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Monolithic *poly* (SPE-*co*-BVPE) capillary columns as a novel hydrophilic interaction liquid chromatography stationary phase for the separation of polar analytes

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ABSTRACT

A novel hydrophilic interaction liquid chromatography (HILIC) stationary phase was prepared by the co-polymerisation of zwitterionic *N,N′*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine (SPE) and the crosslinker 1,2-bis(*p*-vinylphenyl) ethane (BVPE) in the presence of the porogens, toluene and methanol. Monolithic columns were produced by carrying out the α,α'-azoisobutyronitrile (AIBN) initiated reaction for 1, 2, 4, 8 and 12 h inside a 200 μm i.d. fused silica capillary at 75 °C (water bath). The optimum polymerisation time was shown to be 2 h, as this resulted in good porosity, due to enlarged flow-channels and the presence of a higher proportion of mesopores provided a relatively larger surface area than the other columns. The chromatographic properties of the optimised *poly* (SPE-*co*-BVPE) monolithic column were evaluated with test mixtures containing both basic and neutral compounds in the HILIC gradient separation mode. This produced relatively sharp peaks (average peak width at half height = 0.1 min) with average asymmetry factors of 1.4 and baseline resolution was obtained for all the compounds. Using the isocratic separation of the test mixture, the number of theoretical plates (*N*) per metre calculated was between 26,888 and 35,930 by using average values obtained for triplicate injections of the compounds thiourea, toluene and acrylamide.

1. Introduction

Hydrophilic interaction chromatographic (HILIC) has become increasingly popular in separation science due to its complementary selectivity to reversed-phase liquid chromatography (RPLC), and the fact that it also has good compatibility with LC-MS and 2D LC–LC [1]. It makes use of polar stationary phases in combination with apolar aqueous, typically acetonitrile-rich eluents, and allows retention of hydrophilic solutes through interactions with polar functionalities and/or partitioning into an absorbed water layer. The polar zwitterionic monolith provides an environment that is not only capable of hydrophilic interaction with polar and charged analytes but also offers the opportunity for weak electrostatic interaction with analytes carrying either positive or negative charges to occur [2]. Whilst increasing the hydrophilicity of the monolith would enhance HILIC interactions, this type of polymer can be affected by shrinkage in water and swelling in organic solvents and this is related to the lack of or the inhomogeneity of the crosslinking [3]. The resulting instability of the chromatographic support as the eluent composition changes leads to reduced column efficiency and a loss of resolution [4–5]. Therefore, to counteract this problem, a highly crosslinked polymer with good homogeneity is preferred.

A previous study [6–8] described the use of the monomer BVPE, which has a spacer between aromatic rings that allows both vinyl groups to retain the same reactivity for free radical polymerisation, yielding a homogeneous highly crosslinked polymer in a short polymerisation time. The resulting monoliths also had significant proportions of both mesopores and macropores and this allowed for the rapid and high resolution separation of low molecular weight compounds, as well as larger biomolecules, on the same monolithic support. Furthermore, the high fraction of flow-channels provided enhanced column permeability and lower backpressure.

According to Van Deemter et al. [9], column efficiency (height equivalent to a theoretical plate HETP) is directly proportional to the particle diameter of the stationary phase. Therefore, a good monolith should exhibit a good (bimodal) pore-size-distribution of macropores (>75 μm) and mesopores (2–50 nm). By employing an approach

Abbreviations: SPE, *N,N′*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl) ammonium betaine; BVPE, 1,2-bis(*p*-vinylphenyl) ethane; HILIC, hydrophilic interaction liquid chromatography; AIBN, α,α'-azoisobutyronitrile; γ-maps, 3-(trimethoxysilyl)-propyl methacrylate

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introduced by Trojer et al. [10], which uses polymerisation time as a polymerisation parameter for tailoring the porous properties of organic monoliths [11], we used different polymerisation times to evaluate the effect that this would have on the efficiency of the HILIC monolith column prepared by thermal-initiated co-polymerisation of SPE and BVPE inside a 200-μm-i.d. fused silica capillary. The composition of the polymerisation mixture was optimised in order to obtain satisfactory performance for column permeability, efficiency, and separation. The optimised monolithic column was evaluated for the separation of neutral, acid and basic analytes in a HILIC mode. The variations of organic solvent content on separation have been investigated. To date, only a few papers have reported on the fabrication of HILIC monoliths and this is the first to report the formation of such a product by the co-polymerisation of SPE and BVPE.

2. Materials and methods

2.1. Materials

The monomer SPE was a kind gift of Raschig GmBH (Ludwigshafen, Germany). Magnesium ribbon, tetrahydrofuran (THF) inhibitor-free, azobisisobutyronitrile (AIBN), toluene, thiourea, acrylamide, acenapthalene, thymine, adenine, cytosine, uridine, cytidine were all purchased from Aldrich Chemical (Poole, UK). 3-(trimethoxysilyl)-propyl methacrylate (γ-MAPS), ammonium acetate were obtained from BDH Laboratory Supplies (Poole, UK). *p*-vinylbenzyl chloride, uracil and ammonium formate were purchased from Fluka (UK). HPLC-grade methanol and acetonitrile (ACN) were obtained from Fisher Scientific (Leicestershire, UK). The water used throughout all experiments was from Millipore synergy UV (UK). The fused silica capillary with a dimension of 200 μm i.d. (350 μm o.d.) was purchased from Composite Metal Services Ltd. (Hallow, Worcestershire, UK).

2.2. Instrumentation

Experiments were carried out with a laboratory-built HPLC system that comprised an Applied Biosystems 783A programmable absorbance detector (Ramsey), a four-port injection valve with a 100 nl internal loop from Valco (Houston, TA), and a DINA binary pump with software version 1.29 (KYA Technologies Corporation) was used to run both isocratic and gradient conditions. Detection was performed at 214 nm for the standard test mix 1 and 254 nm for test mix 2. A Data Apex chromatographic Clarity data station (Aston Scientific Ltd., Bucks, UK) was used for data acquisition and data handling. Chromatograms were converted to an ASCII file and exported as notepad and redrawn using Microsoft excel.

2.3. Preparation of 1,2-bis(p-vinylphenyl)ethane (BVPE)

BVPE synthesis was accomplished by a Grignard dimerisation of *p*-vinylbenzyl chloride (Fig. 1). A detailed protocol of the preparation of BVPE is described in [6–8]. BVPE was recrystallised from methanol to further increase the purity of the product. The purity of the product was checked and confirmed by GCMS.

2.4. Preparation of poly (SPE-co-BVPE) monoliths

In order to provide anchoring sites for the polymer, each capillary was treated with γ-MAPS, a bifunctional reagent, prior to polymerisation using a method described elsewhere [12]. 50 mg of SPE and 50 mg of BVPE were weighed into a 1.5 ml screw-cap vial, and then 174.2 mg of methanol and 156 mg of toluene were added and mixed in an ultrasonic bath at 40 °C for 15–20 min. When completely dissolved 5 mg of AIBN was then added and completely dissolved. Ultrasonication was also used to remove any dissolved gases. The polymerisation mixture was introduced into a pre-heated silanised fused silica capillary which was immersed in the ultrasonic bath operating at 40 °C. Both the polymerisation mixture and capillary have to be kept warm at all times, as the polymerisation mixture quickly precipitates if it is not warm when filling the mixture, which could result in it precipitating in the capillary. After the capillary was completely filled with the polymerisation mixture, both ends of the capillary were sealed with GC septa, and the capillary was kept in a water bath at 75 °C for 2 h. (reaction as in Fig. 2). The capillary column was then rinsed with methanol to remove the porogenic solvents and any other unreacted soluble compounds. A 2–3 mm detection window was created at a distance of 5 cm from the end of the column using a thermal wire stripper. Monolithic material at this point was pyrolysed and then flushed out with methanol. The resulting capillary column has an effective length of 27.5 cm. A 0.5 cm length of the capillary containing monolith inside was cut for scanning electron microscopy (SEM) analysis (JEOL JSM-5600 LV, Tokyo, Japan).

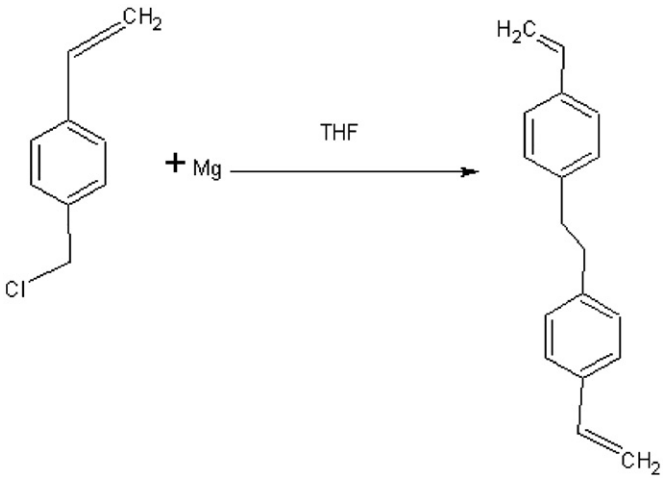

Fig. 1. Synthesis of BVPE by Grignard reaction of *p*-vinylbenzyl chloride and magnesium.

3. Results and discussion

3.1. Effect of polymerisation time

According to Greiderer et al. [7], a shorter polymerisation times for BVPE crosslinked co-polymers leads to more space within the polymer clusters (lower polymer density) with the flow channels that are larger and, consequently, the total porosity is enhanced. For this reason, a study was carried out at different polymerisation times (1, 2, 4, 8 and 12 h) in order to determine if there is an optimal time for producing our monolithic support. The results we obtained have supported Greiderer's observation and, for example, the SEM photographs show that the polymer clusters that are formed become bigger as the polymerisation time increases. At 1, 2 and 4 h polymerisation time, it can be seen (Fig. 3a–c respectively) that the polymer cluster consists of smaller polymer microglobules compared to that from the 8 to 12 h polymerisation times (Fig. 3d and e). Since mesoporosity can be regarded as a function of the microglobule size, the smaller

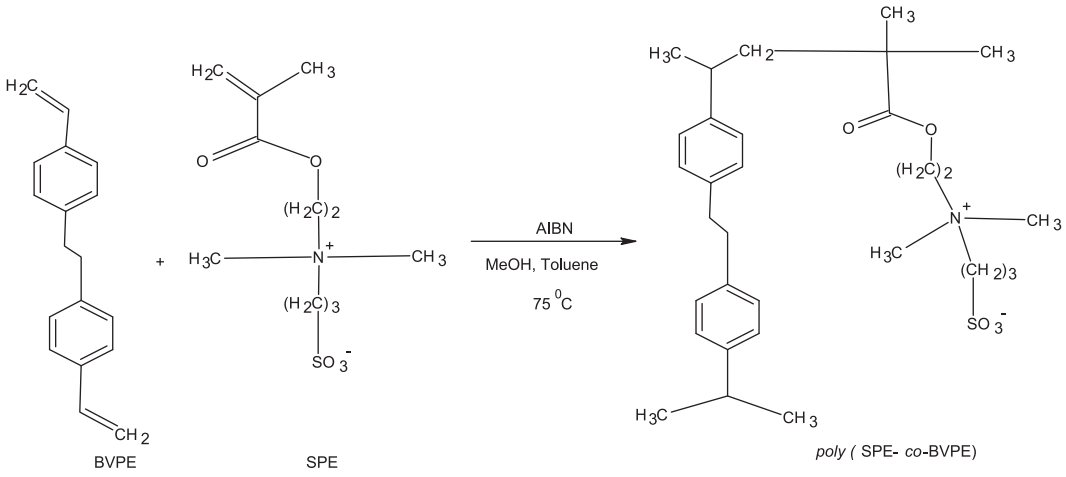

Fig. 2. Preparation of *poly* (SPE-*co*-BVPE) monoliths.

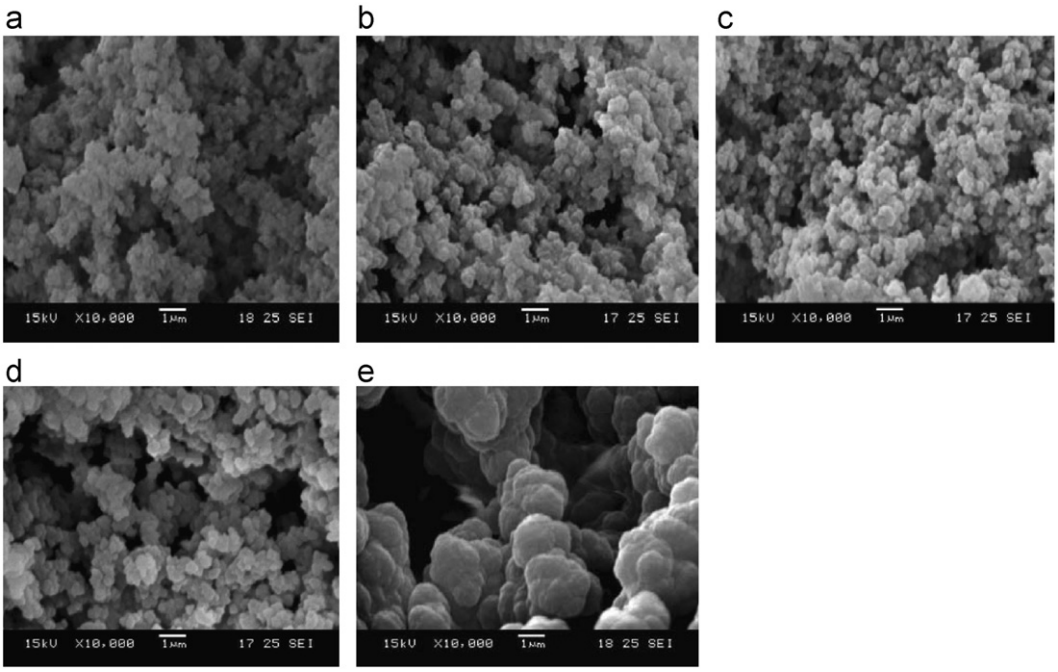

Fig. 3. SEM images of monolithic columns prepared at different polymerisation times (a-1 h, b-2 h, c-4 h, d-8 h and e-12 h).

they are the higher the fraction of mesopores is expected. We can observe from the SEM images that the fraction of mesopores and thus specific surface area is optimised when the polymerisation time is reduced. The five capillary columns obtained from the different polymerisation times were installed on the HPLC system and the pressure of each column was recorded (Table 1) with the mobile phase (95% ACN: 5 mM ammonium formate pH 3) at a flow rate of 5000 nl/min and it is evident from this data that the backpressure due to the capillary column increases with increasing polymerisation time. The column efficiencies of the five capillary columns at different polymerisation times were measured using test mix 1 consisting of toluene, acrylamide and thiourea and a base mix 2 containing uracil, adenine and cytosine (Table 1). We can see that a 2 h polymerisation time has the best column efficiencies. Triplicate injections were carried out using test mix 1 (toluene, acrylamide and thiourea) followed test mix 2 containing the basic compounds uracil, adenine and cytosine. The columns produced with polymerisation times above 4 h generated poor peak shapes, decreased resolution and lower peak intensity. For example, toluene and acrylamide in the first test mix were merged into a single peak and the base test mix showed poor peak shape for the 3 bases with a low intensity and a resolution of <1.5.

The best results were obtained with the column produced with a 2 h polymerisation step. For example, the resolution between uracil and adenine and acrylamide and toluene is twice that for the column produced with the 1 h polymerisation time (see Figs. 4 and 5) and the efficiency, measured by theoretical plates per metre, was also found to be between 3.1 and 7.3 greater. The theoretical plates per metre obtained on testing under isocratic elution conditions (95% ACN+5% 5 mM ammonium formate pH 3) was 35,930, 26,888 and 28,007 for thiourea, toluene and acrylamide respectively. Whilst a base mixture containing uracil (18,034), adenine (11,935) and cytosine (14,496) yielded slightly lower results. Therefore, the 2 h polymerisation capillary was selected as the optimal polymerisation time.

Table 1
Comparison of the column efficiencies and back pressures for the different polymerisation time of the columns.

Column	Sample	Retention time	Efficiency (theoretical plates)	Backpressure (psi)
1 h	Acrylamide	1.250	1610	580.156
	Toluene	1.340	1065	
	Thiourea	2.733	1231	
	Uracil	1.820	1206	
	Adenine	2.160	931	
	Cytosine	2.787	976	
2 h	Acrylamide	1.740	7702	1885.506
	Toluene	1.827	7394	
	Thiourea	3.943	9889	
	Uracil	2.540	4759	
	Adenine	3.037	3184	
	Cytosine	3.843	3983	
4 h	Acrylamide	1.727	929	2074.057
	Toluene			
	Thiourea	3.837	734	
	Uracil	2.407	889	
	Adenine	2.80	630	
	Cytosine	3.617	627	
8 h	Acrylamide	1.900	500	2784.748
	Toluene			
	Thiourea	4.053	989	
	Uracil	2.633	1186	
	Adenine	3.093	784	
	Cytosine	3.930	1940	
12 h	Acrylamide	1.533	1241	3074.826
	Toluene			
	Thiourea	3.543	1280	
	Uracil	2.220	875	
	Adenine	2.703	421	
	Cytosine	3.507	589	

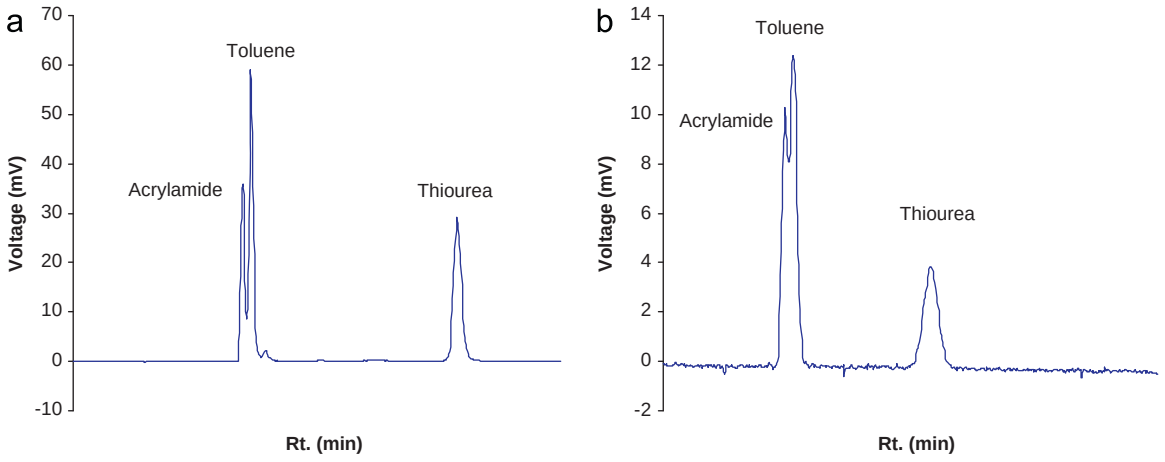

Fig. 4. Separation of the standard test mix for (a) 2 h polymerisation, (b) 1 h polymerisation.

3.2. Separation of bases and neutral compounds

The polar zwitterionic *poly* (SPE-*co*-BVPE) surface provides a hydrophilic environment. A mixture of bases, neutral and acidic compounds were run using a mobile phase gradient starting from 95% ACN to 50% ACN, with 5% buffer in both solvents. Pyrimidines and purines are often used for the evaluation of HILIC columns and a mixture of thymine, uracil, adenine, cytosine, uridine and cytidine was used for the evaluation of *poly* (SPE-*co*-BVPE) monolithic columns in this work. Fig. 6 shows good separation within 10 min for the 6 components of the mixture of pyrimidines and purines (acenapthalene as t_0 marker) producing sharp and symmetrical peaks.

4. Conclusions

A highly hydrophilic porous monolith, *poly* (SPE-*co*-BVPE) formed within capillary columns of 200 μm ID was successfully applied for the rapid and high resolution separation of low molecular weight compounds, such as (pyrimidines and purines). Variation of the polymerisation time has a considerable impact on the porous

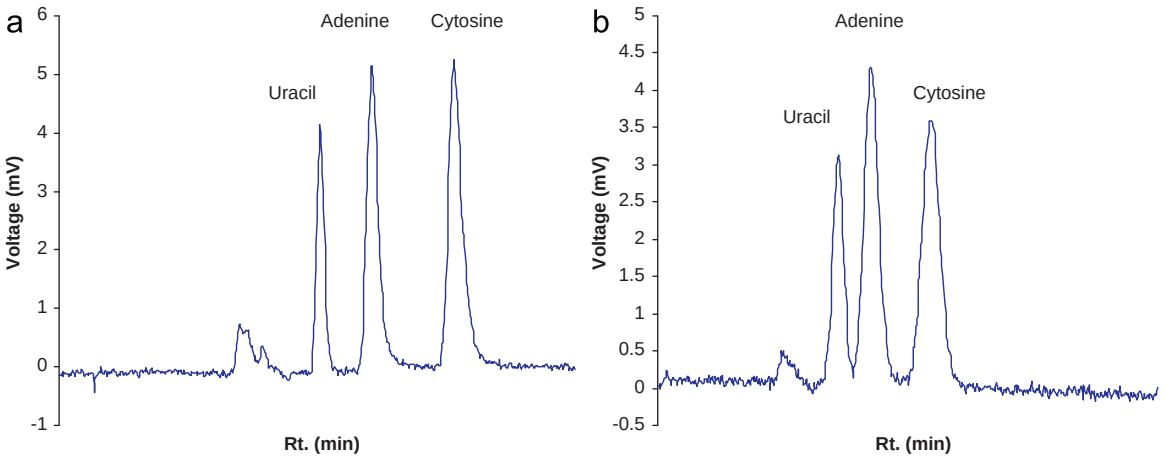

Fig. 5. Separation of base test mix for (a) 2 h polymerisation, (b) 1 h polymerisation.

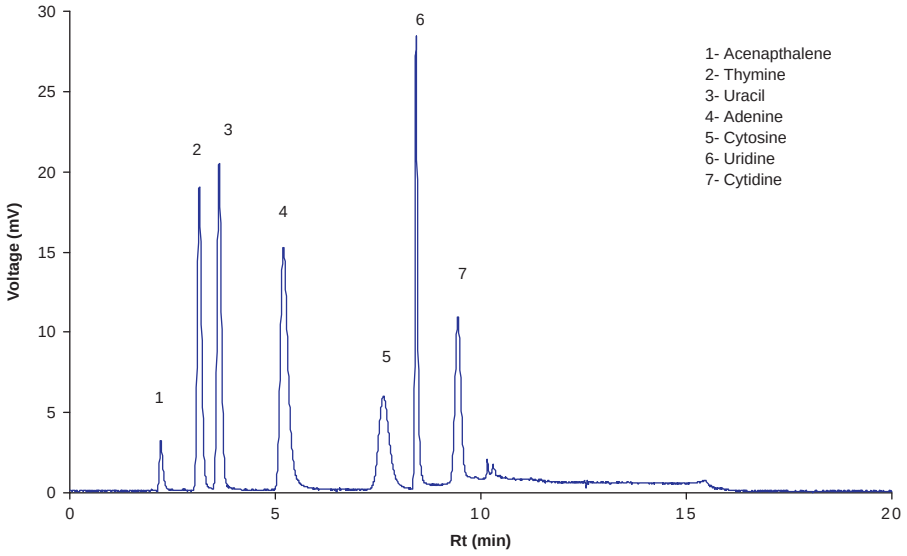

Fig. 6. Separation of pyrimidines and purines.

properties of organic monoliths prepared by thermally initiated free radical polymerisation. The reduction of polymerisation time resulted in HPLC stationary phases that are characterised by the presence of a sufficiently high amount of mesopores to efficiently separate low-molecular weight compounds. At the same time, the total porosity is enhanced due to decreased monomer to polymer conversion. This, in turn leads to the enlargement of the flow channel size and thus to increase column permeability. Their exceptional chromatographic performance towards a range of representative low-molecular weight compounds, confirms the incorporation of accessible mesopores onto the polymeric network surface. Further investigations are underway in order to extend the applicability of HILIC monoliths to include their use during the analysis of peptides generated by enzymatic digestion of proteins.

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