Analytical Methods

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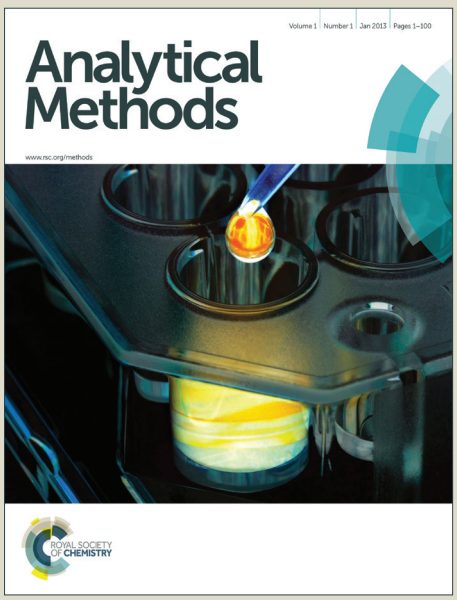

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A novel kind of ionic liquid-based monolithic column and its application to efficient separation of protein and small molecules by high performance liquid chromatography

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ABSTRACT: A novel skeleton porous polymer-based monolith was successfully prepared via in situ free radical polymerization technique in a 50 mm × 4.6 mm i.d. stainless-steel chromatographic column using dodecanol as porogen; ionic liquid (IL), 1-dodecene (C12), and trimethylol propane triacrylate (TMPTA) as monomers; ethylene dimethacrylate as crosslinker. The effects of some variables such as temperature, content of porogen solvent affecting the porous structure were studied in detail. The obtained polymer-based monolith was characterized by scanning electron microscopy, infrared spectroscopy, mercury intrusion porosimetry, and nitrogen adsorption apparatus, respectively. The results showed that the monolithic column had a porous structure, good mechanical stability, high permeability (6.77×10^{-14} m^2), and high specific surface area (155.62 m^2 g^{-1}). Furthermore, the synthesized monolith was undergone liquid chromatographic evaluation by separating lysozyme from egg white and separating different kinds of small molecules mixtures, such as benzene and its analogues and amines. The prepared column showed a good repeatability and reproducibility, of which the column-to-column (n = 7) and batch-to-batch (n = 5) reproducibility were 2.85 and 3.15%, respectively.

Key words: Ionic liquids; High performance liquid chromatography; Monolithic column; 1-Vinyl-3-butylimidazolium chloride

1. Introduction

Polymeric monoliths were introduced about twenty years ago as materials facilitating rapid mass transport driven by convection though the monolith large pores [1]. As the fourth generation chromatographic sorbents, monolithic column possessed unique structure and exhibited some exceptional characteristics which could be described as a continuous porous separation media for separation science [2]. During the past decades, monolithic columns have developed rapidly for their advantages of low back pressure drop, great permeability, fast mass-transfer, simple preparation and easy to be modified [3]. As a result, monolithic columns became an excellent tool in the analytical laboratory, not only covered the separation fields, such as ion-exchange, hydrophobic interaction, size exclusion,

and affinity chromatography etc., but also for sample preparation technique including solid phase extraction (SPE) or solid-phase microextraction (SPME) [4, 5].

Monolithic columns, as a separation media of high performance liquid chromatography (HPLC), based on the different materials, of which, there are mainly two major kinds of monoliths: inorganic silica-based matrices, and organic polymer-based ones such as polystyrenes, polymethacrylate esters, polyacrylamides etc. [6-8]. The silica-based and polymer-based materials showed significant different properties. The silica-based ones allow fast separations of small molecules, and critically, the preparation process was complex and difficult to control [9]. Besides, the silica-based monoliths are suffered from hydrolysis of the Si-O linkage, which resulting in a narrow rage (pH 2-8) of application.

The organic polymer-based monoliths, being post-modified easily, are used widely for their stability in wide pH value (pH1-14) rage [10]. Particularly, polymeric materials have proven to be an excellent stationary phase of HPLC for the rapid separation of large molecules such as proteins, nucleic acids, and peptides etc. [11]. Nevertheless, they are suffered from apparent disadvantages, such as poor reproducibility, poor resolutions, non-uniform structure caused by poor solubility of monomers and porogens. Therefore, a new alternative was introduced using ionic liquid as co-monomer in our work to overcome these problems.

Ionic liquids (ILs) are a class of non-molecular ionic compounds which are chemically inert, stable, and non-volatile organic molten salts. It is worth mentioning that they are liquid at temperature less than 100□ [12-14]. They have attracted wide attention due to their unique properties, including low volatility, tunable viscosity, and good biocompatibility [15, 16]. Today the filed of ILs is of wide interest to many application fields in analytical chemistry, but also outside of that [17, 18]. According to the properties of ILs [19], a new IL-based monolithic material was synthesized.

In this work, a novel HPLC monolithic column was prepared via in situ free-radical polymerization using 1-vinyl-3-butylimidazolium chloride (IL) as co-monomer. The effects of some variables affecting the porous structure were studied in detail. Finally, the optimized monoliths were applied for the separations of lysozyme (Lys) from egg white and aromatic compounds.

2. Experimental

2.1 Materials

1-Dodecene (C12), 1-vinylimidazole, 1-chlorobutane were purchased from Shanghai Aladdin co. (Shanghai, China). Trimethylol propane triacrylate (TMPTA), ethylene dimethacrylate (EDMA), and azobisisobutyronitrile (AIBN) were the products of Tianjin Chemistry Reagent Factory (Tianjin, China). Dodecanol PEG-200 and hexadecanol were product of Shanghai Chemical Plant (Shanghai, China). The aromatic compounds were provided by the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). HPLC-grade methanol and potassium bromide (KBr) were products of Kermel Chemical Reagent Co. Ltd. (Tianjin, China). The stainless-steel columns (50 × 4.6 mm i.d.) were purchased from Beijing Xinyu Instrument Co. Ltd. (Beijing, China). Lys was obtained from Sigma Chemical Co. (St Louis, MO, USA). Triplex distilled water was used for all experiments. All media were filtered through a 0.45 μm membrane before use.

2.2 Instruments

An 1100 system from Agilent Technologies (USA) was applied to chromatographic studies. The HPLC system consisted of a quaternary pump with an online vacuum degasser, an autosampler with variable injection capacity from 0.1 to 100 μL and a UV detector. All sample solutions injected into the chromatographic system were filtered through a millipore membrane (0.45μm) to remove particles and large aggregates. Morphology of the monolithic columns was carried out on a Hitachi S-3400 scanning electron microscopy (Hitachi High Technologies, Tokyo,

Japan). The FT-IR spectra were recorded on an FTIR-8400S IR apparatus in the region of 400-4000 cm^{-1}, (Shimadzu, Kyoto, Japan).

2.3 Preparation of polymer-based monolithic columns

2.3.1 Synthesis of ionic liquids (1-vinyl-3-butylimidazolium chloride, VBC-ILs)

1-Chlorobutane (14.81 g, 160 mmol, 16.6 mL) was added drop-wise to 1-vinylimidazole (8.00 g, 85 mmol, 7.7 mL). The mixture was heated at 70□ under stirring for 24 h. Phase separation occurred and the viscous yellow liquid obtained was washed with ethyl acetate. Then the product was filtered and dried in a vacuum oven until constant weight. Synthesis of processes of VBC-ILs was depicted in Fig. 1(a).

2.3.2 Preparation of IL-based monolithic columns

The monolithic column was directly synthesized via in situ polymerization with a pre-polymerization solution consisting of functional monomers, cross-linker, initiator, and porogen, following sonicated in a bath sonicator for 15 min to degas. The compositions were listed in Table 1, where IL, C12, and TMPTA were used as common monomers; EDMA as crosslinking agent; dodecanol, PEG-200, and hexadecanol as porogens, and AIBN as initiator. The obtained homogeneous solution was manually injection into a clean stainless-steel column (50 × 4.6 mm i.d.) which then was sealed at both ends with closed column heads. The polymerization was incubated in a 60□ water bath for 24 h. The resulting monolithic column was washed online with methanol in conjunction with HPLC to remove unreacted monomers, porogen, and other soluble compounds present in the polymeric rod. The scheme polymerization was shown in Fig. 1(b).

2.4 Characterization methods

2.4.1 Instrumental analytical methods

The preparation conditions have much affected on the structures of monolithic columns. In order to obtain a poly(IL-*co*-C12-*co*-TMPTA-*co*-EDMA) monolithic column with satisfied structure, SEM was used to investigate

the morphologies following different conditions. The porous properties of the monoliths were investigated by mercury intrusion porosimetry, and the specific surface area was calculated from nitrogen adsorption/desorption isotherms. The chemical groups of the monoliths were studied by fourier transform infrared spectroscopy (FT-IR).

2.4.2 Calculation methods

The column permeability *K* was calculated according to the following equation:

$$K = \frac{F \times \eta \times L}{\Delta P \times \pi \times r^2} \quad (1)$$

Where *F* is the mobile phase flow rate, η is the dynamic viscosity of eluent, *L* is the column length, ΔP is the pressure drop across the column and *r* is the column inner radius. The value of the dynamic viscosity for mobile phase methanol was 0.580×10^{-3} kg m^{-1} s^{-1}.

2.5 The separation of Lys from egg white

The IL-based monolithic column was used to separate Lys from egg white by ionic interactions as shown in the following part. Chicken egg white was separated from fresh eggs and diluted to 50% (V/V) with phosphate buffer (50 mmol, pH 7.0). The diluted egg white was homogenized in an ice-bath and centrifuged at 4 □ and 10,000 rpm for 10 min. The separation was carried out under the following chromatographic conditions: UV detector was 280 nm; injection volume was 5.0 μL; Gradient: 0-3 min, 0.02 mol L^{-1} Na_2HPO_4 aqueous solution (pH=12, adjusting with NaOH aqueous solution) was used as the mobile phase; 3.01-10 min, water was used as the mobile phase.

3. Results and discussions

3.1 The optimizationof preparation conditions

3.1.1 The influence of porogens on the monolithic properties

The conditions and results shown in Table 1 showed that columns based on PEG200 (Column J) or hexadecanol (Column K) as porogen showed non-suitable properties, respectively, while dodecanol exhibited good solubility wiht functional monomers and was chosen as the porogen solvent for further optimization.

In order to investigate the influence of dodecanol content on the preparation of poly(IL-*co*-C12-*co*-TMPTA-*co*-EDMA) monolith, different dodecanol contents were investigated as listed in Table 1. The results showed that the column permeability increased, while the hardness and back pressure decreased following the increasing dodecanol proportion (Column A, F-I). However, when the proportion of dodecanol in the pre-polymerization solution increased to 2.4 mL (Column I), the mechanical properties was too poor. Among them, monolithic column exhibited good permeability, moderate hardness, and low back pressure when the amount of dodecanol was 2.0 mL (Column A).

3.1.2 The influence of temperature on the monolithic properties

It is known to all, the preparation temperature affect on the property of the resulting polymeric monolith. So, the polymerization was performed at different temperatures (40, 50, 60, 70□). We found that the permeability of the monolith became worse and mechanical properties were harder, following the increase of the temperature. However, the polymerization could not proceed when the temperature were 40 □ or 50 □. Considering back pressure and mechanical strength, 60 □ was chosen for further experiment.

3.2 Characterizations of the monoliths

3.2.1 SEM figures of monoliths

The SEM was carried out to characterize the cross-section morphology of the resulting monolithic columns, which were shown in Fig. 2 (A-E) and in accordance with different compositions shown in Table 1 (A-E). The results showed that different compositions of the functional monomers affected on the main structure, Fig. 2A obtained from column A possessed expected interconnected and uniform porous structure compared with other monoliths, which was formed by spherical particles accumulation. The SEM photographs of poly(C12-*co*-TMPTA-*co*-EDMA) (Column B) and poly(IL-*co*-TMPTA-*co*-EDMA) (Column C) monolithic columns were shown in Fig. 2B and C, respectively, which both were accumulated with small globules. The density of the pores would be higher and the

pore diameter would be smaller following the increasing amount of EDMA, which was because that more EDMA would lead to highly crosslink. Compared with column A, column D (Fig. 2D) showed a looser pore structure and column E (Fig. 2E) showed a relatively denser structure. The results indicated that the combination of IL, C12, TMPTA, EDMA, and dodecanol could lead to a more porous and uniform structure with high permeability.

Furthermore, the columns A, B, and C underwent liquid chromatographic evaluation by separating small molecules to confirm the performance of the three monoliths. Fig. 3 presented the chromatographic separation of mixed benzene, biphenyl, and anthracene by columns A, B, and C, respectively, of which chromatogram A was much better than that of B and C. The peak band broadening from B and peak tailing from C indicated that the composition of monomers would effect on the structure and then resulting in chromatographic performance. Through optimization and comparison, column A was adopted for the following experiments.

3.2.2 FT-IR characterization of IL-based monolith

The present groups on the monolith were confirmed by the FT-IR. As shown in Fig. 4, the spectrum at 2957-2901 cm^{-1} was due to the C-H bands. A characteristic peak of C=O double bands at 1750 cm^{-1} appeared. Moreover, there was a C-H bond stretching vibration around imidazole ring at 1169 cm^{-1}, which confirmed the presence of IL on IL-based monolith.

3.2.3 Permeability and mechanical strength of the monolith

Permeability (*K*) is an important parameter of HPLC columns. High permeability would result in low back pressure and low mass transfer resistance in HPLC. The permeability of polymer-based monolithic column was determined by pumping methanol through the monolithic column A. According to Equation (1), the calculated permeability values were shown in Table 1. The results demonstrated that different preparations resulted in different permeability. Considering back pressure and hardness of the monolithic columns, IL-based monolith column (column A) with permeability as 6.77×10^{-14} m^2 was selected as the optimized one.

Fig. 5 showed the back pressures of Column A at different flow rate with methanol and water as the mobile phases, respectively. Although the flow rate was raised to 5mL min^{-1} using water as mobile phase, the maximum pressure was 43 bar. Moreover, good linear responses ($r^2 > 0.999$) between the back pressure and the flow rate, which confirmed a good mechanical stability.

3.2.4 Pore size distribution of the monolith

The measurement of the pore size distribution and specific surface area of the monolith was carried out by mercury intrusion porosimetry and nitrogen adsorption-desorption isotherm, respectively. Fig. 6 showed the result obtained by mercury intrusion porosimetry. The total intrusion volume, average pore diameter, and porosity were 1.88 mL g^{-1}, 1.31 μm, and 70.36%, respectively. According to the report of specific surface area, the single point surface area at P/P0 = 0.2999 was 154.74 m^2 g^{-1} and the BET surface area was 155.62 m^2 g^{-1}. The results showed that the IL-based monolithic column had a large surface area.

3.3 Chromatographic behavior of the IL-based monolithic column

3.3.1 Separation of Lys from egg white

The chromatogram was shown in Fig. 9, in which, Lys was separated from egg white successfully. The mechanism of separation was as follows: when Na_2HPO_4 aqueous solution (pH=12, adjusting with NaOH aqueous solution) was used as the mobile phase in the 0-3 min, the pH value of mobile phase was higher than the pI (approximately 11) of Lys, and the Lys was negative charged, which attracted each other with the positive charged monolith. Thus the Lys was retained by the monolithic column. When water was used as the mobile phase in 3.01-10 min, the pH value of mobile phase was lower than the pI of Lys, and the Lys was positive charged, which repulsed each other with the positive charged monolith. Thus the Lys was eluted. The IL-based monolithic column was positive charged because of the present of imidazolium groups. So this monolith could be used to separate proteins by ionic interactions as shown in the following part.

Furthermore, the content of Lys was assayed by ultraviolet spectrophotometry and the purity of Lys was calculated after being dealt with vacuum freeze-drying with the result 92.1%.

3.3.2 The effects of mobile phase on the separation of small molecules

Five mixed compounds were separated with different ratio of methanol/water on the IL-based monolith. Fig. 7 showed that the retention times of the five mixed compounds increased following the decrease of the methanol content, which were 80% (a), 75% (b), and 70% (c), respectively. These analytes were eluted in the order aniline, p-xylene, 1-naphthalene, diphenylamine, and triphenylamine in accordance with their polarities from high to low, which presented the typical reversed phase liquid chromatographic mode. Considering both the analysis time and resolution, the ratio of methanol/water (75/25, v/v) with the flow rate of 1.0 mL min^{-1} was selected as the optimal chromatographic conditions.

Chromatographic behavior of the IL-based monolithic column in the separation of aromatic compounds and the results were shown in Fig. 8, of which, the chromatogram Fig. 8(a) showed the baseline separation of four compounds with the mobile phase methanol/water (75/25, v/v). The analytes were eluted in the following order: aniline, p-xylene, 1-naphthalene, diphenylamine, and triphenylamine, which were correspond to the hydrophobicities of the five analytes from low to high. The result indicated the typical reversed-phase mode in the separation. The retention factors (k) of each sample on the IL-based monoliths were determined at different content of methanol in the mobile phase and the result was shown in Fig. 8(b), of which, a typical reversed phase-liquid chromatographic mechanism was proven. A typical separation of neutral aromatic compounds was shown in Fig. 8(c), in which, it showed the baseline separation of four compounds with the mobile phase methanol/water (68/32, v/v). The four compounds were eluted in accordance with their polarities from high to low as benzene, naphthalene, biphenyl, and anthracene, where the column efficiencies for the four compounds were about 5940-9249 theoretical plates per meter. The retention factors (k) of the four analytes on the IL-based monolith were present in Fig. 8(d),

which also showed the retention factor of each sample decreased following the increase of the methanol content in mobile phase, confirming the typical reversed-phase chromatographic mechanisms of the separation.

3.4 Reproducibility

The reproducibility of monolithic column was obtained through the percent relative standard deviations (RSDs) of the retention factors of the test compounds on column A. The average run-to-run reproducibility (n = 5) of benzene was 1.07%, while the average day-to-day reproducibility (n=3) was 1.75%, respectively. These results demonstrated the stability of the monolithic columns. In addition, the column-to-column and batch-to-batch reproducibility were also investigated with the same or different batch of polymerization mixture, respectively. The column-to-column (n = 7) and batch-to-batch (n = 3) reproducibility were 2.85 and 3.15%, respectively. The result confirmed the resulting monolithic columns had good reproducibility and stability.

4. Conclusions

In this work, a porous and uniform poly IL-based monolithic column with high specific surface area has been successfully prepared using IL as co-monomer via in situ free radical polymerization, which was successfully used to separate Lys from egg white. Besides, the monolithic column exhibited good performance in the reversed phase liquid chromatographic separation. The resulting monolithic column is potentially useful alternative for the efficient separation of proteins and small molecules.

5. Acknowledgement

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Comments of figures and tables

Fig. 1 Synthesis processes of ILs

Fig. 2 Scanning electron microscopy of samples.

Fig. 3 Chromatographic behaviors of basic compounds on the monolith with different monomers.

Fig. 4 The FT-IR spectrum of the poly IL-based monolithic column A.

Fig. 5 The mechanical stability of the IL-based monolithic column at different velocities.

Mobile phase: (a) methanol and (b) water.

Fig. 6 Pore size distribution of the IL-based monolith.

Fig. 7 Effect of methanol content in mobile phase on the chromatographic separation.

Conditions: Monolithic column, 50 × 4.6 mm i.d.; flow rate: 1.0 mL min^{-1}; mobile phase: (A) methanol/water (80/20, v/v); (B) methanol/water (75/25, v/v); (C) methanol/water (70/30, v/v); UV detection wavelength: 254nm; Peak identification: (1) aniline, (2) p-xylene, (3) 1-naphthalene, (4) diphenylamine, and (5) triphenylamine.

Fig. 8 Chromatographic behaviors of the IL-based monolith in the separation of aromatic compounds.

Chromatographic conditions: (a) mobile phase: methanol/water (75/25, v/v); Analytes: 1, aniline, 2, p-xylene, 3, 1-naphthalene, 4, diphenylamine, and 5, triphenylamine. (c) mobile phase: methanol/water (68/32, v/v); Analytes: 1, benzene; 2, naphthalene; 3, biphenyl; 4, antaracene. (b) and (d): mobile phase: methanol/water, and the content of methanol as in this figure; flow rate: 1.0 mL min^{-1}; detection wavelength: 254 nm.

Fig. 9 Chromatogram of the separation of Lys from egg white.

HPLC conditions: UV detector was 280 nm; injection volume was 5.0 μL; Gradient: 0-3 min, 0.02 mol L^{-1} Na_2HPO_4 aqueous solution (pH=12, adjusting with NaOH aqueous solution) was used as the mobile phase; 3.01-10 min, water was used as the mobile phase.

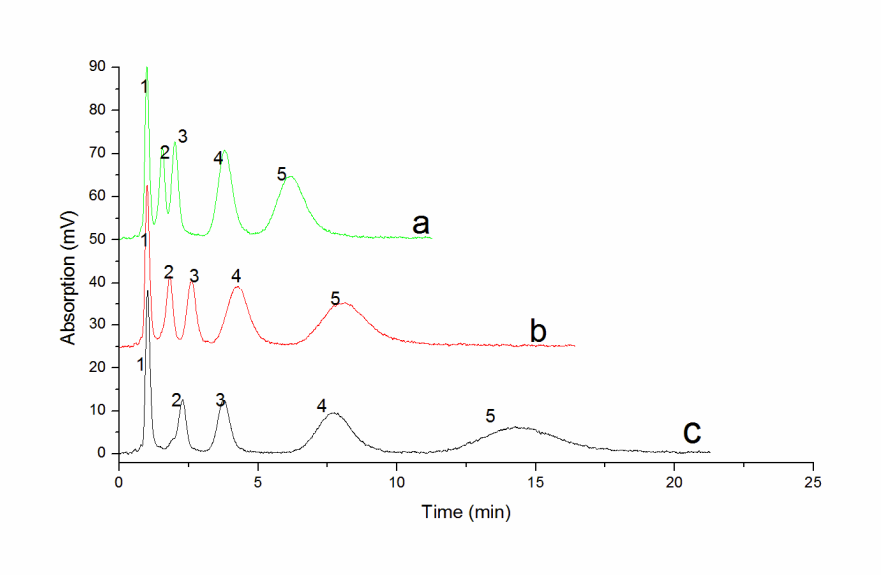
Table 1: Compositions of the pre-polymerization mixtures for the monoliths prepared.

[1] All columns were prepared with 0.01 g AIBN to initiate.

[2] Pressure was obtained with methanol as the mobile phase at 1.0 mL min^{-1}.

Graphical abstract

In this study, ionic liquid was introduced to improve the performance of the polymer-based monolithic column (4.6 mm i.d.) in conjunction with high performance liquid chromatography. The resulting porous monolith with high specific surface area produced improved column efficiency in the separation of small molecules from the mixture compared to the previous works.



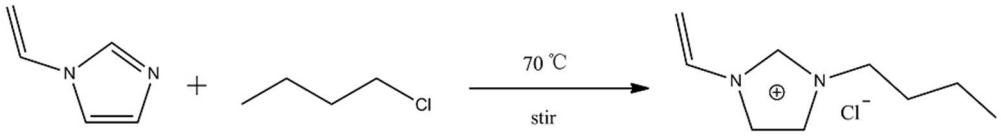


Fig. 1(a)
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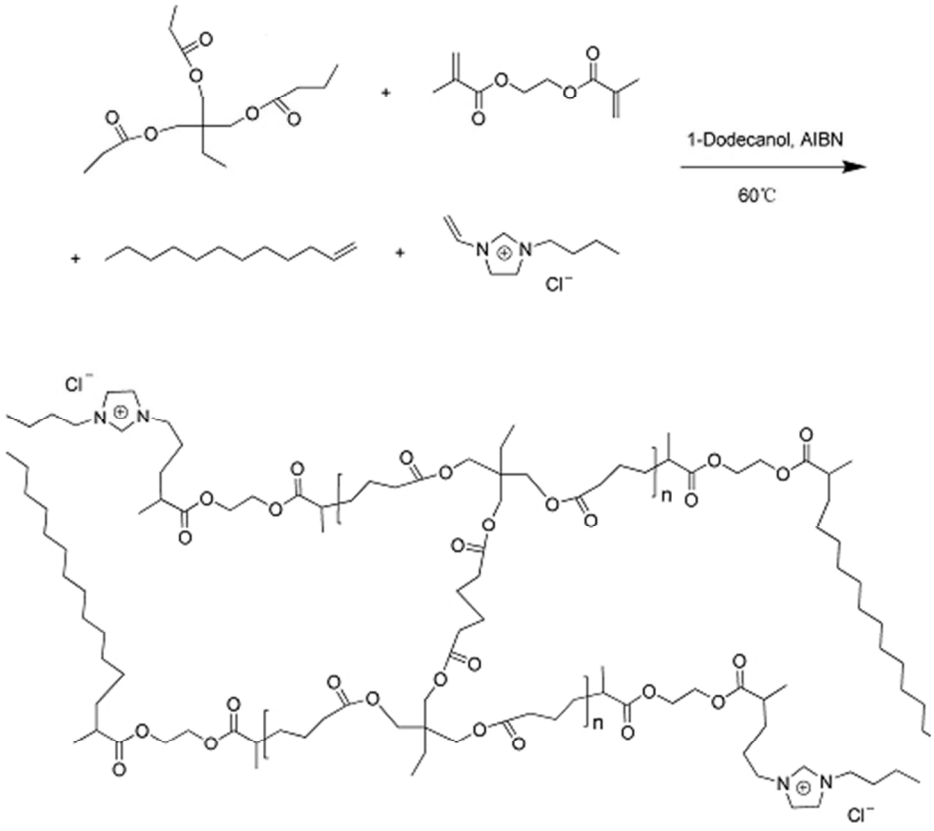

Fig. 1(b)
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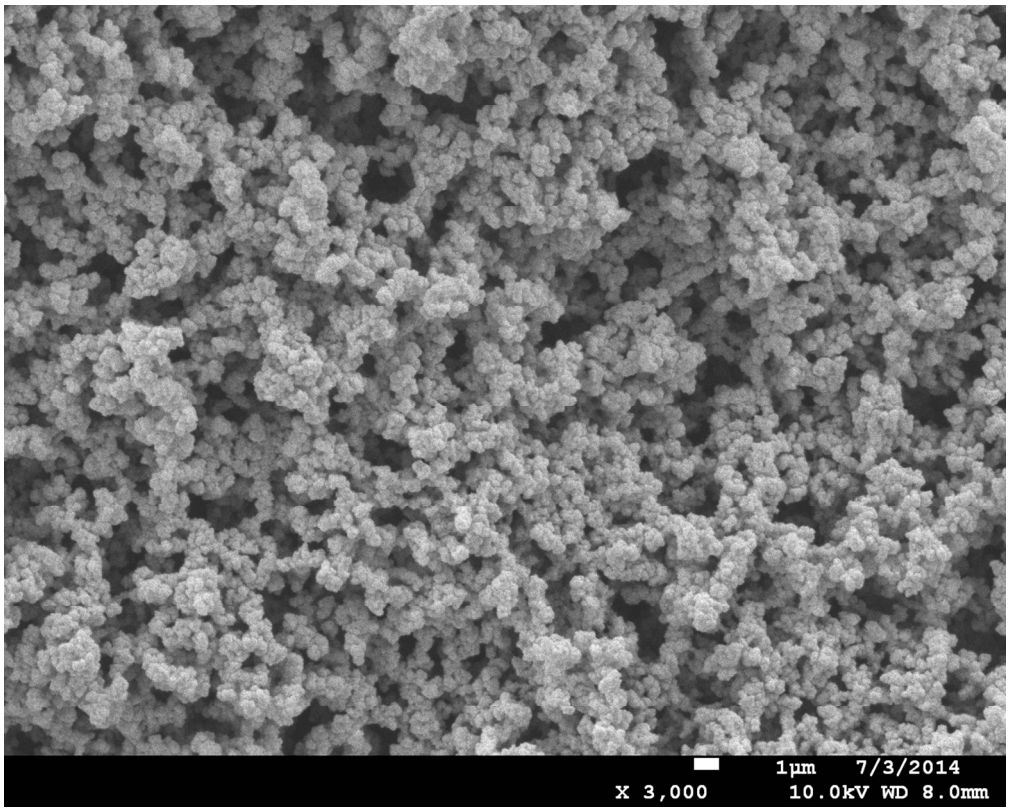

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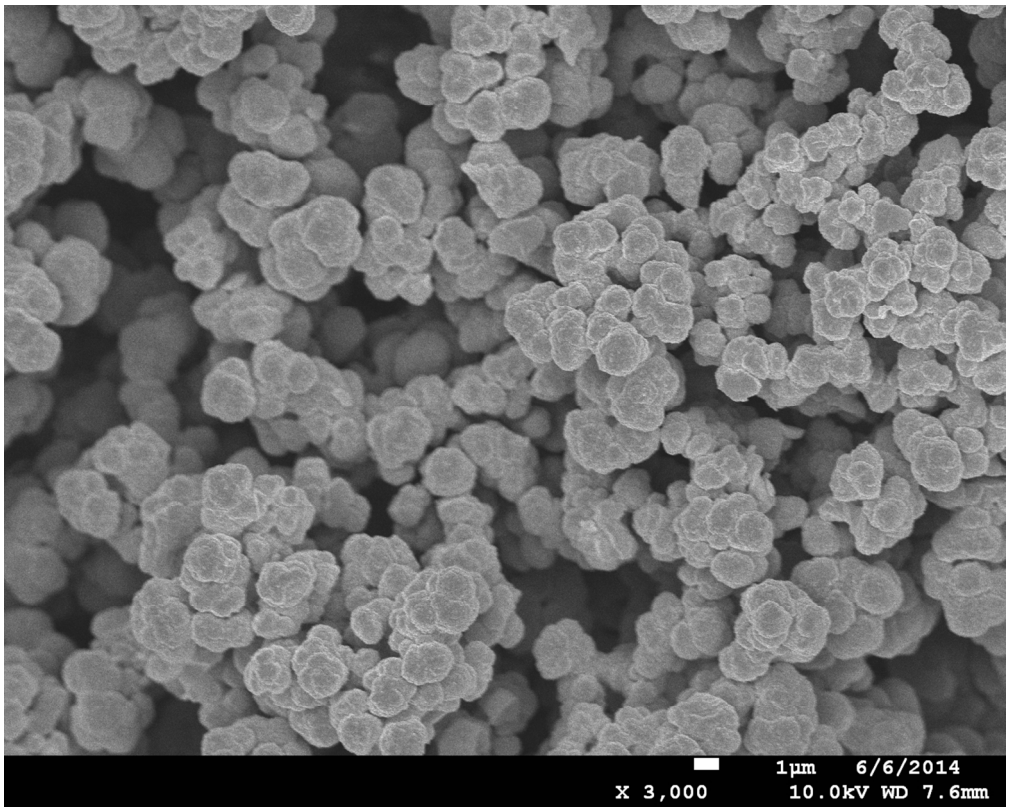

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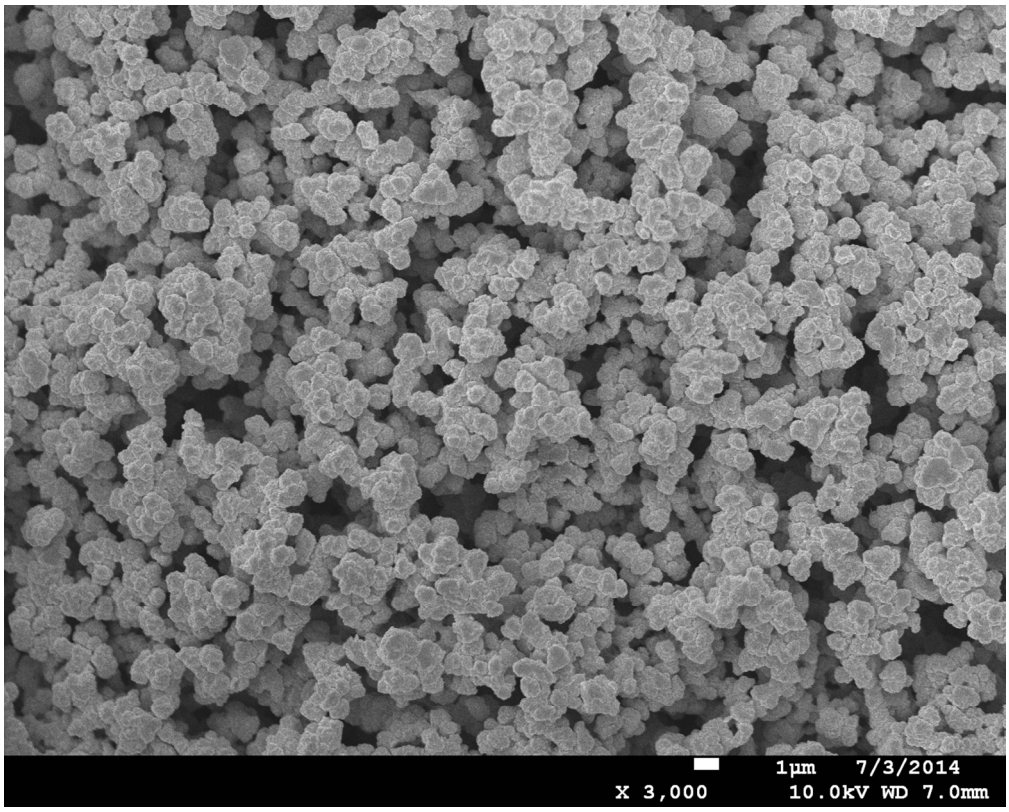

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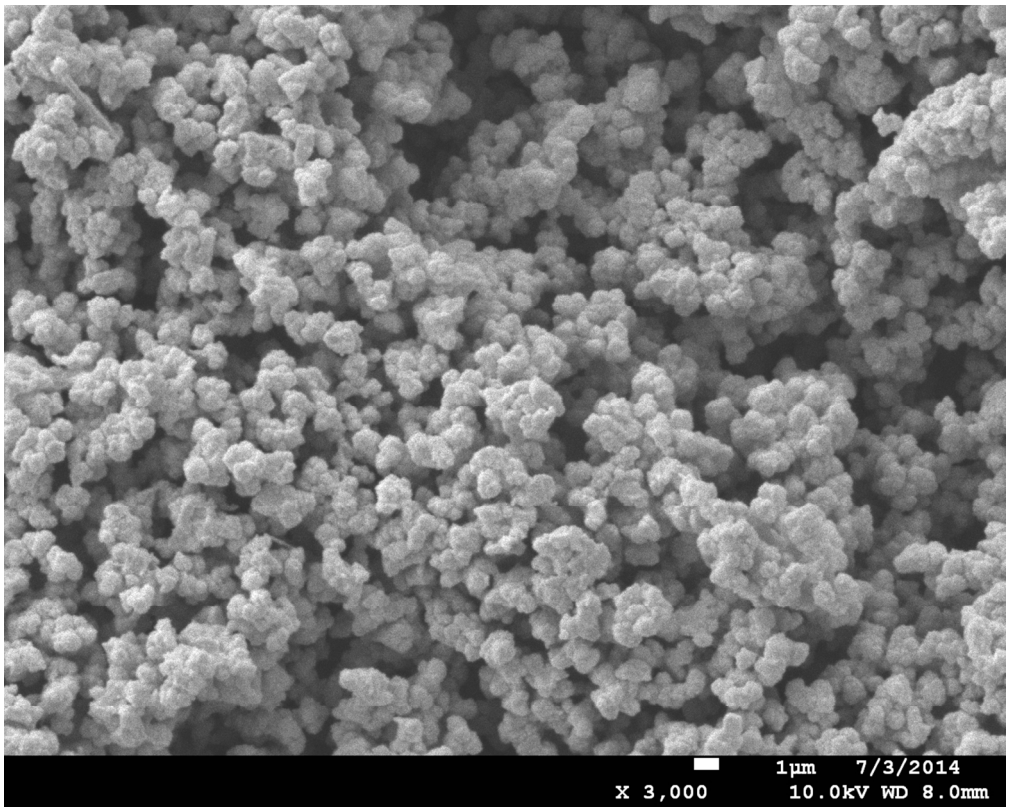

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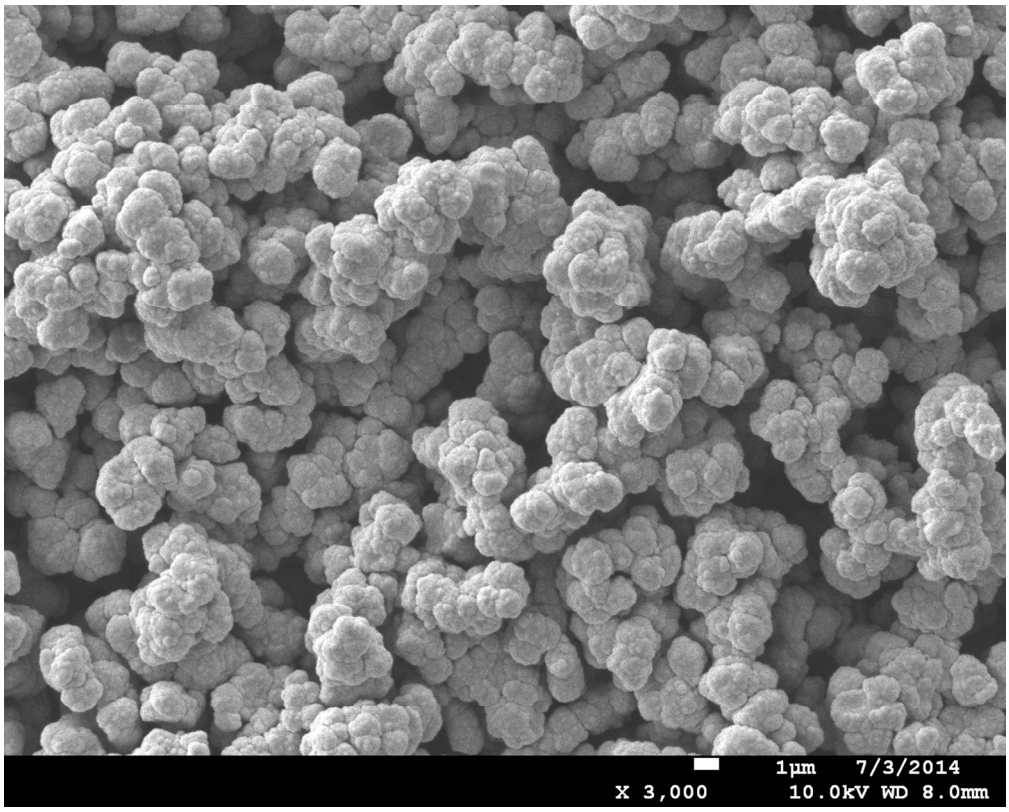

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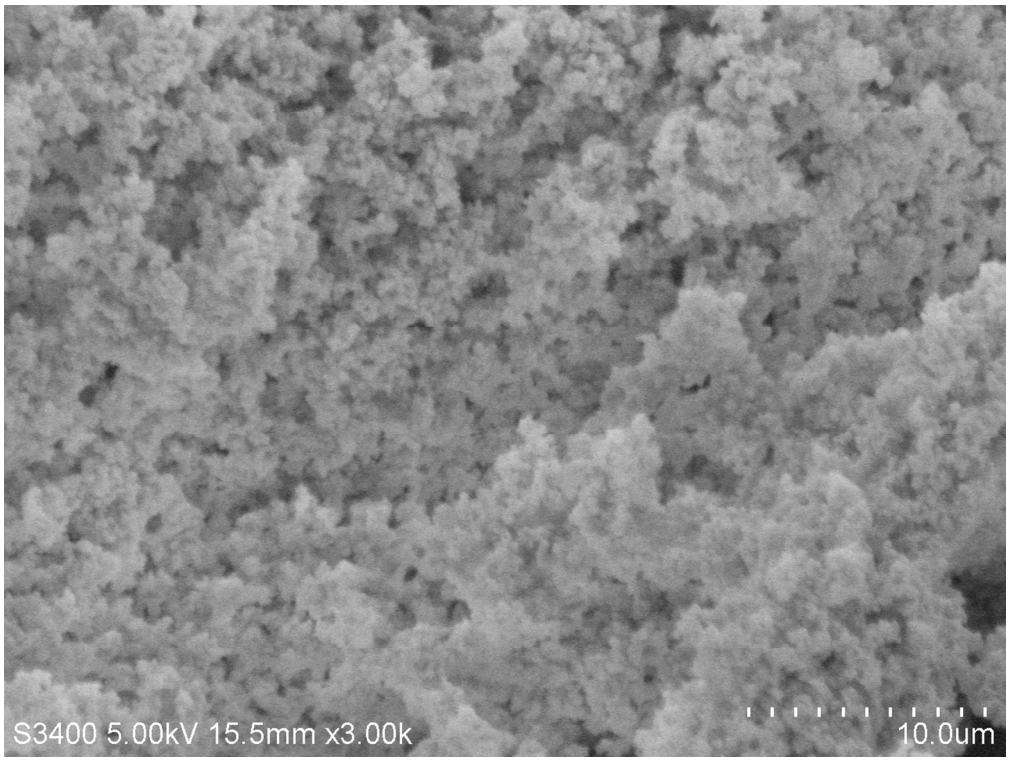

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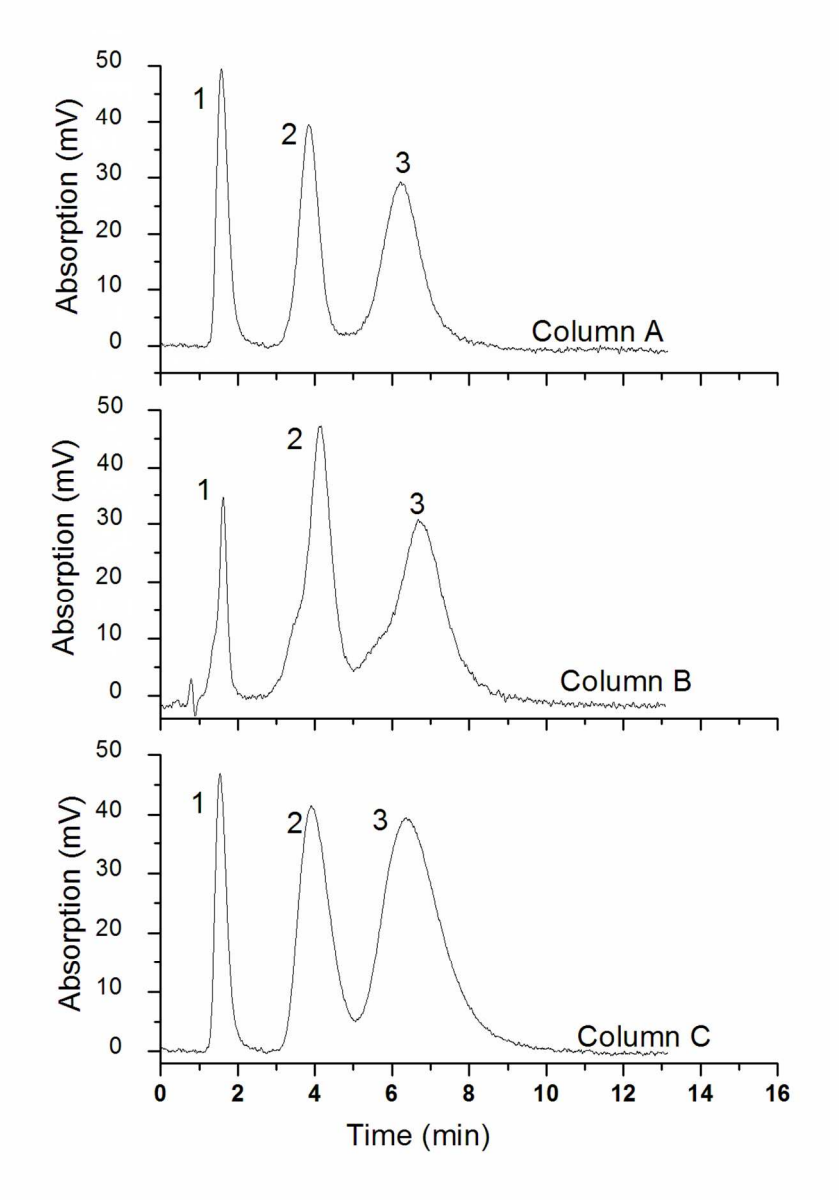

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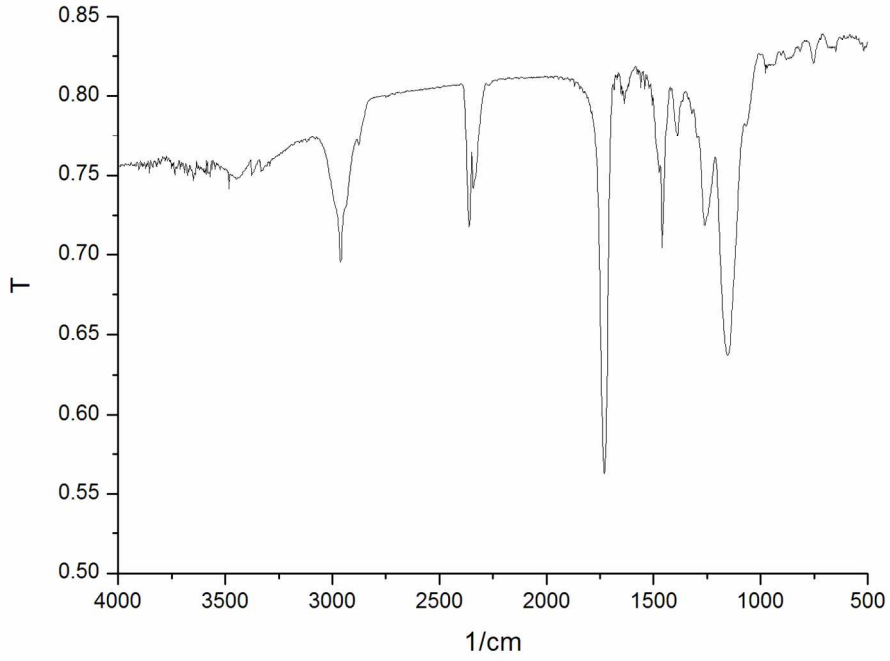

Fig.4
270x208mm (150 x 150 DPI)

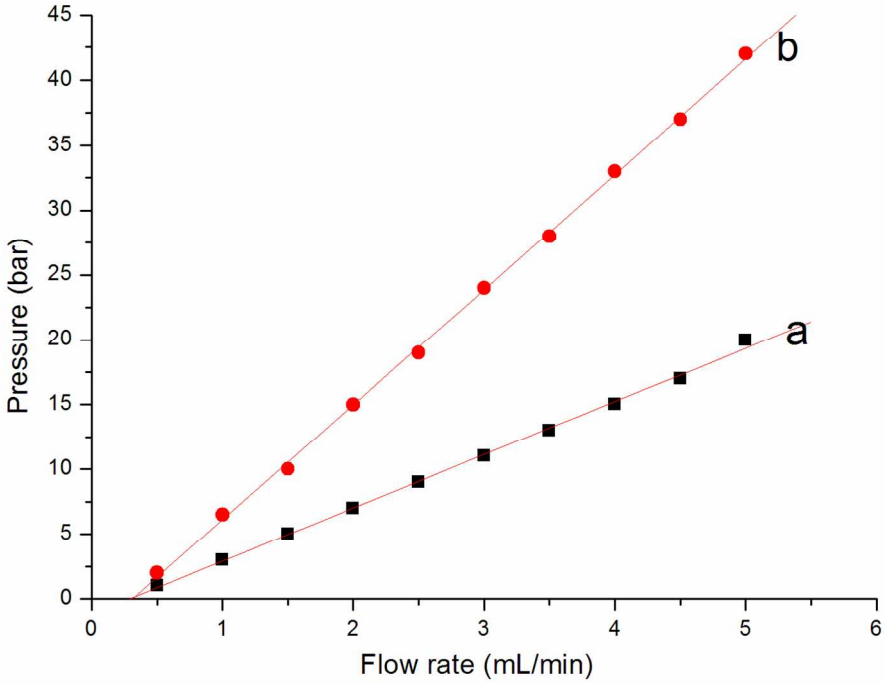

Fig.5
258x206mm (150 x 150 DPI)

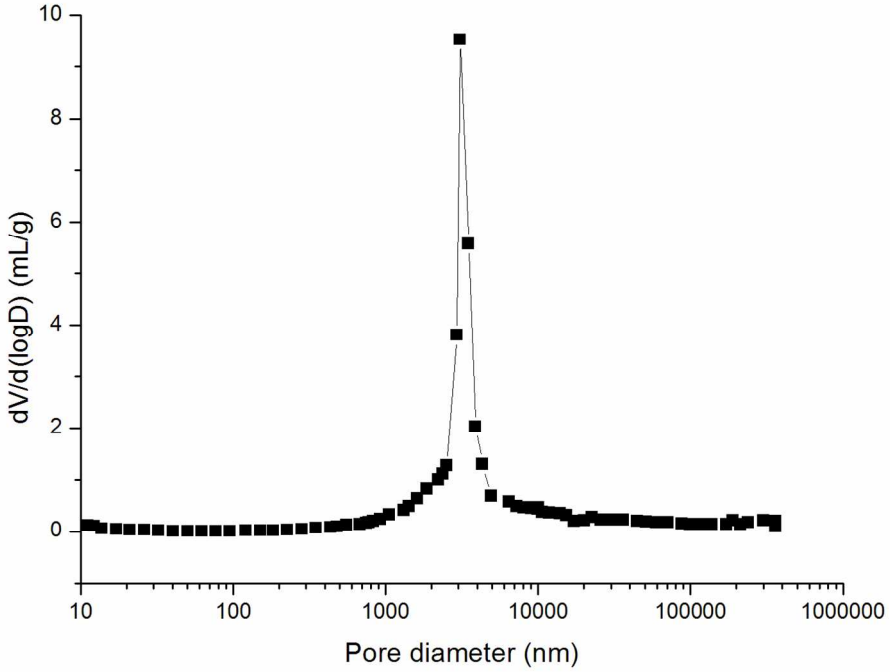

Fig.6
265x208mm (150 x 150 DPI)

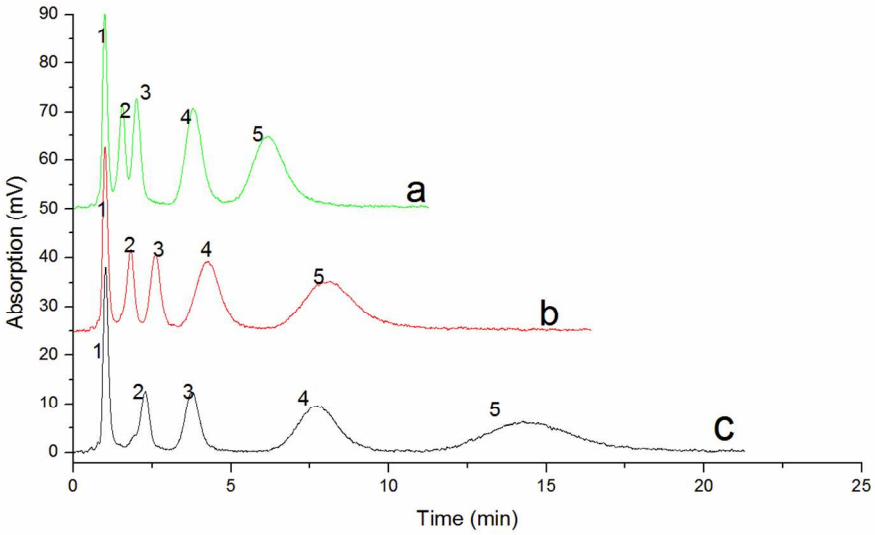

Fig.7
234x154mm (150 x 150 DPI)

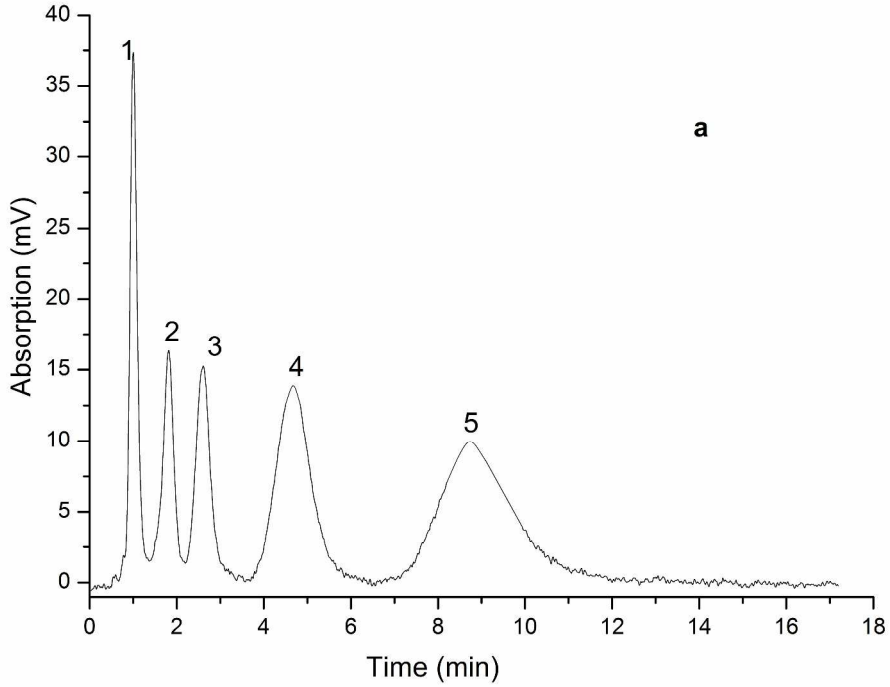

Fig.8(a)
1617x1299mm (96 x 96 DPI)

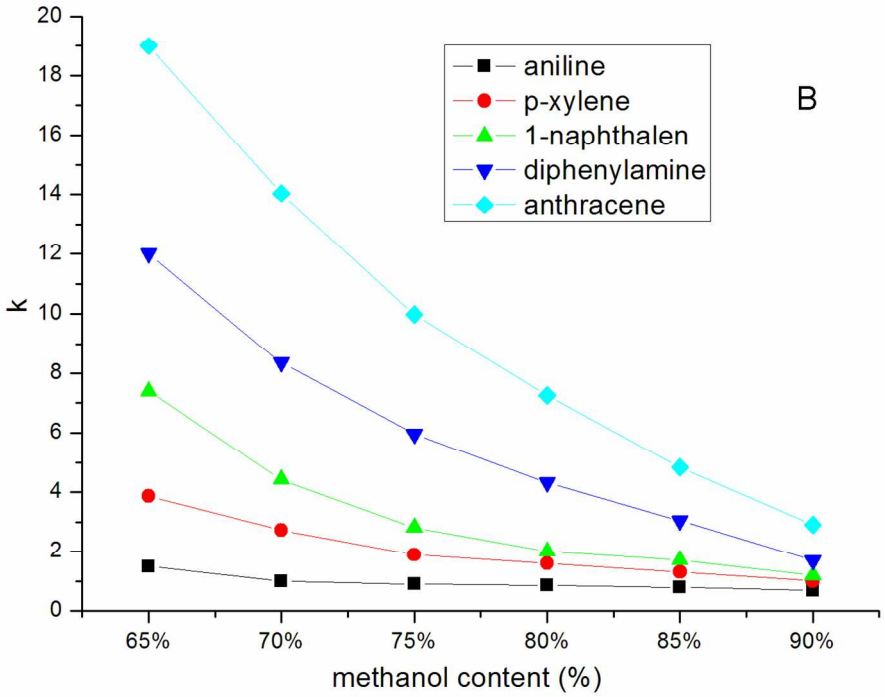

Fig.8(b)
396x322mm (96 x 96 DPI)

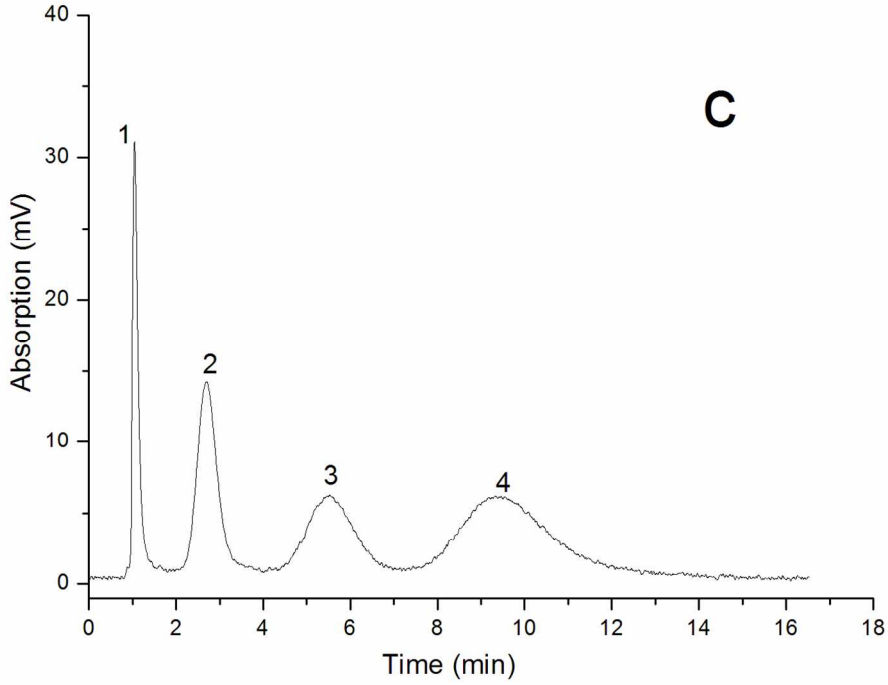

Fig.8(c)
258x206mm (150 x 150 DPI)

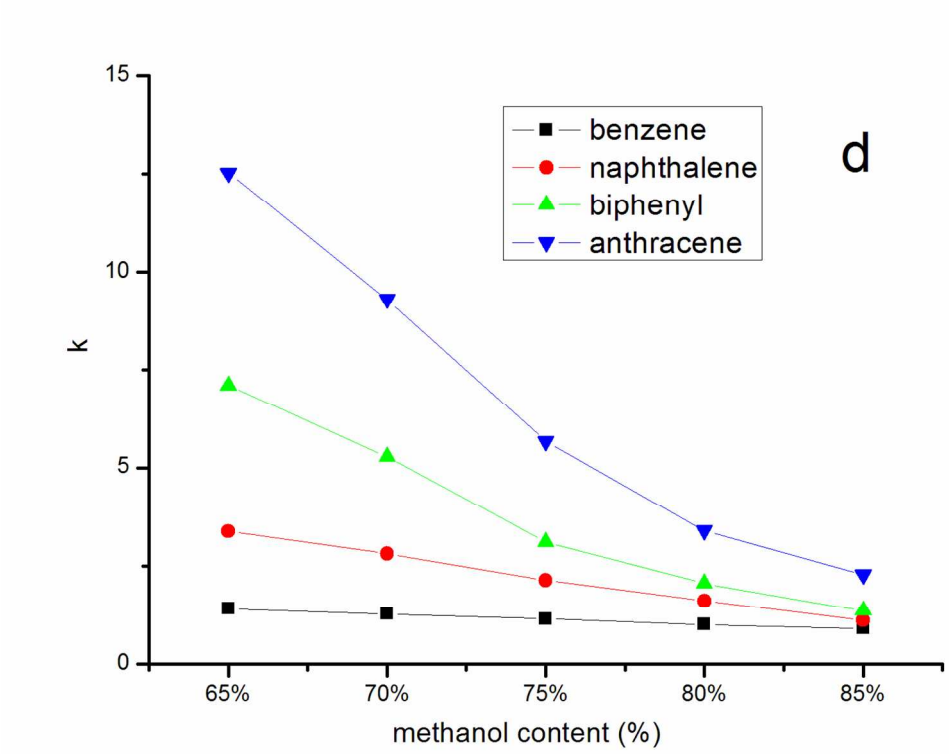

Fig.8(d)
255x207mm (150 x 150 DPI)

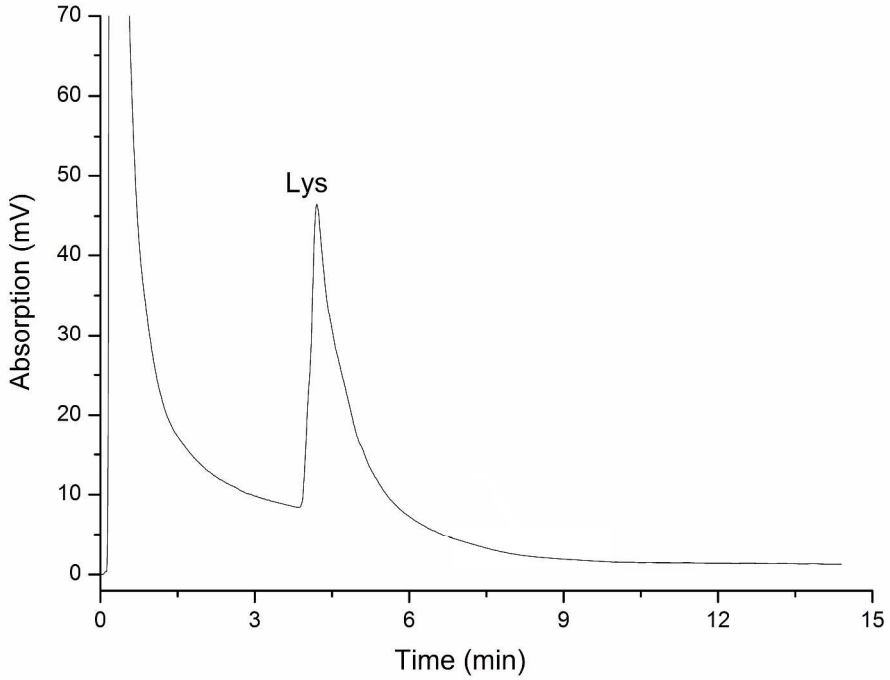

Fig. 9
1639x1299mm (96 x 96 DPI)