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Self-assembled and covalent capillary coating of diazoresin and D-Glucurone for protein analysis in capillary electrophoresis

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ABSTRACT

A simple, environmental and economical capillary electrophoresis method has been developed for the analysis of four model proteins by employing a photoactive and non-toxic diazoresin (DR), D-Glucurone modification of the inner surface of fused-silica capillary. DR/D-Glucurone coated capillary not only effectively eliminated the wall adsorption of proteins, but also showed good stability and repeatability. Instead of using the moisture sensitive and toxic silane coupling agent, the application of photosensitive DR may provide a flexible and environmentally friendly strategy to prepare novel covalently coated capillaries. Thus, DR/D-Glucurone coated capillary has great potential in capillary electrochromatography and may provide a new method for biological compounds separation.

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1. Introduction

Capillary electrophoresis (CE) is now considered a promising and effective technique in separation. Compared with HPLC and GC columns, CE offers several advantages including high separation efficiency [1–3], short analysis time, high resolution, small sample volume, automation and decreased cost of capillaries [4]. However, this technique for proteins analysis is subject to some critical limitations owing to the tendency of protein to be adsorbed onto the negatively charged surface of fused-silica capillaries [5]. This kind of adsorption often results in sample loss, poor resolution, peak broadening, long migration times, and unstable electroosmotic flow (EOF) [6]. Many approaches have been proposed to minimize these untoward interactions of proteins with the capillary wall.

The most common and efficient approach is surface modification with capillary coatings so far [7–8]. Capillary coatings can be broadly categorized as: covalently

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linked polymeric coatings [9], physically adsorbed polymer coatings [10–14]. Physically adsorbed coating refers to the coating material adsorption on the capillary surface by electrostatic, non-covalent adsorption of hydrophobic interaction, van der Waals force, hydrogen bonds or ionic bonds and other non-covalent bonds to form a non-permanently coating layer. Physical adsorption coatings can be prepared simply by flushing the capillary with coating solutions. Both Liu et al. [15] and Allan et al. [16] prepared non-covalent coatings, which improved the separation performance, but the coating is usually unstable and short-lived, resulting in poor separation and analysis. Compared with the physical adsorption coatings, the covalently bonded coatings are very stable and robust. For example, Witos et al. utilized a covalently linked poly ([2 (methacryloyl)-oxyethyl] trimethyl ammonium chloride) coating for the separation of proteins [17] and Tan et al. used a poly (vinylpyrrolidone) coated capillary to reduce protein adsorption to the capillary wall [18]. The covalently linked coatings not only showed very good anti-protein fouling properties, but also demonstrated excellent stabilities for repeatable separations. However, the chemically bonded coatings is critically limited due to the preparation process is usually multi-steps and complicated, such as capillary pretreatment, introducing coupling agents, and inserting target coating reagents, etc. [19–22]. Moreover, the tradition silane coupling agents which are widely used for surface modification are moisture sensitive and have some toxicity that may lead to quality and environmental problems [23–25].

In order to integrate the advantages of the non-covalently and covalently bonded coatings and avoid their disadvantages, in this paper, a novel method for the preparation of covalently linked diazoresin (DR)/D-Glucurone capillary coatings was introduced to obtain a better modification of the inner surface of fused-silica capillary, which was subsequently used to improve the separation of basic proteins. Diazoresin (DR) is a photoactive polymer without toxic [26]. The unique photo-crosslinking reaction of DR has been applied to prepare covalently attached self-assembly films [27], hollow microcapsules [28], and bio chips [29]. DR/D-Glucurone coated on the inner surface of the capillary through lay by lay (LBL) self-assembly, and cured as covalent coating by photosensitive DR. The DR/D-Glucurone-capillary column was successfully employed to suppress Protein adsorption on the inner surface of capillary and achieve baseline separation of Lysozyme (Lys), myoglobin (Mb), bovine serum albumin (BSA) and ribonuclease A (RNase A) within 12 min using capillary electrophoresis (CE).

2. Experiments

2.1. Reagents and solutions

DR (Mn = 2500) was synthesized according to the method described elsewhere [30]. D-Glucurone was obtained from Nanqiao Chemical Reagent Company (Shanghai, China). Lysozyme (Lys), cytochrome c (Cyt-c), bovine serum albumin

(BSA), amyloglucosidase (AMG), myoglobin (Mb) and ribonuclease A (RNase A) were purchased from Sigma (St. Louis, USA). Dendrimer (Mn = 6000) was bought from Guangfu Institute of Fine Chemical Industry (Tianjin, China). N,N-Dimethyl formamide (DMF) was purchased from Yongda Chemical Reagent Company (Tianjin, China). Phosphate acid (H_3PO_4) was purchased from Fuyu Fine Chemical Company (Tianjin, China). Monosodium orthophosphate ($NaH_2PO_4{\cdot}2H_2O$) and dibastic sodium phosphate ($NaH_2PO_4{\cdot}2H_2O$) were bought from Shunqiang Chemical Reagent Company (Shanghai, China). Acetone was obtained from Sanhe Chemical Reagent Company (Tianjin, China). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Hongyan Reagent Company (Tianjin, China). Phosphate buffer was used as separation medium, and the pH value was adjusted by NaOH (0.1 M) and H_3PO_4 (40 mM). The concentrations of Lys, Cyt-c, BSA, AMG, Mb, and RNase A in the testing samples were all 0.5 mg mL^{-1}. All solutions were filtered through a 0.45 μm membrane before use.

2.2. Preparation of the DR/D-Glucurone-coated capillary

The preparation of the D-Glucurone covalently coated capillary was as follows: (1) a new bare fused silica capillary was activated: First rinsed with 0.1 M NaOH for 30 min, deionized (DI) water for 10 min, then rinsed with 0.1 M HCl for 30 min, DI water for 10 min and eventually dried with N_2. (2) The capillary was coated by D-Glucurone: Coating was performed by flushing the capillary with aqueous solution of DR (2 mg mL^{-1}) for 5 min, and then flushed with DI water for 2 min. Subsequently, the capillary was flushed with aqueous solution of D-Glucurone (2 mg mL^{-1}) for 5 min, and then flushed with DI water for 2 min. A self-assembled DR/D-Glucurone bilayer coating was completed. The coating cycle was repeated for several times to obtain multilayer DR/D-Glucurone coated capillary. Afterwards, the coated capillary was exposed to 365 nm UV light with an intensity of 350 μW cm^{-2} for 15 min. Following the decomposition of the diazo group in the film under UV irradiation, the hydrogen bonds converted into covalent bonds [31]. The covalently linked D-Glucurone capillary coatings formed successfully. The schematic illustration of these preparation steps above was shown in Figure 1.

2.3. Instrumentation and characterization

UV-vis spectrometer (TU-1810, China) was used for monitoring the LBL self-assembly coating process. The photo-crosslinking of the DR/Dendrimer coating on the capillary was carried out using a 365 nm UV curing system (EXFO Omnicure S1000) with a lamp power of 100 W. Atomic force microscope (AFM, CSPM 5500, China) was used for surface characterization of the coatings. The CE experiments were performed on a CL1020 high performance capillary electrophoresis instrument (Huayang liming instrument Co., China). Fused-silica capillaries of 75 μm ID and 375 μm OD were provided by Yongnian Optic Fiber (Hebei, China). The EOF

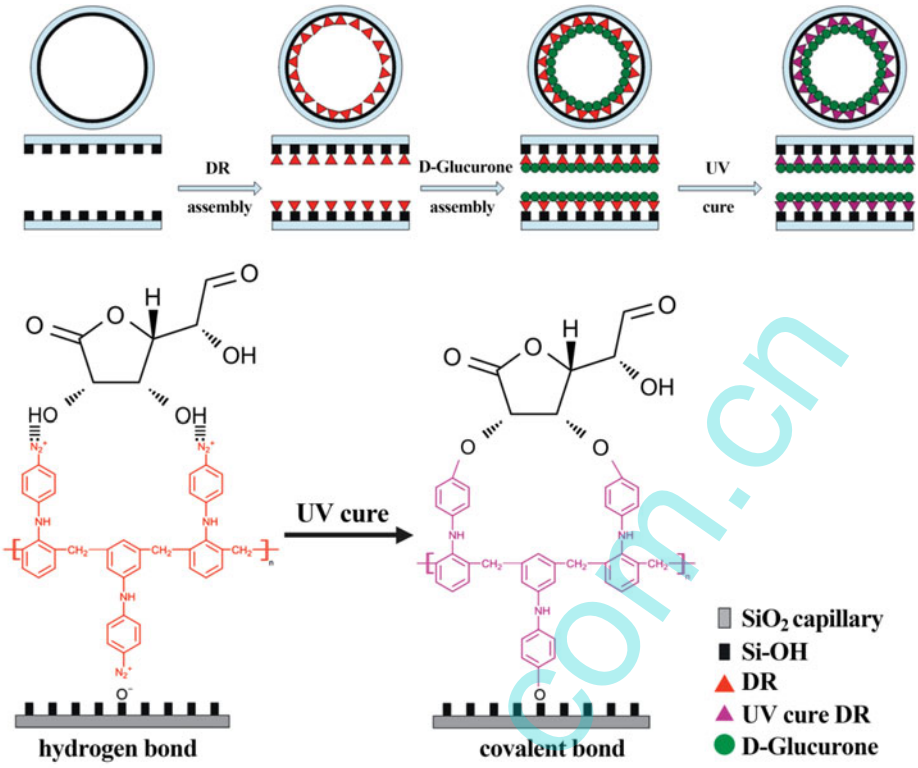

Figure 1. Schematic illustration for the preparation of DR/ D-Glucurone capillary coatings.

measurements were carried out using a method reported elsewhere [32]. Phosphate buffers (40 mM) of pH 3.0–9.0 were prepared to determine EOF at different pH values. DMF with a concentration of 0.5 vol % was used as the EOF marker.

3. Results and discussion

3.1. Formation of DR/D-Glucurone coatings on the inner wall of capillary

3.1.1. LBL self-assembly

The self-assembly process of DR/D-Glucurone coating was monitored by UV-vis spectrometer. As shown in Figure 2, the peak at 380 nm is attribute to the characteristic $\pi-\pi^*$ transition absorption of the diazo group of DR. The UV-vis absorption intensity at 380 nm increases linearly with the increasing number of bilayers (Figure 2, inset). The result indicates that the DR/D-Glucurone coating assembled on the inner wall of capillary successfully and uniformly.

3.1.2. UV cross-linking

As shown in Figure 3, the absorbance of the coatings at 380 nm reduces gradually with the increase of irradiation time (from 0 s to 35 s). This trend is due to the decomposition of the diazo groups that involved in the DR/D-Glucurone

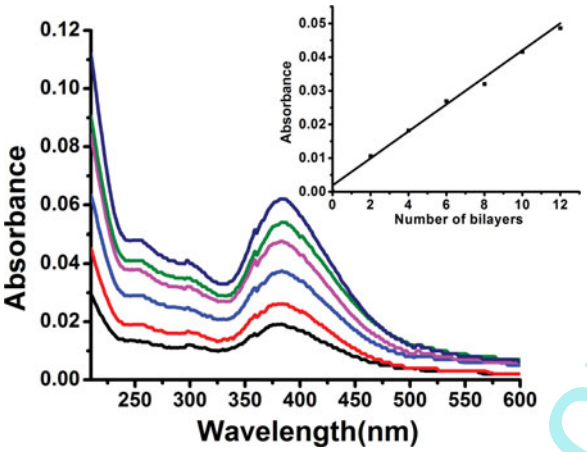

Figure 2. UV–vis spectra of the coating assembly from the DR and DR/D-Glucurone. Number of assembly cycles (bottom to top): 1, 2, 3, 4, 5 and 6. The inset plot shows that the absorbance of the films at 380 nm changes linearly with the number of assembly cycles.

multilayer coatings under UV irradiation and the hydrogen bonds were converted into stable and robust covalent bonds. The photoreaction, which originates from the diazonium decomposition, is a first-order reaction: $\ln[(A_0-A_e)/(A_t-A_e)]$ is linear with irradiation time (Figure 3, inset), where A_0, A_t and A_e represent the absorbance of the coating before irradiation, after irradiating for predetermined time, and at the end of irradiation, respectively.

3.1.3. Stability

As shown in Figure 4, the UV-vis spectrum of the non-irradiated coating changes dramatically after immersion in DMF for 30 min (Fig. 4a), while the spectrum of the UV-irradiated film changes little after the DMF etching (Fig. 4b). It indicates that the hydrogen bonds between the DR and D-Glucurone turn into covalent bonds

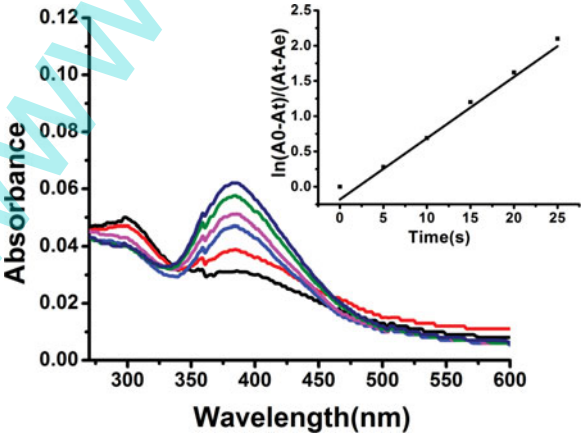

Figure 3. UV-vis spectra of DR/D-Glucurone multilayer coatings at different irradiation times. Irradiation time (s) (top to bottom): 0, 5, 10, 15, 25 and 35 s; Irradiation intensity (at 365 nm): 350 μW/cm^2. Inset: relationship between $\ln[(A_0-A_e)/(A_t-A_e)]$ and irradiation time.

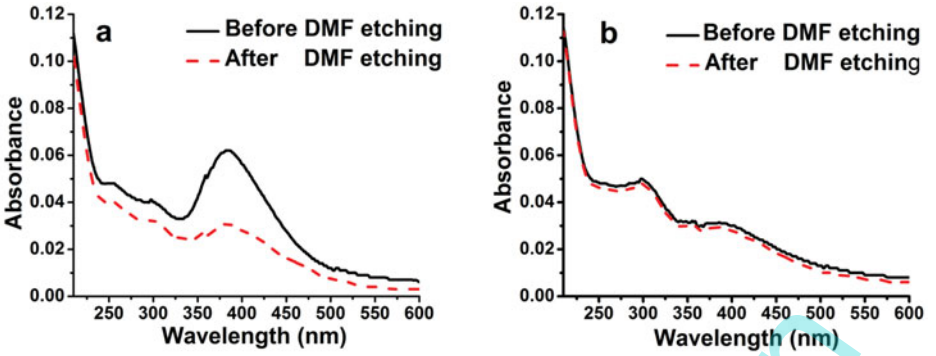

Figure 4. UV-vis spectra of non-irradiated (a) and irradiated (b) DR/D-Glucurone multilayer coatings before (solid lines) and after (dash lines) etching with DMF at 25°C for 30 min.

after irradiation, and the covalent bonding is much more stable than the hydrogen bonding.

3.1.4. Morphology

Atomic Force Microscope (AFM) experiments were carried out to characterize the features of the inner wall of D-Glucurone-capillary column. Figure. 5a shows the AFM images of bare capillary, which possesses a smooth inner surface and has an average surface roughness (Ra) of 0.225 nm. After surface modification with 2 and 4 layers of DR/D-Glucurone covalent coatings, the Ra increases to 0.532 and 1.386 nm, respectively (Figure. 5b and 5c). The increased Ra with layer numbers indicates that the LBL coating process is carried out successfully.

3.2. Performance of the DR/ D-Glucurone covalent coatings for CE analysis of proteins

3.2.1. EOF

EOF is the driving force for the CE separation. Effective measurement of the EOF in CE plays an important role in the separation of model analytes. In this work, we evaluate the effect of pH on the EOF for bare, non-covalently and covalently

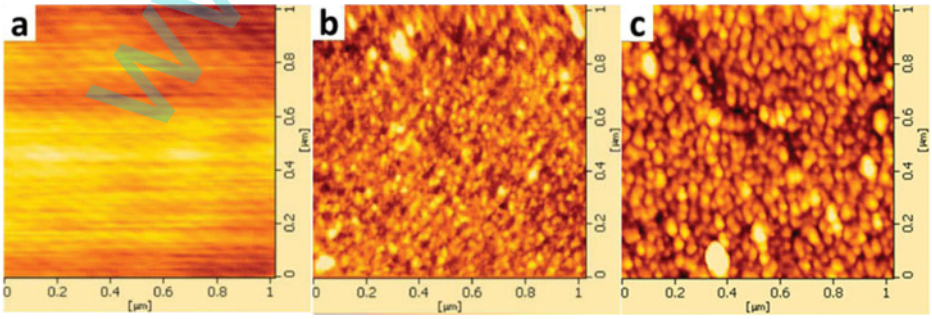

Figure 5. AFM images of inner surface of (a) bare capillary, (b) two layers of DR/D-Glucurone covalently coated capillary, and (c) four layers of DR/D-Glucurone covalently coated capillary.

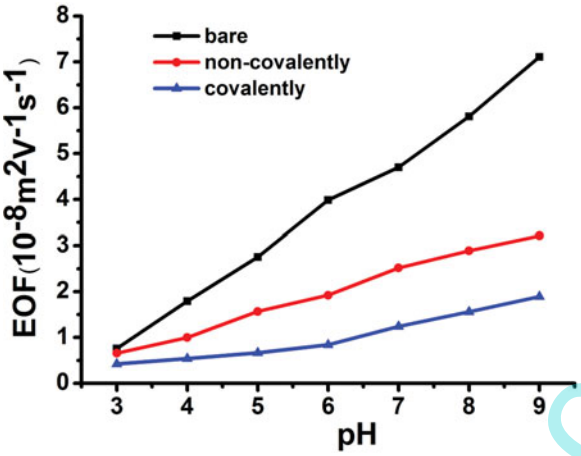

Figure 6. Influence of pH on EOF of bare, DR/D-Glucurone non-covalently and covalently coated capillary columns. Test conditions: buffer, 40 mM phosphate; injection, 20 s with a height difference of 20 cm; applied voltage, +15 kV; UV detection, 214 nm; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25°C.

DR/D-Glucurone coated capillary columns. Figure 6 compares the EOF at different buffer pH in bare, DR/D-Glucurone non-covalently and covalently coated capillary columns. For bare capillary, the rationale for the generation of EOF is the dissociation of silanol groups on the inner surface of the capillary column. With the increase of buffer pH value, the magnitude of EOF also increased, because the alkaline condition favored the dissociation of silanol groups. When the capillary is coated with DR/D-Glucurone layers, especially the covalently coated layers, the silanol groups are mainly reacted or shielded by the coatings resulting in the EOF lowering down.

3.2.2. Effect of coating types

Under the optimum operating conditions, CE separation performance of four proteins with bare capillary, DR/D-Glucurone non-covalently and covalently coated capillaries is shown in Figure 7. The bare capillary performs a strong adsorption to the proteins, having a bad separation result with only two characteristic peaks (Fig 7b). Although the non-covalently DR/D-Glucurone coated capillary column has a better performance, the four proteins still can not be fully separated. This defective experiment result is caused by the poor stability of the non-covalently bonded coatings on the inner surface of capillary. However, the covalently DR/D-Glucurone coated capillary column has a wonderful performance, and baseline separation of the four proteins is attained in 12 min (Fig. 7c).

3.2.3. Stability and reproducibility

Stable coatings can improve the separation efficiency and extend the life span of capillary columns. The stability of the covalently DR/D-Glucurone coated capillary columns was evaluated by using the relative standard deviation (RSD) of the degradation ratio of EOF and migration time. Table 1 summarized the EOF of the DR/D-Glucurone covalently capillary columns that treated with long time flush of

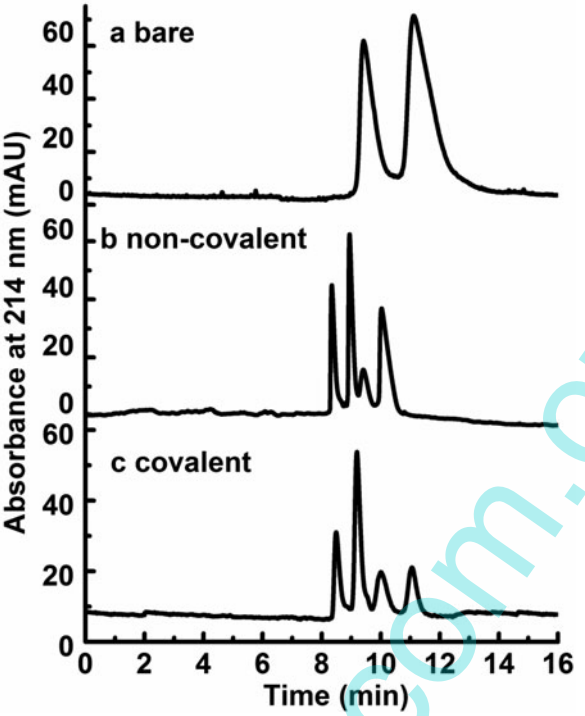

Figure 7. Separation of four proteins using the bare capillary (A), three-layer D-Glucurone non-covalent coated capillary (B) and three-layer D-Glucurone covalently coated capillary (C). Separation conditions: buffer, 40 mM phosphate (pH = 4.0); injection, 20 s with a height difference of 20 cm; applied voltage, +15 kV; UV detection, 214 nm; sample, 0.5 mg/mL for each protein; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25°C. Peak identification: 1, Lys; 2, BSA; 3, Mb; 4, RNase A.

0.1 M NaOH, 0.1 M HCl, and DMF. The minor degradation ratio of EOF (within 15%) indicates the good stability and well tolerance to the strong alkaline, acid and organic solvent of the DR/D-Glucurone covalent coatings. Just as show in figure 4b, the spectrum of the irradiated covalent coating does not change after immersion in the organic solvent DMF for 30 min. As summarized in Table 2, the run-to-run (n = 5) relative standard deviation (RSD) of migration time for the proteins is less than 1%, day-to-day (n = 5) RSD is less than 3%, and capillary-to-capillary (n = 5) RSD is less than 4%, respectively. After continuous 60 times running, the RSD of migration time of each protein are still less than 3%. These results imply that DR/D-Glucurone covalent coatings have good stability and repeatability performance.

Table 1. The chemical stability of three-layer DR/ D-Glucurone covalent capillary coatings.

Rinse solvent	EOF before rinsing ($\times 10^{-8}m^2V^{-1}S^{-1}$)	EOF after rinsing 15 min ($\times 10^{-8}m^2V^{-1}S^{-1}$)	Degradation ratio (%)
0.1 M HCL	0.3205	0.3631	13.29
0.1 M NaOH	0.4499	0.5143	14.31
Acetone	0.5629	0.6281	11.58
DMF	0.4984	0.5596	10.23

EOF test conditions: the same as Figure 6, and buffer pH = 3.0, NaOH, 0.1 M; HCl, 0.1 M.

Table 2. Separation performance of the three-layer DR/D-Glucurone covalent capillary coatings.

	Migration time RSD (%)			
Protein	run to run (n = 5)	day to day (n = 3)	capillary to capillary (n = 3)	continuous 60 times running
Lys	0.68	1.34	2.15	1.25
BSA	0.73	2.49	3.33	2.35
Mb	0.79	2.87	3.79	2.87
RNaseA	0.86	2.15	3.54	2.46

Separation conditions: the same as Figure 7.

4. Conclusions

In this paper, a novel kind of covalently linked DR/D-Glucurone coated capillary column was fabricated successfully. The D-Glucurone was first introduced to be coated on the inner surface of the capillary by LBL self-assembly through unique photochemistry reaction of DR. The hydrogen bondings between the DR and D-Glucurone or capilary were converted into covalent bonding after curing with UV light. The DR/D-Glucurone covalently linked capillary column exhibit better stability and repeatability than bare capillary and non-covalently bonded DR/D-Glucurone columns. Using photosensitive DR instead of highly toxic and moisture sensitive silane coupling agent, which makes the fabrication process of DR/D-Glucurone covalently linked capillary column is more environmental and simple than traditional method. Baseline separation of Lys, BSA, RNase A and Mb can be achieved within 12 minutes using the covalently linked capillary column under the optimum separation conditions. The covalently linked DR/D-Glucurone coated capillary column would provide a green and simple model for effective CE applications.

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