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Nanochitosan crosslinked with polyacrylamide as the chiral stationary phase for open-tubular capillary electrochromatography

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Abbreviations: ACN, acetonitrile; **AIBN,** 2,2'-azobis(2-methylpropionitrile); **BHT**, butylhydroxytoluene; **Bis**, N,N'-methylenebisacrylamide; **CEC,** capillary electrochromatography; **CS,** chitosan; **CSP**, chiral stationary phase; **DMSO**, dimethylsulfoxide; **DPPH**, 2,2-diphenyl-1-picryl-hydrazyl; **EtOH**, ethanol; **GMA**, glycidyl methacrylate; k'', retention factor; k_e'', velocity factor; **MAA**, methacrylamide; **γ-MAPS**, 3-(trimethoxysilyl) propylmethacrylate; **OT**, open tubular; **PVA**, polyvinyl alcohol; **Tris**, tris(hydroxymethyl)aminomethane

Abstract

Nanoparticles exhibiting favorable surface-to-volume ratios create efficient stationary phases for electrochromatography. New nanomaterials derived from chitosan (CS) were immobilized onto modified capillaries for use as the chiral stationary phase (CSP) in open-tubular electrochromatography. This immobilization was achieved through the copolymerization of glycidyl methacrylate-modified nano-CS with methacrylamide (MAA) and bis-acrylamide crosslinkers (forming the MAA-CS capillary) rather than the attachment of nano-CS to the copolymer of GMA, MAA, and bis-acrylamide (forming the MAA+CS capillary). The completed MAA-CS capillary and its precursors were examined by SEM and ATR-IR measurements. Before separating chiral samples, the MAA-CS capillary was characterized by electroosmotic flow measurements at varying pH values, concentrations, and volume percentages of organic modifiers in the running buffers. Tryptophan enantiomers were well separated by the MAA-CS capillary, whereas no enantioselectivity was observed in the MAA+CS capillary. With the addition of 80% MeOH into the phosphate buffer, the chiral separation of (±)-catechin was accomplished in a normal-phase mode. However, the new CSP has its limitations, as only two groups of α-tocopherol stereoisomers were separated.

Keywords: Capillary electrochromatography / Chiral stationary phase / Chitosan / Nanoparticles / Open-tubular / Polyacrylamide /

1 Introduction

Growth in chiral separation techniques is based on the continued development of new chiral stationary phases (CSPs) and better mechanistic understandings of them. Among these techniques, capillary electrochromatography (CEC) providing various column technologies is well-suited to the discovery of new phases with proper formats, and its many successful implementations for enantiomer separations are often reviewed [1–4]. Traditionally, CSPs or chiral selectors used in HPLC columns have been used in CEC, which has resulted in the production of three major categories of column technologies: packed, open-tubular (OT), and monolithic columns.

Due to the lack of phase ratios, the OT column format attracts less attention compared to the other two column technologies. However, it is a comparatively straightforward approach that does not require the arduous fabrication of any frits that is required in particulate-packed column creation or the blending of monomer reagents with suitable porogens in precise proportions that is required for monolith. Strategies to increase the loadability of the designed chiral selectors have been developed and include the techniques of polyelectrolyte multilayer coating [5–7], layer-by-layer assembly [8,9], high-affinity incorporation into biolayers [10,11], and immobilization on silica gel or organic polymer gel as achiral supported layers (brush-type CSPs) [12–15]. The OT-CEC capillaries modified with chemically bonded chiral selectors have longer lifetimes and better reproducibilities than capillaries with physical coatings that were created by any of the first three strategies. In addition to brush-type CSPs, chiral selectors are chemically bonded to the polymer-type CSPs. Examples of OT-CEC capillaries with polymer-type CSPs are a molecular imprinted polymer [16–18] and a bonding of avidin protein [19,20].

Nanoparticles exhibiting favorable surface-to-volume ratios create efficient stationary phases for electrochromatography [21]. Some nanomaterials (including silica [22], titanium oxide [23,24], gold [25], and carbon nanotubes (CNT) [26,27]) have previously been

chemically immobilized in OT-CEC capillaries. In a similar manner, chiral nanoparticles are potential CSPs for use in enantioseparations. Chitosan (CS) particulates and their derivatives have been employed in a wide range of biomedical applications including drug-delivery and gene-delivery systems [28,29], and their natural properties meet the requirements of polymer-type CSP. Chitosan, poly-β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose is a functional linear polysaccharide and the *N*-deacetylated derivative of chitin, the second most abundant biopolymer in nature. Because of its specific basic properties, CS and its derivatives have mainly been used as coating reagents that were adsorbed on bare capillaries to separate bioactive molecules by OT-CEC [30,31]. For enantiomeric separations by CEC using CS-immobilized CSPs, a monolithic phase composed of sol–gel/organic hybrid materials containing CS and bovine serum albumin (BSA) has been the only successful example studied to date [32]. Here, BSA and CS both are possessed of chiral selectivity. As regards HPLC, CS-immobilized CSPs have been successfully applied in some studies [33-35].

In this study, CS nanoparticles prepared by a precipitation method were copolymerized with methacrylamide (MAA) monomers and N,N'-methylenebisacrylamide crosslinkers (Bis) to create an OT-CEC capillary with CSP functionality. The complete MAA-CS capillary and its precursor materials were characterized by SEM and ATR-IR. Further, the effects on its electroosmotic mobilities (μ_{eo}) caused by changes in pH values, buffer concentrations, and the volumetric addition of organic modifiers to the running buffers were investigated. Chiral samples including tryptophan, catechin, and α-tocopherol were prepared to test the CSP functionality of the new capillaries.

2 Materials and methods

2.1 Reagents and chemicals

Most chemicals used were of analytical or chromatographic grades. Chitosan (CS; from shrimp shells, practical grade, ≥ 75% deacetylated), 2,2-diphenyl-1-picryl-hydrazyl (DPPH),

methacrylamide (MAA), N,N'-methylenebisacrylamide (Bis), glycidyl methacrylate (GMA), 2,2'-azobis(2-methylpropionitrile) (AIBN), sodium tetraborate, phosphoric acid, sodium dihydrogenphosphate, hydrochloric acid, acetonitrile (ACN), and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Boric acid, acetic acid, ammonium carbonate, methylene chloride, methanol and ethanol (EtOH) were obtained from Panreac Quimica S.A. (Barcelona, Spain). Sodium hydroxide, butylhydroxytoluene (BHT), polyvinyl alcohol (PVA), disodium hydrogenphosphate, tri-sodium phosphate, citric acid, sodium dihydrogen citrate, disodium hydrogen citrate, and trisodium citrate were supplied by Merck (Garmstadt, Germany). Acetone, sodium sulfate, sodium acetate were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Tris(hydroxymethyl)aminomethane (Tris) was received from TEDIA (Carson City, CA, USA). The chemical 3-(trimethoxysilyl) propylmethacrylate (γ-MAPS) was obtained from Acros Organics (Geel, Belgium). Finally, *m*-Methylbenzoic acid was purchased from TCI (Tokyo, Japan).

The chiral analytes consisted of tryptophans (L, D, and DL forms), the catechins of (+)-(2R,3S)- and (—)-(2S,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol, and the α-tocopherols of (+)-2*R*,4'*R*,8'*R*-α-tocopherol and its racemate (±). Their respective chemical structures are illustrated in Fig. 1. All chiral analytes were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Sample concentrations were 1.0 mg/mL tryptophan in H_2O, 25 µg/mL catechins in MeOH, and 10 mg/mL vitamin E in a preservation solution (98 ml MeOH, 2 ml H_2O and 5 mg BHT). Purified water (18 MΩ cm) from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to prepare samples and buffer solutions.

2.2 Instrumentation

The laboratory-built electrophoresis apparatus consisted of a ±30 kV high-voltage power supply (Model: TriSep TM-2100, Unimicro Technologies, CA, USA) and a UV-Vis detector (Model: LCD 2083.2 CE, ECOM, Prague, Czech). Electrochromatograms were recorded

using a Peak-ABC Chromatography Data Handling System (Kingtech Scientific, Taiwan). A Joel JSM-6700F Scanning Microscopy at National Chung Hsing University acquired the SEM images at an accelerating voltage of 3.0 kV. The ATR-IR spectra were obtained by a Shimadzu Prestige-21 IR spectrometer (Kyoto, Japan) that was equipped with a single reflection horizontal ATR accessory (Model: MIRacle, PIKE Technologies, WI, USA).

2.3 Preparation of capillary columns

The scheme of major reactions concerning with the synthesis of the CS-immobilized capillary is shown in Fig. 2.

2.3.1 Preparation and derivatization of CS nanoparticles

According the precipitation method [36], CS (0.25%, w/v) was dissolved in an aqueous solution of acetic acid (2%, v/v) containing PVA (10%, w/v). Sodium sulfate solutions (20%, w/v) were added dropwise (5 mL/min) to CS solutions while stirring at 400 rpm under ultrasonication. After adding sodium sulfate solutions, stirring and sonication were continued for another 1 h. CS nanoparticles (nano-CS) were collected by centrifugation at 3000 rpm, rinsed for several times with distilled water until the pH value decreased to 7, and dried at 80^{o}C for 1 day.

The purified, dry nano-CS (5 mg), *m*-methylbenzoic acid (50 mg), and potassium acetate (50 mg) were dispersed in methylene chloride (5 mL) at room temperature for 6 h under ultrasonication. The resulting acid-treated nano-CS could be easily obtained by evaporating the methylene chloride and wash with water for the measurements of SEM and ATR-IR. For attaching vinyl groups on the nano-CS, the GMA (5 mL) reagent was directly added into the above methylene chloride mixture and reacted at 60°C for 2 h under ultrasonication. After the derivatization, the GMA-nano-CS derivative with allyl groups was obtained and offered its reactivity during the polymerization required for MAA-CS column preparation.

2.3.2 Preparation of MAA-CS and MAA+CS capillaries

The preparation of a silanized phase proceeded according to previously described protocols

[27]. A new, bare capillary column (Polymicro Technologies, Phoenix, AZ, USA) with 375-μm O.D. x 75-μm I.D. was treated with 1.0 M NaOH and successively washed with pure water, 0.1 M HCl, pure water, and then acetone. The clean, bare capillary was then filled with a solution composed of DPPH (0.02 g), γ-MAPS (1.0 mL), and MeOH (1.0 mL). This capillary was kept at room temperature for 24 h to undergo the silanization, which was completed after a series of rinses with MeOH, H_2O, and acetone.

For preparation of the MAA-CS capillary, a polymerization solution was mixed with MAA (0.03 mol), Bis (0.01 mol), AIBN (30 mg), and GMA-nano-CS derivatives (3 mL of methylene chloride solution). After the silanized capillary was filled with the polymerization solution, most of the mixed solution was purged out of capillary by blowing nitrogen through the tube at 40 psi for 5 min, but a thin layer of solution was left on the capillary wall that was ready to react with the silanized capillary. The capillary was left at room temperature for 30 min to complete the polymerization reaction. Finally, the completed MAA-CS capillary was washed successively with H_2O, ethanol, and acetone for 30 min. It was then ready for CEC testing.

For the preparation of the MAA+CS capillary, a silanized capillary was filled with a polymerization solution containing MAA (0.03 mol), Bis (0.01 mol), AIBN (30 mg), and GMA (3 mL). After purging at 40 psi for 5 min, the capillary was stored at room temperature for 30 min. A portion of the methylene chloride solution (5 mL) containing 5 mg of CS nanoparticles, *m*-methyl benzoic acid (50 mg), and potassium acetate (50 mg) was put inside the capillary. The capillary was plugged with a septum and placed in an oven at 60°C for 2 h. Finally, the completed MAA+CS capillary was cleaned in the same way as the MAA-CS capillary.

2.4 CEC conditions

Most experiments were conducted using the common CZE buffers of Tris, acetate, citrate, phosphate, ammonium carbonate, and borate buffers within a pH range of 5.0 to 10.5 and an

ionic concentration range of 10 to 300 mM. ACN and MeOH were used as organic modifiers added in the buffers. All prepared buffer solutions for CEC analysis were filtered through a 0.45-μm cellulose ester membrane (Adventec MFS, Pleasanton, CA, USA). DMSO was used as the neutral marker. At the end of the analysis, the studied capillary was washed with methanol, pure water, and running buffer, sequentially, between each run. Prior to a sample injection, a working voltage was applied for 5 min to condition the charge distribution in the column. The prepared test samples were introduced by siphoning using a height difference. The samples were detected by UV light absorption measurements at 214 nm for DMSO, 214 nm for tryptophans, 280 nm for catechins, and 200 nm for vitamin E.

3 Results and discussion

3.1 Characterization of MAA-CNT phase

3.1.1 SEM images and ATR-IR spectra

The nano-CS was primarily prepared by a precipitation method and was reported with the size distribution ranging from 40 to 100 nm measured by TEM micrographs [36]. Afterward the amino end groups of nano-CS reacted with *m*-methyl benzoic acid in methylene chloride under ultrasonication. This amidzation increased the hydrophobicity of CS chains, which tended to be straightened due to the intermolecular interactions with concurrent self-association of *m*-methyl benzoic acid units of neighboring chains. The sizes of the CS self-aggregates were mainly controlled by the molecular weight of the CS backbone chains. According to the study on deoxycholic acid-modified CS, its self-aggregates may form a cylindrical bamboolike structure when the chitosan backbone is higher than 40 kDa and had the mean diameter in the range of 130-300 nm measured by dynamic light scattering method [37]. In Fig. 3(A), the SEM image of *m*-methyl benzoic acid-treated nano-CS shows the bamboolike structure with aggregation of some basic rod-like units, which diameter was nearly 150 nm. The ATR-IR spectrum of the acid-treated nano-CS is shown in Supporting

information. The ATR-IR peaks correspond to an amide bond formation between the amine groups of CS and the acid groups of the *m*-methyl benzoic acid.

The acid-treated nano-CS was further modified with GMA to anchor vinyl groups on the CS chain. This modification required an epoxide ring-opening reaction and proceeded from the nucleophilic attack of the CS hydroxyl groups. The ATR-IR of the resulting products, GMA-nano-CS, is shown in Supporting information. The prepared GMA-nano-CS was added to the polymerization solution that was ready for free-radical polymerization. This polymerization occurred in a beaker and in the silanized capillary. The SEM images of the polyacrylamide/nano-CS composite present in the beaker and in the capillary are shown in Fig. 3(B) and 3(C), respectively. In both images, acrylamide polymers tied bundles of bamboolike nano-CS together. The C=C bonds in GMA-nano-CS and γ-MAPS, a silanizing agent bonding on a capillary, were successfully engaged in the polymerization of MAA in a close silanized capillary as in an open beaker without the engagement of γ-MAPS. Inner surface microphotographs of a length of ground MAA-CS capillary were taken and are shown in Fig. 3(D). Some bundles of nano-CS were still attached to the capillary, but many were lost during grinding. A scatter of rod-like CS particles was observed in the right bottom of Fig. 3(D). For the MAA-CS capillary shown in Supporting information, no IR absorption was found at 1640 cm^{-1}. This result means that the vinyl groups of all involved monomers were completely used. Further, some absorption peaks were found between 1050 and 1110 cm^{-1}, which correspond to the Si–O stretching mode, and around 1700 cm^{-1}, which correspond to the C=O stretching in the polyacrylamide phase.

3.1.2 The EOF profiles with varying buffer pHs, ionic strengths, and ratios of organic modifier

Before applying the MAA-CS capillary for electochromatography and the chiral analyses, the characterization of the EOF driven by the capillary under different buffer conditions was performed. Some of the chemical properties of the MAA-CNT phase were revealed by these

measurements. The two curves shown in Fig. 4(A) illustrate the dependence of μ_{eo} on the pH levels of the phosphate buffer for both the bare fused-silica capillary and the MAA-CS capillary. Although the curve patterns of the MAA-CS were not the same as those of the bare capillary, the effect of the residual silanol groups on the surface charges of the MAA-CS capillary should be taken into consideration, which is especially true when the completeness ratio for the silanization using γ–MAPS reagent was only around 20% [38]. The dissociation of the residue silanols was more hindered by the coating layers of MAA-CS than the free silanols on a bare capillary as the EOF of a bare capillary exceeded that of the MAA-CS capillary at the high pH levels (pH > 9). The hydrogen of the silanols might be associated with some atoms, such as oxygen and nitrogen, on the MAA-CS layers through hydrogen bonding, and thus resulted in an EOF profile different from that of a bare capillary. Due to conversion of the amine groups on the nano-CS to amide derivatives by their reaction with *m*-methyl benzoic acid, the positive charge that formed on the MAA-CS phase surface could be largely reduced. Similar phase structures and cathodic EOF patterns were found in CEC columns with immobilized polysaccharides [39,40].

The dependence of the EOF mobility on the logarithmic electrolyte concentration is known to be linear in open tubes with thin double layers [41-43]. The trend of the curve of μ_{eo} values along with the log *C* axis for the bare fused-silica and MAA-CS capillaries is shown in Fig. 4(B). For the MAA-CS column, one point at the 10-mM concentration did not fit the line. This phenomenon can be explained by the presence of a surface conductance, which could induce the buildup of excess local charge in the double layer. The double layer might then slip under the influence of the electric field applied to the tortuous surfaces, which were caused by the modification process [44,45]. These overlapping double layers contributed to the deviations from linearity and from the estimated slope value of -2×10^{-4} cm^2 V^{-1} S^{-1} mM^{-1} [43]. In the range from 30 mM to 100 mM, the curve for the MAA-CS capillary was linear ($R^2 = 0.9373$, slope = -14×10^{-4} cm^2 V^{-1} S^{-1} mM^{-1}). In this same range, the bare fused-silica

capillary curve was also linear (0.9382, -3.3) [38]. The slope value of -14 x 10^{-4} is far from the estimated value of -2 x 10^{-4} and is much larger than the slope value of -8.2 x 10^{-4} that is predicted for a PLOT capillary with a polyacrylate phase [38]. This difference is considered to be a manifestation of the "openness" of the column, as packed columns typically have higher negative slopes than OT columns [46].

The effect of adding the organic solvents of ACN and MeOH in the buffer solution on the μ_{eo} values is highlighted in Fig. 4(C), which shows the presence of concave curves that have minimums around 40-60% volume content of organic modifier. A similar trend is observed in many OT-CEC formats including direct polymer coating, stepwise fabrication, and in-situ polymerization [47–49,38]. This is primarily the result of a change in the ratio of the dielectric constant to the viscosity of the running buffer (see the Y_2 axis in Fig. 4(C)). This situation suggests that the DMSO solute was a good EOF probe and that its chromatographic interactions with the modified phases could be ignored.

The reproducibility of the capillary fabrication was evaluated from the μ_{eo} values measured at pH 7.6 for five runs of the MAA-CS capillary. The RSD values were 3.3, 3.7, and 4.2% for three newly replicated capillaries. At the 95% confidence level, no significant differences between the columns were observed by *t*-test. The MAA-CS capillaries could be used for more than 400 times within 6.0% RSD in half a year in the studies on the chiral separations of different samples under various running buffers across wide pH ranges (pH 2–10) and volume ratios of organic modifier (5–100%). This indicated the fabrication of the modified capillaries was pretty robust.

3.2 Separation of tryptophan enantiomers

Tryptophan enantiomers were used as chiral probes to assess the CEC enantioselectivities of the MAA-CS and MAA+CS capillaries. After trying several buffers (described in section 2.4), the best peak shape and resolution of racemic tryptophan were achieved with a Tris buffer system using the MAA-CS capillary. The electropherograms shown in Fig. 5(A), (B), and (C)

resulted from use of the Tris running buffer at a fixed molarity (100 mM) with different pH values (pH = 8.5, 9.5, and 10.5). Figure 4(B) demonstrates the buffer at pH 9.5, which had a much more satisfactory resolution than either pH 8.5 or 10.5. Changing the buffer concentration from 50 mM to 200 mM verified the suitability of using a 100-mM concentration. An organic modifier, MeOH, was added to the Tris buffer (pH 9.5, 100 mM) but did not increase the selectivity (Fig. 5(D)).

In another report by Kato et al. [32], a CSP composite of sol−gel, bovine serum albumin, and chitosan for monolithic CEC of enantiomeric tryptophans reached the α′ values, t_D/t_L = 1.10−1.15, higher than 1.02 obtained in the conditions of Fig. 5(B), but Kato et al. considered the enantioselectivity by chitosan was negligible. However, our MAA-CS capillary had higher theoretical plate numbers of tryptophans, 650,000 and 930,000 (N/m), than the monolithic CEC capillary with 130,000 (N/m) of thioruea. Moreover, a promising resolution, 3.8, was observed in the Fig. 5(B), even compared to a resolution of 1.2 observed from the CSP based on covalently bonded chitosan for ligand-exchange liquid chromatography [33]. We thus see that MAA-CS is a potential component for CSP as long as a proper buffer condition is used. After more than 400 trials in half a year, the MAA-CS capillary could still afford the 3% and 1% RSD values (n=5) for the resolution and selectivity respectively.

The MAA+CS capillary, which was prepared by an alternative approach for the immobilization of nano-CS on the column, did not achieve enantioseparation of the tryptophans under any of our buffer conditions. This outcome might be attributed to the lacks of CS chiral selectors immobilized in the MAA+CS capillary. Before CS molecules nucleophilically attacked the epoxide rings of the polymerized GMA, some rings might have been opened during the preparation of the polymerization solution, the formation of the polymeric phase with the GMA units, and the waiting time necessary for the polymerization. Another potential reason could arise from the epoxide rings exposed on the solid surface of the polymeric phase. These fixed rings might have been fewer or less reactive to the

well-dispersed nano-CS in the methylene chloride solution (in the MAA+CS capillary) when compared with the free GMA molecules that homogeneously reacted with nano-CS solution prior to polymerization (in the MAA-CS capillary). In any cases, the MAA+CS capillary would not be used in the following separations.

3.3 Chiral separation of catechin

The (+)-(2R,3S)-catechin and (—)-(2S,3R)-catechin belong to the flavonoid group and have different bioavailabilities and bioactivities [50,51]. Their separation is often achieved using cyclodextrin chiral selectors with HPLC, CE, and MEKC methods [52]. Using a CEC method with the MAA-CS capillary, the effect of the pH levels of the phosphate buffer (50 mM, 80% (v/v) MeOH) on the chiral separation of these catechins was found that the optimal pH level seemed to be pH 6.6, at which the electrochromatograms showed the best selectivities. The migration times did not decrease when the EOF magnitude was increased by increases in pH levels of the running buffer, as shown in Fig. 4(A). Thus, a chromatographic retention is likely involved in the separation mechanism. The effect of the ionic strength of the phosphate buffer (pH 6.6, 80% (v/v) MeOH) on the chiral separation of the catechins is shown in Fig. 6. Here the migration times for the MeOH solvent peak and the two catechin peaks increased as the buffer concentration increased, which can be simply explained by the decreased EOF, as shown in Fig. 4(B). Obviously, the selectivity between the (+)-catechin and the (−)-catechin was increased with increasing buffer concentration. However, electrodispersion due to mismatched sample and buffer conductivities resulted in the peak tailing and fronting. After one and half year trial, the resolution, Rs = 3.6 (±0.2 (n=5)), and selectivity, α = 2.5 (±0.1 (n = 5)), were observed in the conditions of Fig. 6(C) with better peak shape.

The effect of different volume ratios of MeOH in the phosphate buffer (pH 6.6, 50 mM) on the separation of the (±)-catechin in the MAA-CS capillary showed the shortened migration times when increasing the volume percentages of MeOH from 70% to 90% would increase the EOF, as shown in Fig. 4(C). However, the role of the organic modifier not only

altered the EOF, but it also affected the chromatographic partitioning between the catechin molecules and the MAA-CS phase. Differentiating between the electrophoretic and chromatographic contributions to the CEC separation is essential, particularly in this study, which focuses on the chiral selectivity induced by nano-CS. Adopting the definition formulated by Rathore and Horváth, measures of electrophoretic migration and chromatographic retention in CEC can be described by a velocity factor (k_e'') and a retention factor (k''), respectively [53,54]. In brief, they are expressed by equations (1) and (2):

$$k_e'' = \frac{\mu_{ep}}{\mu_{eo2}} \quad (1)$$

$$k'' = \frac{\left[t_{M2} \times \left(1 + k_e''\right) - t_{02}\right]}{t_{02}} \quad (2)$$

where μ_{ep} and μ_{eo2} are the electrophoretic and electroosmotic mobilities. These mobilities can be obtained from open-tubular CE experiments on a bare capillary (column 1) and from the CEC experiments on the MWNT immobilized capillary (column 2), respectively, as follows:

$$\mu_{ep} = \frac{L_1 \times L_{d1}}{V_1} \times \left(\frac{1}{t_{M1}} - \frac{1}{t_{01}}\right)$$

$$\mu_{eo2} = \frac{L_2 \times L_{d2}}{t_{02} \times V_2}$$

where L is the total column length, L_d is the distance between the inlet and the detection point, V is the applied voltage, t_M is the migration time of solute, and t_0 is the migration time of DMSO. The plots of velocity and retention factors versus the MeOH modifier percentage are shown in Fig. 7.

In Fig. 7, the k_e'' values increase with the increase in the MeOH percentage within the BGE from 70% to 100%. The electrophoretic properties of analytes should be taken into account when their pK_a values can increase and thus result in higher effective charges (q_{eff}).

Further, the BGE viscosity (η) values would decrease as more organic modifier was blended with the aqueous medium [55,56]. Based on the equation $\mu_{ep} = q_{eff}/6\pi\eta r$ where r is the radius of the analyte ion, the electrophoretic migration of the catechin analytes toward the cathode will be increased while q_{eff} is increased and η is decreased. This enhanced electrophoretic migration is seen in the upward curve of the k_e'' values shown in Fig. 7, however, the discrimination between the k_e'' values is not sufficient to separate the isoomeric pair at any MeOH percentage. On the contrary, there is discrimination between the k'' values in the cases of clear chiral selectivity with 80% and 90% MeOH levels. Moreover, the k'' values increased with increases in the MeOH percentage. Thus, chromatographic retention was observed with a normal-phase mode, which is in agreement with similar results found via HPLC with polysaccharide CSPs [57].

3.4 Chiral separation of α-tocopherol

While α-tocopherol, a vitamin E class, has 3 chiral centers at the 2, 4' and 8' positions, the naturally occurring 2R,4'R,8'R-α-tocopherol (RRR-α-tocopherol) is characterized by the very powerful, biologically active antioxidants. The stereoisomers were determined by off-line HPLC and GC after their derivatization by acetylation or methylation [58,59]. To examine the ultimate chiral selective abilities of our developed MAA-CS phase, a racemic all-rac-α-tocopherol solution without derivatization was used as a test sample. However, only two peaks were found in the electrochromatograms, as shown in Fig. 8. The pH level and the addition of the ACN modifier in the borate buffers greatly helped in separating the two peaks. This situation is analogous to using a chiral polyacrylate and a Chiralpak OT as HPLC stationary phases to separate α-tocopherol racemate into two peaks, which correspond to 2R and 2S stereoisomers [60-62]. In our case, RRR-α-tocopherol was validated as a member of the latter group corresponding to the second peak. For a better resolution of other group members, several potential means of improvement are possible including the derivatization of the samples and the nano-CS.

4 Concluding remarks

Comparison of SEM and ATR-IR spectra taken during the fabrication of an OT-CEC capillary from the graftation of acid-treated nano-CS onto polyacrylamide proved that this new chiral stationary phase was successfully bonded to the capillary wall. The completed MAA-CS capillary was characterized by the measurement of EOF profiles under different running buffer pHs, ionic strengths, and ratios of added organic modifier prior to the separation of chiral samples. For tryptophan, the MAA-CS capillary had satisfactory resolution, which was in contrast with the MAA+CS capillary that had poor resolution. This poor resolution might be caused by the poor loading of CS chiral selectors in the capillary during fabrication. An improved chiral selectivity between (±)-catechins was achieved in a non-aqueous mode by addition of 80% MeOH into the phosphate buffer. As only two peaks were found during the separation of α-tocopherol stereoisomers, we have recognized that the composition and construction of the MAA-CS capillary may need to be further modified in the future.

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Figure captions and legends

Figure 1. Chemical structures of the chiral samples.

Figure 2. Schemes to synthesize the CS-immobilized capillary.

Figure 3. SEM images. (A) Acid-treated nano-CS; (B) nano-CS/polyacrylamide composite (MAA-CS powder); (C) coatings around a cut rim of the MAA-CS capillary; and (D) coatings on the inner wall of the MAA-CS capillary.

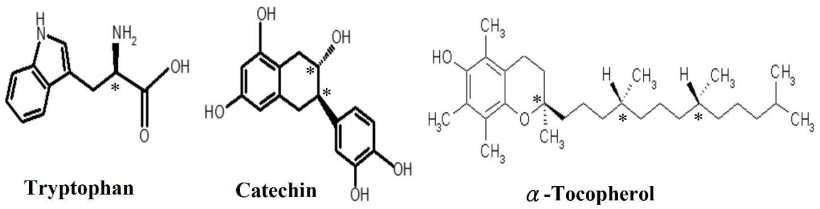
Figure 4. Dependence of electroosmotic mobility on buffer pH, ionic strength, and the ratio of organic modifier. Columns: (□) a bare fused-silica capillary; (○) and (●) the MAA-CS capillary. BGE conditions: phosphate buffer, (A) 50 mM; (B) pH 7.5; (C) 10 mM, pH 7.5. Sample: DMSO; hydrostatic injection of 10 cm for 1 sec and detection at 214 nm. The applied voltage was 10 kV. The symbols (○) and (●) in (C) represent the BGE mixing with ACN and MeOH, respectively, and their corresponding ε/η values are denoted by (◊) and (♦).

Figure 5. Enantioseparations of the tryptophans in the MAA-CS capillary of (60 cm (55 cm) x 75 μm I.D.). Conditions: BGE, Tris buffer, 100 mM at pH equals (A) 8.5, (B) 9.5, (C) 10.5, and (D) 9.5 with the addition of 10% (v/v) MeOH. The applied voltage was 15 kV. Samples: hydrostatic injection of 10 cm for 5 sec and detection at 214 nm. Peak correspond to (1) D-tryptophan and (2) L-tryptophan.

Figure 6. Chiral separations of catechins with different buffer concentrations in the MAA-CS capillary (45 cm (42 cm) x 75 μm I.D.). Conditions: BGE, MeOH (80%, v/v) and phosphate buffer, pH 6.6, at buffer concentration equals (A) 10 mM, (B) 50 mM, (C) 70 mM, and (D) 90 mM. The applied voltage was 15 kV. Samples: hydrostatic injection of 10 cm for 5 sec and detection at 280 nm. Peak correspond to (S) MeOH, (1) (−)-catechin, and (2) (+)-catechin.**Figure 7.** Effect of the addition of MeOH into the phosphate buffer (pH 6.3, 100 mM) on the velocity factor (k_e'') and the retention factor (k'') of (±)-catechins . CEC conditions are the same as in Fig. 6(B). (○) and (□) represent the k_e'' values of (−)-catechin and (+)-catechin, respectively. (●) and (■) represent the k'' values of (−)-catechin and

(+)-catechin, respectively.

Figure 8. Chiral separations of racemic α-tocopherols in the MAA-CS capillary (52 cm (47 cm) x 75 μm I.D.). Conditions: BGE, borate buffer, 100 mM, at pH equals (A) 7.5, (B) 8.5, (C) 9.5, and (D) pH 8.5 with addition of 10% (v/v) ACN. The applied voltage was 10 kV. Samples: hydrostatic injection of 10 cm for 5 sec and detection at 200 nm. Peak assignments: (1) Group 1, (2) Group 2 containing (+)-2*R*,4'*R*,8'*R*-α-tocopherol.



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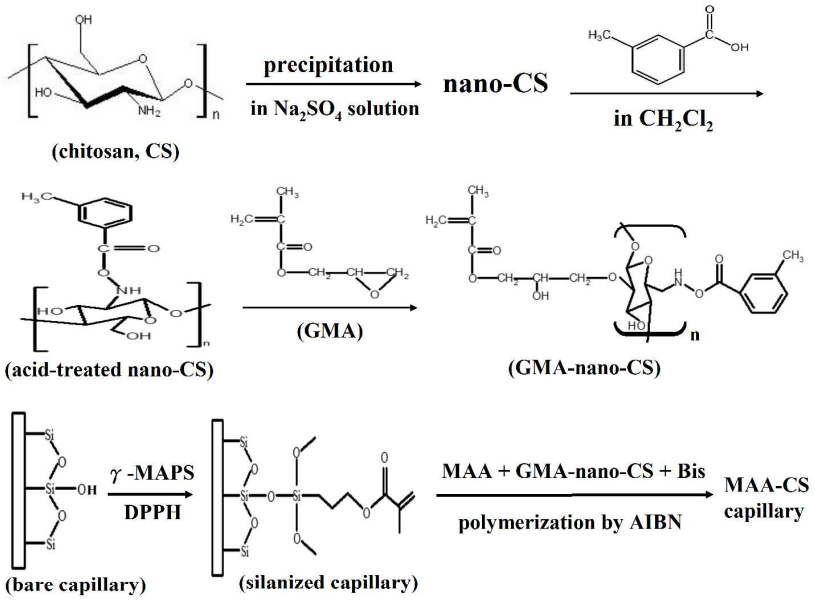

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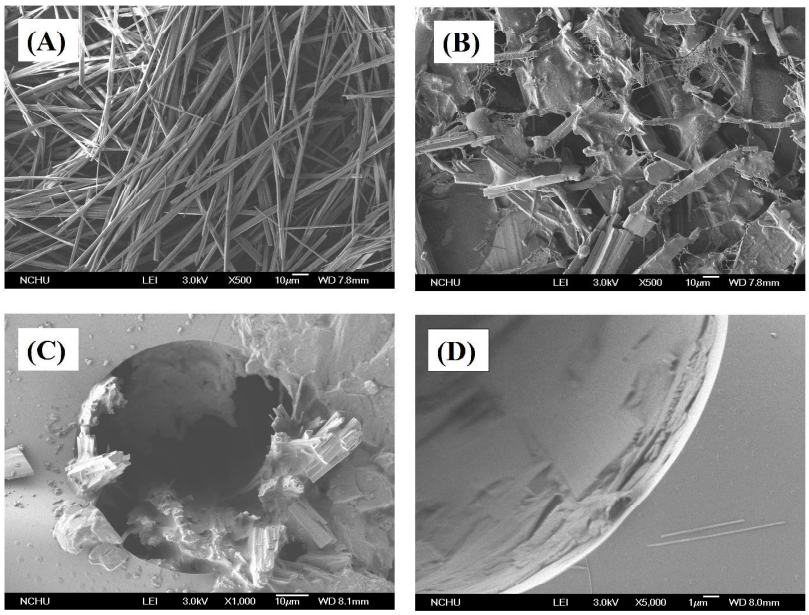

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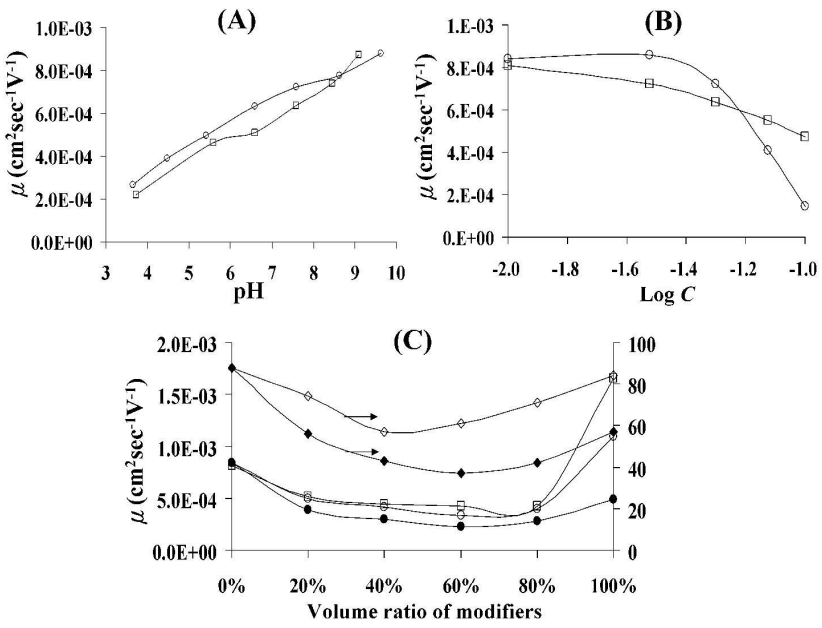

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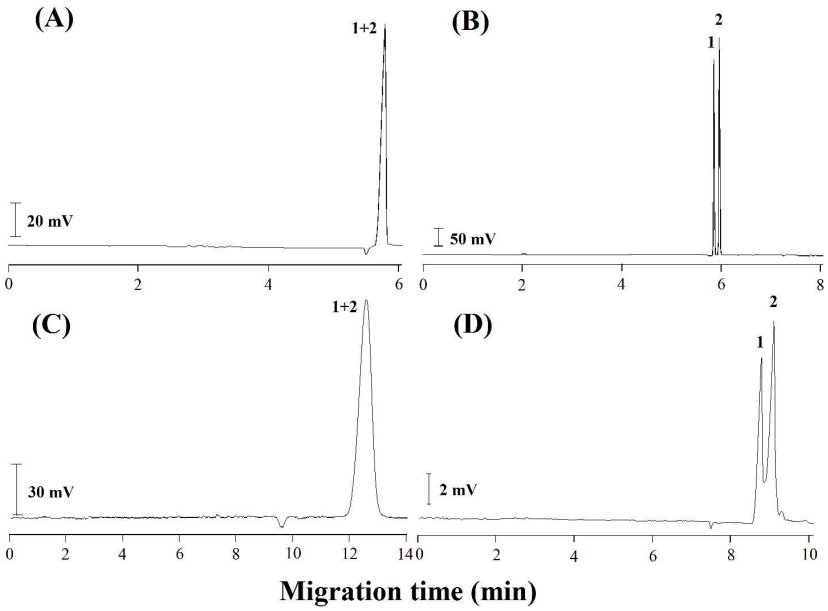

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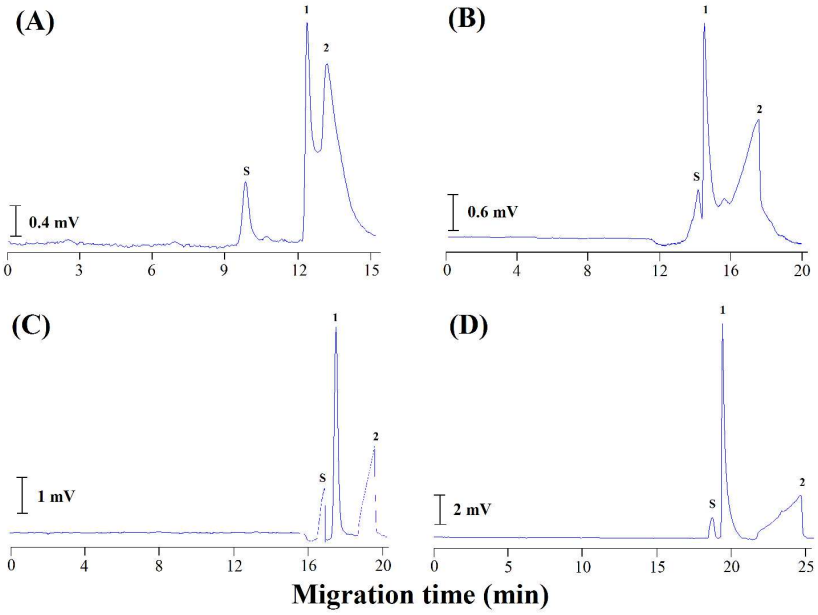

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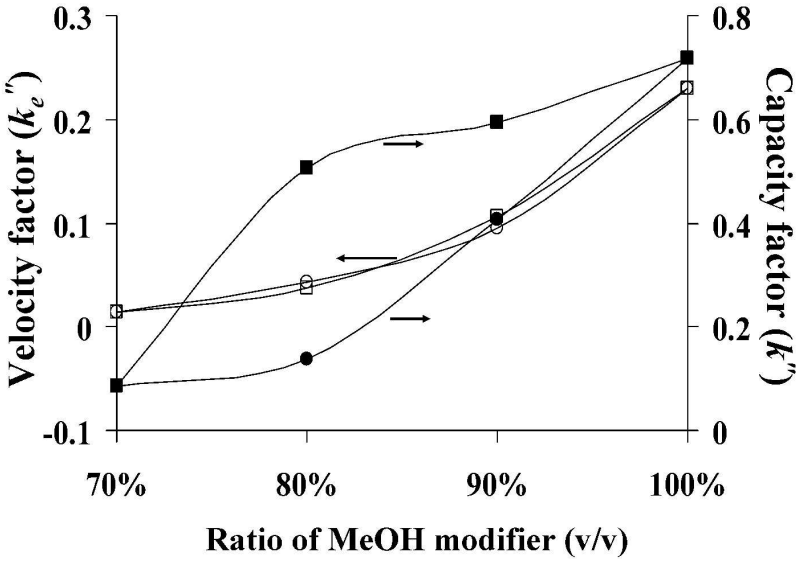

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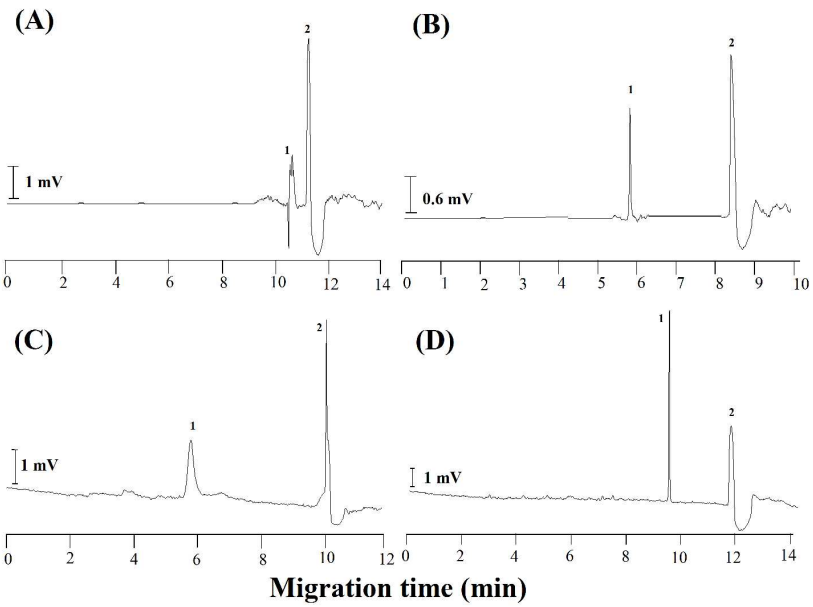

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