxy)-2-hydroxypropyl methacrylate as the cross-linker in a binary porogenic solvent consisting of PEG 400 and a 0.05 M sodium hydroxide aqueous solution. The alkaline solution were found to be useful for the improvement of the mechanical stability of the porous monoliths. The monolithic column showed excellent reversed-phase selectivity and various compounds, such as alkylbenzenes, phenols and polycyclic aromatic hydrocarbons, were separated successfully. The highest column efficiency was 1.75 × 10^6 N m⁻¹. The relative standard deviations of the migration time for thiourea and four alkylbenzenes were all less than 5.0%, which indicates the monolithic column has good stability. The application of the monolithic column for the analysis of polycyclic aromatic hydrocarbons in spiked lake water samples illustrated its great potential for practical application.

**Keywords** Capillary electrochromatography, monolithic column, reversed-phase, N-benzylquininium chloride, practical application

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**Introduction**

Reversed-phase chromatography is a versatile and popular method in separation sciences. In high performance liquid chromatography (HPLC) and capillary electrochromatography (CEC), it has very wide applications, such as for pharmaceutical analysis,1,2 food analysis,3–5 environment analysis6 and biological chromatography (HPLC) and capillary electrochromatography (CEC), it has very wide applications, such as for pharmaceutical analysis,1,2 food analysis,3–5 environment analysis6 and biological analysis.7 CEC is a micro-scaled separation technique, which combines the high efficiency of capillary electrophoresis (CE) and high selectivity of HPLC.3,8 For CEC, organic polymer monolithic columns containing a well-established continuous porous bed are an excellent column type in CEC. They have exhibited great application potential because of the advantages of ease of preparation, high column efficiency, high selectivity and good pH stability.10–12 The common monolithic stationary phases with reversed-phase mode mainly include C18,13 C12,14 C4,15 styrene,16,17 4-vinylbiphenyl,18 and grapheneoxide.18 CEC with monolithic stationary phase mentioned above has been successfully applied for the separation of polycyclic aromatic hydrocarbons (PAHs), alkylbenzenes, amino acids, phenols, anilines, food additives, etc. For the reversed-phase stationary phases modified with C18,13 C12,14 C4,15 and grapheneoxide,18 the strong reversed-phase retention performance requires a high proportion organic phase (more than 80% acetonitrile) in mobile phase to achieve the elution of analytes. The excessive consumption of organic phase is not cost-saving or environmentally friendly. Therefore, the development of novel monolithic columns that can exhibit good reversed-phase selectivity under chromatography conditions with a small amount of organic phase is valuable.

Because of the introduction of charged functional monomers, the new type styrene-based16,17 and vinylbiphenyl-based18 reversed-phase monolithic columns make a compromise between the amount of organic phase and the excellent reversed-phase selectivity. For the separation of various compounds with different polarity, the amount of organic phase decreases to 40%. The previous work provides very meaningful reference for the preparation of innovative reversed-phase monolithic columns, which can achieve the successful separation of hydrophobic compounds with the mobile phase containing a low proportion of organic phase. N-Benzylquininium chloride (NBQ) is an amphiphilic functional monomer. It has quaternary ammonium, quinolone and benzyl groups in its structure. The hydrophobic portion, quinoline and benzyl groups, can provide excellent hydrophobic and π–π interactions while the charged group, quaternary ammonium, is necessary for the generation of electroosmotic flow (EOF) in CEC. In addition, the charged group is beneficial for weakening the strong reversed-phase

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retention performance. On the choice of cross-linker, a new compound 3-(acryloyloxy)-2-hydroxypropyl methacrylate (AHM, log \( P \) 1.121) with moderate nonpolarity is introduced. For the fabrication of the monolithic column, the cross-linkers ethylene dimethacrylate (EDMA)\(^{19-21}\) and divinylbenzene (DVB)\(^{22,23}\) are always used. The log \( P \) values of EDMA and DVB are 1.931 and 3.478, respectively. The log \( P \) values are from SciFinder. The hydrophobicity of these cross-linkers is very strong. The organic polymer monoliths synthesized with these cross-linkers result in that the analytes cannot be eluted easily. Therefore, AHM is more suitable for the preparation of innovative monoliths with moderate reversed-phase retention performance.

In this study, a novel reversed-phase monolithic column poly(N-benzylquininium chloride-co-3-(acryloyloxy)-2-hydroxy-propyl methacrylate) (poly(NBQ-co-AHM)) was prepared successfully. The monomer NBQ and cross-linker AHM were firstly used for the fabrication of the reversed-phase monolithic column. The monolithic column had good permeability and excellent mechanical stability. The monolithic column poly(NBQ-co-AHM) shows good separation selectivity for alkylenzines, phenols and PAHs. The combination of NBQ and AHM provides a promising option for the development of a novel reversed-phase monolithic column.

### Experimental

#### Reagents and materials

NBQ and thiourea were obtained from J&K Company (Beijing, China). AHM was bought from TCI Company (Shanghai, China). 3-(Triethoxysilyl)propyl methacrylate (γ-MAPS) and 2,2-azo-bis(isobutyronitrile) (AIBN) were purchased from Aladdin Reagent Factory (Shanghai, China). Methylbenzene, ethylbenzene, propylbenzene, butylbenzene, and aromatic solvents. In addition, a monolithic column can be prepared by the copolymerization of NBQ, AHM, and porogenic solvents. In the experiments, the monolithic column was firstly flushed with 1.0 M NaOH for 1 h, H\(_2\)O for 0.5 h, 1.0 M HCl for 1 h, H\(_2\)O for 0.5 h and methanol for 0.5 h, respectively. Subsequently, the bare capillary was dried by nitrogen stream for 2.0 h. For the vinyl modification of the inner wall of the capillary, a methanol solution of 50% \( γ \)-MAPS (v/v) was introduced into the preconditioned capillary. After sealing both ends of the capillary with a piece of Teflon tubing, the capillary was put into a 45°C water bath overnight. Finally, the capillary was rinsed using methanol to clear away the residuals and dried with nitrogen for later use.

#### Monolithic column preparation

For the preparation of the monolithic column, 20 mg NBQ, 80 mg AHM, 240 mg PEG 400, 160 mg aqueous sodium hydroxide (NaOH-H\(_2\)O, 0.05 M) and the initiator ammonium persulfate (1.5 wt% of the monomer and cross-linker) were carefully weighed and put together to obtain a copolymerization mixture solution. The solution was kept in an ultrasonic bath for 10 min to form a homogeneous solution and degas. Then the copolymerization mixture solution was introduced into the vinyl-modified capillary of a certain length. The capillary with both ends sealed by rubber stoppers was put into a 80°C water bath for 16 h. Finally, the monolithic columns were flushed with methanol for 10 h to remove the unreacted monomers and cross-linkers, and porogenic solvents. In addition, a monolithic column was prepared with 20 mg NBQ, 80 mg AHM, 240 mg PEG 400, 160 mg H\(_2\)O and the initiator ammonium persulfate (1.5 wt% of the monomer and cross-linker) to investigate the influence of alkaline solution on the mechanical stability of the monolithic stationary phases. Before the CEC experiments, a detection window of approximately 3.0 mm was made behind the monolithic stationary phases by burning away the coating of the capillary. The total length and effective length of the monolithic columns were 34.0 and 25.5 cm, respectively.

#### CEC experiments

In the experiments, the monolithic column was firstly flushed using the mobile phase for 1.0 h on the mechanical syringe pump. Then it was equilibrated on the CE system at a voltage of –10 kV for 30 min. All the separations were carried out at 25°C.

### Results and Discussions

#### Preparation and characterization of monolithic columns

The amount of monomers, cross-linkers and porogens have
strong influence on the permeability and separation performance of the monolithic columns. The permeability \(K\) is calculated using the equation: 
\[ K = (F \times \eta \times L)/(\Delta P \times \pi \times r^4), \]
where \(L\) is the total length of the monolithic column and \(\Delta P\) is the pressure drop across the monolithic column. \(F\) and \(\eta\) are the linear velocity and the dynamic viscosity of the mobile phase methanol \((F: 0.5 \mu \text{L min}^{-1}; \eta: 0.580 \times 10^{-3} \text{ kg m}^{-1} \text{s}^{-1} \text{ at } 25^\circ \text{C})\). A series of monolithic columns were synthesized by changing the weight ratio of monomers, cross-linkers and porogens (Table S1, Supporting Information). Firstly, the weight ratio of porogens including the PEG 400 and NaOH–H\(_2\)O solution was optimized. When the weight ratio of PEG 400 is 40\%, the monomer NBQ and cross-linker AHM are not completely soluble in the porogenic solvent. When increasing the weight ratio of PEG 400 to 48\% (Column 1), a homogenous copolymerization solution was obtained, which indicates that PEG 400 is a good solvent and NaOH–H\(_2\)O solution is a poor solvent. The permeability of the monolithic column (Column 1) is \(0.23 \times 10^{-13} \text{ m}^2\). When further increasing the weight ratio of PEG 400 to 56\%, the monolithic column was blocked and the mobile phase cannot pass through the monolithic column. Therefore, the optimal weight ratio between PEG 400 and NaOH–H\(_2\)O solution was determined to be 48:32.

The weight ratio of monomer to cross-linker is another important factor for the preparation of monolithic columns. When the weight ratio of NBQ to AHM changed from 2:18 to 6:14 (Columns 3, 1 and 4), the permeability of the monolithic columns decreased from \(0.29 \times 10^{-13}\) to \(0.13 \times 10^{-13} \text{ m}^2\). From the scanning electron microscope (SEM) images (Figs. 1A-a, B-b and C-c correspond to Columns 3, 1 and 4, respectively), it is observed that the morphology change of these three monolithic columns is obvious. The morphology becomes more and more tight and the polymer spheres become increasingly smaller. The amount of through-pores obviously decreases, which is the reason for the decrease in permeability of Columns 3, 1 and 4 in turn. In particular, due to the poor permeability, the CEC experiments could not be carried out stably using Column 4. Furthermore, Columns 3 and 1 were used for the separation of four alkylbenzenes to compare their chromatography performance. As can be seen in Fig. 2, the perfect baseline separation is obtained on Column 1 with the resolution of more than 1.58. The column efficiencies for methylbenzene, ethylbenzene, propylbenzene and butylbenzene were \(1.69 \times 10^5, 1.54 \times 10^5, 1.57 \times 10^5\), and \(1.47 \times 10^5 \text{ N m}^{-1}\), respectively. Using Column 3, methylbenzene and ethylbenzene are not separated completely, and the resolution is 1.13; the column efficiencies for methylbenzene, ethylbenzene, propylbenzene and butylbenzene were \(1.14 \times 10^5, 1.02 \times 10^5, 0.92 \times 10^5\), and \(0.85 \times 10^5 \text{ N m}^{-1}\), respectively. The hydrophobic selectivity factor of ethylbenzene \((\alpha_{\text{ethylbenzene}}\)) can be obtained by taking the ratio between the retention factors \((k)\) of methylbenzene and ethylbenzene. On Column 1, the ratio of \(k\) of ethylbenzene to that of methylbenzene \((\alpha_{\text{ethylbenzene}}\)) is 1.183, which is larger than the hydrophobic selectivity factor of ethylbenzene obtained from Column 3 \((\alpha_{\text{ethylbenzene}} = 1.178)\). Therefore, the optimal weight ratio of monomer to cross-linker is 4:16 (Column 1).

In the experiment, the concentration of aqueous sodium hydroxide greatly affects the copolymerization reaction. When increasing the concentration of aqueous sodium hydroxide from 0 to 1.0 mM, the monolith morphology is changed from maple polymers to sorrel gels (Fig. S1, Supporting Information). When the concentration of aqueous sodium hydroxide is 0 or 0.05 M, the permeability of the monolith is suitable for CEC experiments. However, due to the absence of aqueous sodium hydroxide, the mechanical stability of the monolith is poor. When measuring the backpressure, there are some suspensions flushed out constantly from the capillary and the backpressure is unstable. The suspensions are some detached stationary phases of the monolithic column. The morphology of the monolith using H\(_2\)O as a porogen instead of H\(_2\)O–NaOH is shown in Fig. S2 (Supporting Information). The SEM images in Figs. S2B and S2C were taken sequentially in the same site. Some cracks appeared in the cross-section of the monolith and the cracks became larger. The reason is that the mechanical stability of the monolith is so poor that the structure of the monolith is easily torn apart due to bombardment of electron beam from SEM. For the monolith using H\(_2\)O–NaOH as a porogen, the mechanical stability is stronger and no cracks appeared (Figs. 1A and 1a). All these results show that 0.05 M aqueous sodium hydroxide is useful for the improvement of mechanical stability of the monolithic column poly(NBQ-co-AHM).

The successful synthesis of the monolithic column (Column 1) was confirmed by the FT-IR spectra (Fig. 3). The peaks of stretching vibration of quinoline ring in NBQ structure are observed in 1386 – 1477 cm\(^{-1}\) region. The peak at 1732 cm\(^{-1}\) is assigned to the carbonyl (C=O) stretching vibration of AHM. The characteristic peak of O–H bond of NBQ and AHM is at 3462 cm\(^{-1}\).
Reversed-phase chromatography

The retention behavior of the monolithic column poly(NBQ-co-AHM) (Column 1) was investigated using methylbenzene (log P 2.72), ethylbenzene (log P 3.23), propylbenzene (log P 3.73) and butylbenzene (log P 4.24) as analytes. Thiourea is the EOF mobility marker. The mobile phase consists of acetonitrile and ammonium formate buffer (10 mM, pH 3.0). The retention factor was calculated with the formula: $k = (t_r - t_0)/t_0$, where $t_r$ and $t_0$ is the retention time of the analyte and EOF marker, respectively. The relationship between retention factors of the four alkylbenzenes and acetonitrile content in the mobile phase is shown in Fig. 4. The retention factors decrease obviously when the acetonitrile content increases from 25 to 60% (v/v). When the acetonitrile content is 40%, the alkylbenzenes are separated successfully and the elution order is thiourea < methylbenzene < ethylbenzene < propylbenzene < butylbenzene according to the hydrophobicity from low to high. All these results demonstrate that the poly(NBQ-co-AHM) monolithic column possesses a typical reversed-phase retention mechanism.

EOF

The monolithic column poly(NBQ-co-AHM) generates an anodic EOF because of the presence of quaternary ammonium groups on the surface of the stationary phases. The pH value, buffer concentration and organic solvent of the mobile phase have a great effect on EOF mobility. The EOF mobility ($v_{eo}$) is evaluated by the following formula: $v_{eo} = \varepsilon_0 \varepsilon \zeta E/\eta$, where $\varepsilon_0$ and $\varepsilon$ is the permittivity of a vacuum and the dielectric constant of the eluent, respectively; $\zeta$ is the zeta potential, $E$ is the applied field strength, and $\eta$ is the viscosity of the eluent.\(^{26}\)

There was a strong EOF in a wide range of pH values (2.0 – 9.0). The influence of pH value on EOF was investigated with thiourea as the neutral marker by changing the pH value of the mobile phase. As shown in Fig. S3 (Supporting Information), EOF decreased with the increase of pH values, buffer concentration and ACN content. With the increase of pH values, the enhanced ionization of residual silanol groups on the capillary inner wall generates an opposite EOF, which weakens the anodic EOF generated by the monolith. In addition, $\zeta$ decreases with the increase of buffer concentration from 10 to 30 mM, which results in the decrease of $v_{eo}$. For ACN content, it influences $\varepsilon$, $\zeta$ and $\eta$. With the increase of ACN content, $\varepsilon \zeta/\eta$ decreases.\(^{23}\) This is the reason for the decrease of $v_{eo}$.

Column efficiency

For a novel monolithic column (Column 1), column efficiency is an important parameter to evaluate the chromatography performance. The flow velocity of the mobile phase has great influence on the plate height. The column efficiency was investigated using toluene as the analyte under the chromatography condition with 40% acetonitrile in the ammonium formate buffer (10 mM, pH 3.0), and the applied voltage was changed from -10 to -25 kV. As can be observed in Fig. S4 (Supporting Information), the variation trend of the plate height is U-shaped and the lowest plate height is 5.72 μm, which corresponds to a column efficiency of 1.75 × 10^5 N m^-1. The high column efficiency was a great advantage for the application of the resultant monolithic column on chromatographic separation.

Mechanical stability

For the organic polymer monolithic column, the mechanical instability is the inherent drawback.\(^{25}\) The sodium hydroxide aqueous solvent is useful for the improvement of mechanical stability of monolithic column poly(NBQ-co-AHM). The relationship between flow rate of the mobile phase and backpressure of the monolithic column is used to evaluate the mechanical stability.\(^{28}\) A monolithic column (total length, 34.0 cm; effective length 25.5 cm) was connected to a NANO pump with the flow rate changing from 0.2 to 0.6 μL min^-1 using methanol as the mobile phase. As can be seen in Fig. S5 (Supporting Information), with the increase of flow rate, the backpressure is linearly increased. The result suggests that the monolithic column has good mechanical stability.

Reproducibility

For a new monolithic column, reproducibility is very important. The reproducibility of the monolithic column poly(NBQ-co-AHM) was evaluated by measuring relative standard deviations (RSDs) of the migration time for thiourea and alkylbenzenes (Table 1). The intra-day (n = 5), inter-day (n = 5) and column-to-column (n = 3) reproducibility are all less than 5.0%. The result demonstrates that the monolithic column (Column 1) has good reproducibility.

Applications
Separation of phenols. The monolithic column poly(NBQ-co-
Table 1 Day-to-day (n = 5), column-to-column and batch-to-batch (n = 3) RSDs of Column 1 for separation of thiourea and alkylbenzenes (the experimental conditions are the same as in Fig. 2).

| Analyte          | Intra-day (n = 5) | Inter-day (n = 5) | Column-to-column (n = 3) |
|------------------|-------------------|-------------------|-------------------------|
| Thiourea         | 0.69              | 0.92              | 1.32                    |
| Methylbenzene    | 0.85              | 1.03              | 1.75                    |
| Ethylbenzene     | 1.29              | 1.56              | 2.31                    |
| Propylbenzene    | 1.68              | 1.88              | 3.07                    |
| Butylbenzene     | 1.86              | 2.12              | 3.87                    |

Fig. 5 Separation of phenols. Experimental conditions: mobile phase, different content acetonitrile in ammonium formate buffer (10 mM, pH 3.0); applied voltage, –20 kV; electrokinetic injection, –5 kV × 5 s; detection wavelength, 214 nm. Peaks: 1, phenol; 2, naphthalene; 3, phenanthrene; 4, pyrene; 5, benzo[a]fluoranthene.

Fig. 6 Separation of PAHs in lake water. (A) Lake water sample; (B) PAHs spiked lake water sample; (C) standard PAHs mixture. Experimental conditions: mobile phase, 50% acetonitrile in ammonium formate buffer (10 mM, pH 3.0); applied voltage, –20 kV; electrokinetic injection, –5 kV × 5 s; detection wavelength, 214 nm. Peaks: 1, thiourea; 2, naphthalene; 3, phenanthrene; 4, pyrene; 5, fluoranthene; 6, benzo[a]fluoranthene.

The PAHs are separated completely on the poly(NBQ-co-AHM) monolithic column with lower amounts of organic phase. In particular, Zhang et al.15 used stearyl methacrylate as the monomer to synthesize a C18 monolithic column and achieved the separation of PAHs just based on the hydrophobic interaction. For the poly(NBQ-co-AHM) monolithic column, not only the hydrophobic interaction but also the π–π interaction from the benzene ring and quinoline structure both contribute to the separation of PHAs. Therefore, the poly(NBQ-co-AHM) monolithic column shows more powerful separation selectivity than the C18 monolithic column. In Fig. 6, it can be seen that the baseline separation of the PAHs are achieved with the mobile phase containing 50% ACN in ammonium formate buffer (10 mM, pH 3.0). The elution order is naphthalene < phenanthrene < pyrene < fluoranthene < benzo[a]fluoranthene according to the hydrophobicity from low to high, which indicates that success separation is based on the reversed-phase mechanism.

The poly(NBQ-co-AHM) monolithic column was used for the analysis of PAHs in a lake water sample. Before use, the lake water sample was filtered using a 0.22-μm nylon membrane filter. It can be seen in Fig. 6A that there are no PAHs in the lake water. The lake water sample spiked with standard PAHs mixture was used to evaluate the separation selectivity, and good separation was obtained (Fig. 6B). The elution time and elution order are in good agreement with that of the standard PAHs mixture (Fig. 6C). This monolithic column shows promising prospects for practical applications.

Conclusions

In the experiment, a novel reversed-phase monolithic column poly(NBQ-co-AHM) was firstly prepared successfully for CEC. The copolymerization method was very simple. Due to the abundant quinoline and benzyl groups in the NBQ structure, the reversed-phase selectivity is powerful and good separations can be achieved with lower amounts of organic phase. The low consumption of organic phase is cost-saving and environmentally

AHM) (Column 1) is suitable for the reversed-phase separation of phenols. A mixed sample of five phenols including phenol (log \( P_{\text{phenol}} = 1.54 \)), hydroquinone (log \( P_{\text{hydroquinone}} = 0.62 \)), resorcinol (log \( P_{\text{resorcinol}} = 0.81 \)), pyrocatechol (log \( P_{\text{pyrocatechol}} = 0.84 \)) and phloroglucinol (log \( P_{\text{phloroglucinol}} = 0.21 \)) are used to evaluate the separation performance (Fig. 5). When the ACN content increases from 20 to 40%, the resolutions between the five phenols decrease, and their \( k\) values also become smaller. When the ACN content is 10%, excellent baseline separation of phenols is achieved with higher resolution, but phenol and pyrocatechol cannot be eluted because of the enhanced retention. Under the chromatography condition with 20% ACN in ammonium formate buffer (10 mM, pH 3.0), the phenols are almost separated completely with the elution order phloroglucinol < hydroquinone < resorcinol < pyrocatechol < phenol. The elution order is according to the hydrophobicity of phenols from low to high. All the results demonstrate that the poly(NBQ-co-AHM) column offers good reversed-phase performance and is very suitable for the separation of phenols.

Separation of PAHs. The standard PAHs mixture comprising naphthalene, phenanthrene, fluoranthene, pyrene and benzo[a]fluoranthene were used for the further demonstration of the reversed-phase separation ability of the poly(NBQ-co-AHM) monolith (Column 1). Compared to the previous work,13,21,28,29 the standard PAHs mixture is suitable for the reversed-phase separation ability of the poly(NBQ-co-AHM) monolith (Column 1). Therefore, the poly(NBQ-co-AHM) monolithic column was used for the analysis of PAHs in a lake water sample. Before use, the lake water sample was filtered using a 0.22-μm nylon membrane filter. It can be seen in Fig. 6A that there are no PAHs in the lake water. The lake water sample spiked with standard PAHs mixture was used to evaluate the separation selectivity, and good separation was obtained (Fig. 6B). The elution time and elution order are in good agreement with that of the standard PAHs mixture (Fig. 6C). This monolithic column shows promising prospects for practical applications.

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friendly. Various compounds, including alkylbenzenes, phenols and PAHs, were separated successfully. The monolithic column has good porous structure, mechanical stability and reproducibility, which make it very promising for practical applications.

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Supporting Information

The preparation and characterization of monolith poly(NBQ-co-AHM) were shown in Figs. S1 and S2. The influence factors on EOF mobility, plate height and mechanical stability of Column 1 were shown in Figs. S3 to S5. The compositions of the polymerization mixtures for the different monolithic columns were shown in Table S1. Supplementary data to this article can be found online at doi.org/10.2116/analsci.20P075.

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