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Homogeneous reversed-phase agarose thermogels for electrochromatography

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Abstract

A method for the derivatization of agarose by covalent attachment of hydrophobic ligands for reversed-phase (RP) chromatographic separation and ionic groups for generation of electroosmosis under electrochromatographic conditions in the capillaries or microfluidic channels filled with the thermogel of this agarose derivative is described. The product renders a capability of reversible thermogelation. The thermogels formed provide sufficient hydrophobicity and electroosmosis for the separations of the analytes under RP mobile-phase conditions and electric field applied. The gels may be used repeatedly without loss of resolution. They are thermally replaceable and UV transparent (providing possibility in column/in-gel detection), require no covalent attachment to the capillary inner wall (or microchip channel), and are suitable for isocratic or gradient operation in the aqueous–organic mobile phases.

Keywords: Agarose thermogels; Thermogels; Stationary phase, electrochromatography; Electrochromatography; Electroosmosis; Hydroxybenzoic acids; Benzoic acids

1. Introduction

According to theoretical implications, the particulate or non-particulate (continuous bed, monolithic) packings of uniform morphology will never be perfect and will cause the chromatographic band broadening, for instance due to eddy diffusion. Resistance to mass transfer in the particulate or non-particulate packings is dependent on the size of the flow channels. Although being of limited skeleton porosity [1], the non-particulate beds may result some chromatographic zone broadening due to the diffusion into the micropores/mesopores in the skeletons, which may cause resistance to the mass transfer, separating small molecules. All these drawbacks may be overcome using macroscopically homogeneous media such as non-dispersed gels for chromatographic separations without the flow channels. Since the gel integrates the liquid and the solid polymer forming a uniform structure, it can be regarded as homogeneous phase at a molecular level.

The majority of the chromatographic separations are performed using the reversed-phase (RP) systems [2], also in the case of electrodriven separations of small molecules [3]. It is difficult to synthesize lipophilic homogeneous gels compatible with common aqueous RP mobile phases as constituent of the homogeneous gel. Hydrophilic gels as anti-turbulent or sieving media are commonly applied in electrophoresis. Due to mechanical weakness and small pores, the liquid cannot be pressed through the gel at velocities sufficient for chromatographic separations. Electroosmosis is an undesirable feature in the gel electrophoresis; however, it has an advantage of being applied [4–6] for the propulsion of the mobile phase in the charged gels of sufficient porosity (pore size) in capillary (or microchannel) electrochromatography.

There are two possibilities to form a gel inside the microfluidic channel or capillary. The first possibility is to use monomeric precursors of the gel and polymerize them in situ or the polymer and cross-linker to form a cross-linked gel in situ. The second possibility is to prepare a solution of the thermoreversible gel, draw or press it into capillary and let it cool down.

The first technique was described by Fujimoto et al. [4,7–9]. It is similar to the continuous bed technique using free radical polymerization of the water-soluble acrylic co-monomers. Much lower concentrations of the monomers and cross-linker were used to form a gel in the fused silica capillary. *N*-Isopropylacrylamide,

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2-acrylamido-2-methylpropanesulfonic acid as electroosmosis generating agent and *N*,*N*′-methylenebisacrylamide as cross-linker were used to form the gels. Total monomer concentration (%T) in the gels ranged from 6.9 to 10%, cross-linking concentration with respect to monomers (%C) was from 5.8 to 10%. Digitalized images of zone front of electroosmotic flow (EOF) performing the frontal analysis of rhodamine B were recorded [4]. Almost flat EOF front was documented in the 75 μm i.d. capillary at the field strength 285 V/cm. Isocratic separations of several ketones, steroid mixture and polyaromatic hydrocarbons were performed. The plate numbers for the homogeneous gel beds were reported from 107 000 to 160 000 plates per metre. The decrease in the retention factors and an increase in the fraction of the organic modifier in the mobile phase indicated, that gels have had typical RP stationary-phase properties. In all the experiments with the homogeneous gels, the "in-capillary" (in-gel) detection was performed due to a low concentration of the acrylic copolymer; therefore, low UV absorbance of the gel. Similar acrylic gels were formed by Palm and Novotny [5] and used for capillary electrochromatography (CEC). High efficiencies were reported for a mixture of alkylphenones—up to almost 400 000 plates per metre. "On-capillary" (off-gel) UV and fluorescence detection was used.

The poly(vinyl alcohol) gel was formed in situ by Hjertén et al. [6] via drawing a solution of 4% polyvinyl alcohol into a capillary pre-coated with linear polyacrylamide and then electrophoretically driving through the borate ions, which cross-link the polymer by complexation and form a gel. Separation of parabenes mixture was performed using the resultant gel. Enantiomer-selective gel was synthesized using human serum albumin derivatized with acrylate groups via protein reaction with glycidylacrylate [6]. The derivatized protein solution was mixed with ammonium peroxodisulfate and tetramethylethylenediamine and drawn into the linear acrylamide polymer-pre-coated fused silica capillary for in situ polymerization. Due to high UV absorbance, the protein solution was filled up to the detection window and "on-capillary" detection in the segment without the gel was performed. Chiral separation of D,L-kynurenine in the protein-based homogeneous gel was demonstrated using the prepared capillary column. Hjertén et al. [10,11] have developed a method where negatively and positively charged polyacrylamide gels copolymerized with 2-hydroxy-3-allyloxy-propyl-β-cyclodextrin were prepared for enantiomer separations using CEC.

Hjertén et al. [6] have proposed several approaches to obtain thermoreversible gels for CEC. They prepared charged or charged-polymer-entrapped homogeneous gels as electroosmosis-generating stationary phases for capillary electrochromatography. Versatile method was developed providing a possibility to use the replaceable low-melting point agarose in automated runs with the entrapped acrylic polymer, bearing phenyl boronate and acrylic acid groups. Boronate ligands had dual property to generate electroosmosis parallelly to acrylic acid groups and to interact by complexation with the compounds having OH groups in the *cis*-configuration (for instance ribonucleosides). The ribonucleosides were separated using 0.3% agarose gel entrapped with the boronate groups containing acrylic polymer. The sample compounds were neutral at this pH; therefore, they were driven by electroosmosis in the homogeneous gel.

The aim of the present study is to synthesize a lipophilic polymer capable to form homogeneous thermogels compatible with aqueous–organic RP mobile phases and providing sufficient zeta potentials for electroosmotic propulsion of the mobile phases to make these gels useful for highly efficient RP capillary electrochromatography.

2. Materials and methods

2.1. Materials

D-5 LE (low electroosmosis) agarose was from Hispanagar (Spain), 3-chloro-2-hydroxypropyldimethyldodecylammonium chloride (Quab342) (40% aqueous solution) and 3-chloro-2-hydroxypropyldimethylstearylammonium chloride (Quab426) (40% solution) were kindly supplied by Degussa Hüls (Germany). Methanol (MeOH) of HPLC grade, glacial acetic acid (HOAc), triethylamine (TEA), Tris, boric acid, methyl-, ethyl-, propyl-, butyl-, pentyl- and hexyl-4-hydroxybenzoic acid esters were purchased from E. Merck (Darmstadt, Germany). Fused silica capillary (50 μm i.d. × 365 μm o.d.) was obtained from Polymicro Technologies (Phoenix, USA). 1,2-Epoxyoctadecane was from Aldrich-Chemie (Steinheim, Germany).

2.2. Instrumentation

Chromatographic experiments were performed using modular capillary electrochromatography system. Linear 206 PHD (Reno, NV, USA) modified by installing ball lenses for on-capillary detection and high-voltage source DA-30 (SpectroVision, USA), generating up to 30 kV were used. Diffusion–extraction-based injection of the analytes was used dipping the capillary into the sample vial for 10 s.

2.3. Synthesis of lipophilic agarose

Agarose was suspended in NaOH aqueous solution and cationizing reagent was added dropwise to the stirred suspension as described in Table 1. The reaction mixture was stirred for 20 h at 20 °C. Alternatively, the reaction was accomplished in 2 h stirring the suspension at 50 °C. In the latter case, two portions of 100 mg of sodium borohydride were added, one at the beginning and the second 15 min prior to finishing the reaction. Derivatized agarose was neutralized with acetic acid solution and washed on the 0.22 μm cellulose acetate membrane filter with distilled water. The products did not differ with respect of their electroosmotic

Table 1
Derivatization of agarose

Column	Agarose (g)	NaOH concentration (%)	NaOH (ml)	Reagent portions (ml)	Reagent added
AG1	0.2	1.5	10	1 + 1	Quab342 added at 30-min interval
AG2	0.2	2.0	10	1 + 1	Quab342 added at 30-min interval
AG3	0.2	1.5	10	0.5 + 0.5 + 0.5 + 0.5	Quab342 added at 3-h interval
AG4	0.2	1.63	11	2	0.4 ml water, 1.4 ml Quab342, 0.2 g 1,2-epoxyoctadecane
AG5	0.3	1.33	15	1.2	Quab342
AG6	0.2	1.33	15	0.8	Quab426

or hydrophobicity properties. In this work, we used 20 h at 20 °C derivatization procedure. In order to vary electroosmotic and hydrophobic properties of the resultant gel, different ratios of reagent to agarose were selected or agarose derivative was premixed with underivatized agarose (in order to reduce hydrophobicity of the gel).

3. Results and discussion

Recently, we have synthesized alkyl-derivatized hydrophobic agarose-based homogeneous gels, which can be used for capillary electrochromatography in common RP conditions [1,12]. In contrast to particulate silica gels or cellulose [13], no light scattering was observed owing to the homogeneous consistency of RP agarose gel. "In-capillary" (in-gel) UV detection; therefore, is easy to perform with these gels. Agarose derivative containing ionic groups and hydrophobic moieties was used to prepare the gels for RP capillary electrochromatography. Principally, any conventional agarose used for the gel electrophoresis can be used for the derivatization. It is difficult to derivatize gel-forming polymers (namely agarose) in order to generate desired chromatographic properties, simultaneously rendering thermoreversible properties and the pore size high enough to permit the electroosmotic flow in the resultant gel. Derivatization of the agarose may prevent hydrogen bond formation and gelation of the polymer. The pore size in the agarose gel is a function of the concentration for instance, 2% common agarose gel has pore size of ca. 150 nm [14]. It also depends on the M_r (relative molecular mass) of the agarose polymer and kind and density of the derivatization [15].

The amphiphilic compounds soluble in the aqueous medium that afford cationic functionalities were used to derivatize agarose. In addition to 3-chloro-2-hydroxypropyldimethyl-dodecylammonium chloride or 3-chloro-2-hydroxypropyldimethylstearylammonium chloride, similar reagents containing ionic, hydrophobic, and reactive groups (e.g. epoxy or chlorohydrin) such as 3-chloro-2-hydroxypropyltrimethylammonium chloride and 2,3-epoxypropyltrimethyl-ammonium chloride were found useful for generation of both EOF and lipophilicity in the thermoreversible agarose gels (results not shown).

Solution of the derivatized agarose was prepared in common manner by heating the suspension to 60–80 °C. The hot solution was than drawn by vacuum or pushed into the capillary using pressure up to 0.5–1.0 MPa. Pressure mode is preferable when gels with higher concentration should fill narrow and/or long capillaries. This procedure is compatible with the operation conditions of the commercially available automated CE/CEC apparatus equipped with thermostated capillary cassette, sample tray, and external pressure source [1]. To avoid long conditioning period, the solution of the RP agarose was prepared in the aqueous/aqueous–organic buffer typical of the CEC experiment. The capillary can be used after a brief conditioning (ca. 20 min) with the mobile phase, gradually increasing the electric field strength up to 200–300 V/cm. An electrical field strength of up to 500 V/cm was sometimes applied during the chromatographic experiments. Stepwise gradients with the 20–30% increments in the organic modifier were electroosmotically pumped through the gels to achieve conditioning of capillary columns or elution of the analytes. The RP agarose gels tolerate mobile phases consisting of up to 100% methanol or acetonitrile with no shrinkage or rejection of the gel out of the fused silica capillary observed even after applying the mobile-phase gradients. Repeated chromatographic analyses could be accomplished with the gels. Some capillary columns were successfully used for over 70 consecutive chromatographic experiments. Thorough de-gassing of the mobile phases was of crucial importance. No gel aging effects were observed during storage of the capillary columns in the refrigerator for a month with the ends dipped in the mobile phase.

The hydrophobicity of the beds was modulated by the degree of derivatization of agarose with hydrophobic ligands or, alternatively, by mixing the derivatized with non-derivatized agarose. The electroosmotic mobility and hydrophobicity of the gel gradually decreased with decreasing fraction of the modified agarose (Fig. 1). When the fraction of unmodified agarose reached 0.95, the electroosmotic mobility was still observed. However, it was about four times smaller, since the low percentage of modified agarose in the gel significantly decreased the concentration of ionizable groups. Since the density of alkyl chains in the gel also decreased, this led to the decrease in hydrophobicity (Fig. 2). Electroosmotic flow velocities in the gels were comparable with that of common particulate RP silica gels used for CEC [16]. The 0.5–3.5% gels were employed in this study. These were entirely UV transparent for the wavelength range from 220 to 280 nm with no need for the frits

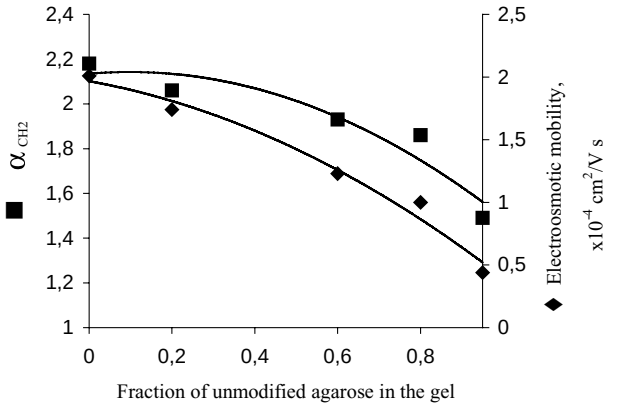

Fig. 1. Effect of the ratio of modified to unmodified agarose in the gel on electroosmotic mobility and hydrophobicity. Agarose gel AG5. Total gel concentration is 1.8%; mobile phase: 30% MeOH in 0.35% TEAA; pH: 3.9 and voltage: −3 kV.

or covalent binding of the gels to the wall of fused silica capillary.

Electrochromatographic separations of parabene homologues using capillary columns, filled with different agarose gels, AG1 1.2%, AG2 1.2%, AG3 1.5% and AG4 1.5%, are depicted in Fig. 3. Agarose derivatization conditions are presented in Table 1. Agarose derivatives AG1 and AG2 were synthesized at different sodium hydroxide concentrations, which catalyzed the reaction. Agarose gel AG3 synthesis was similar to AG1; however, the cationizing reagent was added at smaller portions and longer intervals. The functionalizing reagent was added in several portions in order to insure the uniformity of derivatization in the heterogeneous reaction medium and prevent self polymerization of epoxy or chlorohydrine compounds. Agarose derivative AG4 was obtained using mixed reagent Quab342 and 1,2-epoxyoctadecane. Since Quab342 is a detergent, it solubilized the highly non-polar 1,2-epoxyoctadecane in the aqueous derivatization medium. As shown in Fig. 3a and b, the increase of the sodium hydroxide concentration in the derivatization reaction mixture increased the hydrophobicity of the agarose derivative. The capillary column filled

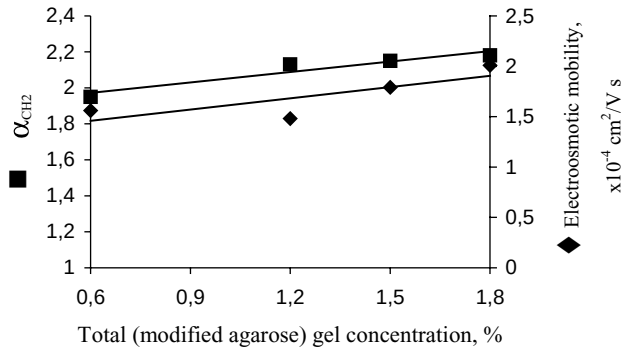

Fig. 2. Effect of concentration of modified agarose gel on electroosmotic mobility and hydrophobicity. For conditions, see Fig. 1.

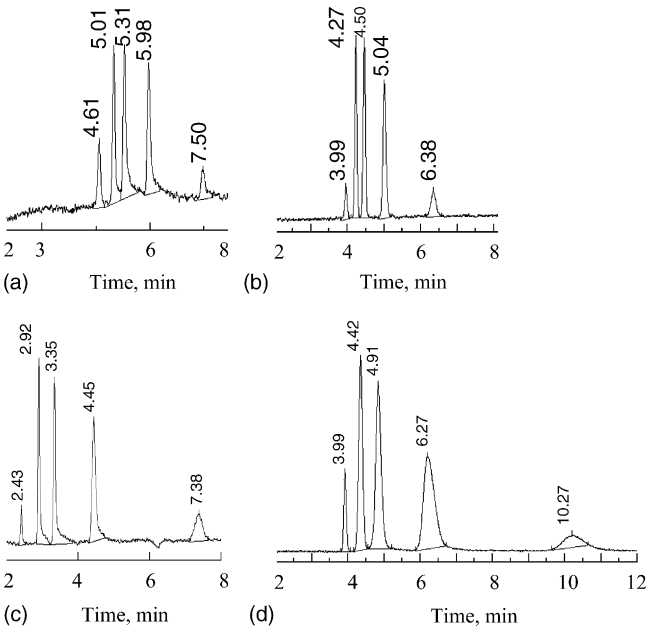

Fig. 3. Separation of unretained marker (acetone) and parabene mixture. Stationary phase: (a) agarose gel AG1 1.2% (b) AG2 1.2% (c) AG3 1.5% (d) AG4 1.5%. Mobile phase: 30% MeOH in 0.35% TEAA, pH: 3.9 and voltage: −3 kV.

with AG3 (Fig. 3c) demonstrated higher electroosmosis and hydrophobicity (observed that gel concentration was slightly higher: 1.5% instead of 1.2% used for AG1-filled capillary column, so it could result as a combined effect of the agarose derivatization and the gel concentration). The capillary filled with agarose AG4 derivatized with alkyl and alkylammonium ligands was the most hydrophobic. Chromatographic characteristics calculated for these capillaries are listed in Table 2. It confirms the observations discussed above. The hydrophobicities of the gels expressed as the methylene group selectivity were calculated for the parabene homologues while the plate numbers were determined for unretained marker. The highly hydrophobic agarose gel AG4 ($\alpha_{CH_2} = 2.5$) containing mixed ligands was less efficient (N = 130 000 plates per metre). A possible explanation is that hydrophobic ligands prevent formation of hydrogen bonds between the agarose macromolecules and decrease uniformity of the formed gel, which is reflected in the column efficiency.

Table 2
Characteristics of different RP agarose gel capillary columns

RP agarose gel	α_{CH_2} [a]	Efficiency (plates per metre)	Column length (cm)[b]
AG1 1.2%	1.94	300 000	10.3 (15.1)
AG2 1.2%	2.05	300 000	9.5 (12.9)
AG3 1.5%	2.20	270 000	10.6 (15.1)
AG4 1.5%	2.50	130 000	10.6 (15.1)

[a] Methylene group selectivity.
[b] Effective length and total length (in parenthesis) of capillary column.

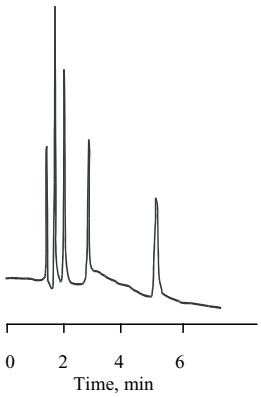

Fig. 4. Separation of parabenes using RP CEC. Agarose gel: AG5 1.8%. Mobile phase: 30% MeOH, pH: 3.9, voltage: −3 kV, current: 6 μA. Effective column length is 6.9 cm. Peaks: acetone, methyl-, ethyl-, propyl- and butyl *p*-hydroxybenzoic acid ester (in elution order).

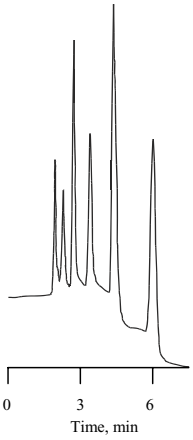

Fig. 5. Step-gradient elution of parabenes using RP CEC. Agarose gel column: AG6 1.8%. Mobile phase: 50% MeOH in 20 mM Tris–borate buffer pH 7.1 for 150 s then changed in single step to 65% MeOH in 20 mM Tris–borate buffer pH 7.1, voltage: −5 kV. Peaks: methyl-, ethyl-, propyl-, butyl-, pentyl- and hexyl *p*-hydroxybenzoic acid ester (in elution order).

Electrochromatogram obtained using a short capillary filled with RP agarose gel is shown in Fig. 4. The separation of standard mixture of parabenes is accomplished in almost 6 min in the capillary column AG5 1.8% with an effective length of 6.9 cm at a field strength of 325 V/cm. This application also illustrates that derivatization using single-step addition of cationizing reagent (see Table 1) is sufficient to form a gel with both high efficiency and sufficient hydrophobicity.

Fig. 5 exemplifies a step gradient elution of the standard mixture of parabenes using the RP agarose capillary column. It also illustrates the applicability of different cationizing reagents, since 3-chloro-2-hydroxypropyldimethylstearylammoniumchloride (Quab426) was used in this case. The efficiency for the unretained neutral EOF marker (uracil) using the same capillary column under isocratic conditions (70% methanol in 20 mM Tris–borate buffer, pH 7.1) was 280 000 plates per metre.

4. Conclusions

We have demonstrated here the possibility to derivatize agarose with alkyl ligands containing ionic group. The agarose derivatives are capable of thermoreversible gelation in aqueous or aqueous–organic solvents. The method is attractive, since it is compatible with the most commercially available CE/CEC machines as well as microfluidic devices. The gels provide a possibility of operation in common RP electrochromatographic conditions and feature high UV transparency and possibility of "in-capillary" (in-gel) detection (no light scattering effect was observed) and high separation efficiencies (up to 300 000 plates per metre).

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References

[1] A. Maruška, in: F. Švec, T.B. Tennikova, Z. Deyl (Eds.), Monolithic Materials: Preparation, Properties, and Applications, Elsevier, Amsterdam, 2003, pp.143–172.
[2] R.E. Majors, LC–GC 4 (1991) 686.
[3] P. Myers, in: K.D. Bartle, P. Myers (Eds.), Supports and Stationary Phases for Capillary Electrochromatography (RSC Chromatography Monographs), Royal Society of Chemistry Cambridge, 2001, pp. 33–42.
[4] C. Fujimoto, Y. Fujise, E. Matsuzawa, Anal. Chem. 68 (1996) 2753.
[5] A. Palm, M.V. Novotny, Anal. Chem. 69 (1997) 4499.
[6] S. Hjertén, Á. Végvári, T. Srichaiyo, H.-X. Zhang, C. Ericson, D. Eaker, J. Capillary Electrophor. 1–2 (1998) 13.
[7] C. Fujimoto, Anal. Chem. 67 (1995) 2050.
[8] C. Fujimoto, J. Kino, H. Sawada, J. Chromatogr. A 716 (1995) 107.
[9] C. Fujimoto, Analusis 26 (1998) M49.
[10] A. Vegvari, A. Foldesi, O. Kochegarova, M.G. Schmid, V. Kudirkaite, S. Hjertén, Electrophoresis 21 (2000) 3116.
[11] Á. Végvári, S. Hjertén, J. Chromatogr. A 960 (2002) 221.
[12] O. Kornyšova, A. Maruška, in: V. Janickis, J. Šukytė (Eds.), in: Proceedings of the Conference on Inorganic Chemistry and Technology, Technologija, Kaunas, 2000, p. 55.
[13] A. Maruška, U. Pyell, J. Chromatogr. A 782 (1997) 167.
[14] P. Serwer, S.J. Hayes, Anal. Biochem. 158 (1986) 72.
[15] M. Dahmani, M. Ramzi, C. Rochas, J.-M. Guenet, Int. J. Biol. Macromol. 31 (2003) 147.
[16] I.S. Krull, R.L. Stevenson, K. Mistry, M.E. Swartz (Eds.), Capillary Column Technology, HNB Publication, New York, 2000, pp. 34–59.