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A novel diazoresin/polyethylene glycol covalent capillary coating for analysis of proteins by capillary electrophoresis†

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A novel method for the preparation of covalently linked capillary coatings of polyethylene glycol (PEG) was demonstrated using photosensitive diazoresin (DR) as the coupling agent. Layer by layer (LBL) self-assembly film of DR and PEG based on hydrogen bonding was first fabricated on the inner wall of capillary, then the hydrogen bonding was converted into covalent bonding after treatment with UV light through a unique photochemistry reaction of DR. The covalently bonded coatings suppressed protein adsorption on the inner surface of the capillary, and thus a baseline separation of lysozyme (Lys), cytochrome c (Cyt-c), bovine serum albumin (BSA) and ribonuclease A (RNase A) was achieved using capillary electrophoresis (CE). Compared with the bare capillary or non-covalently bonded DR/PEG coatings, the covalently linked DR/PEG capillary coatings not only improved the CE separation performance for proteins, but also exhibited good stability and repeatability. Due to the replacement of highly toxic and moisture sensitive silane coupling agents by DR in the covalent coating preparation, this method may provide a green and easy way to make the covalently coated capillaries for CE.

Introduction

Capillary electrophoresis (CE) presents a tool that is growing in importance for biochemical analysis because the method can be operated under physiological buffer conditions and offers various advantages over conventional methods, including short analysis times, low sample consumption, high efficiency, high sensitivity and economy.[1,2] However, when analyzing proteinaceous samples, protein adsorption onto fused-silica capillary walls is one of the major challenges of CE.[3–5] This leads to sample loss, peak broadening, poor resolution, unstable electroosmotic flow (EOF), and long migration times.[6] In order to minimize protein adsorption onto the capillary surface, surface modification with capillary coatings is the most efficient and commonly used approach.[7–10]

Capillary coatings can be classified into non-covalently and covalently bonded coatings. The non-covalent coating can be produced simply by flushing the capillary with coating solutions, and the coating molecules absorb on capillary surface by weak interactions such as electrostatic, van der Waals, and hydrogen bonding, *etc.*[11–13] Furthermore, the layer-by-layer (LBL) self-assembly technique can also be used to prepare the non-covalently bonded capillary coatings, which provides the coating with new structures and functions.[14–18] For example, Haselberg *et al.*[19] prepared polybrene–dextran sulfate–polybrene (PB–DS–PB) triple layer coatings by the LBL self-assembly technique, and the coatings were fully compatible with mass spectrometry (MS) detection, causing no background signals and ionization suppression. The coatings were used for the analysis of α-chymotrypsinogen, ribonuclease A, Cyt-c and Lys by CE-MS, and the detection limits for them were 16, 11, 14 and 19 nM, respectively. Compared with the non-covalently bonded coatings, the covalently bonded coatings are very stable and robust. For example, Timperman *et al.*[20] prepared chemically bonded PEG coatings which were used for high efficiency separation of four basic proteins (BSA, alcohol dehydrogenase, carbonic anhydrase and trypsin inhibitor). The covalently linked coatings of PEG not only showed very good anti-protein fouling properties, but also demonstrated excellent stabilities for repeatable separations. However, the preparation process of covalently bonded capillary coatings is usually complicated which includes multi-steps such as capillary pretreatment, introducing coupling agents, and inserting target coating reagents, *etc.*[21–25] Moreover, highly toxic and moisture sensitive silane coupling agents are traditionally used in the covalent coatings, which often cause environmental and quality problems during the manufacture and application.[26,27]

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In the fabrication process of capillary coatings with high quality and performance, how to combine the advantages of the non-covalently and covalently bonded coatings together, and avoid their disadvantages, is becoming one of the main development directions. In this work, we reported a novel method for the preparation of covalently linked PEG capillary coatings using the LBL self-assembly technique combined with photochemistry reactions. The fabrication, structure and property of the coatings were studied and discussed preliminarily.

Experimental

Chemicals

DR (M_n = 2500) was synthesized according to a method described elsewhere.[28] Lysozyme (Lys), cytochrome c (Cyt-c), bovine serum albumin (BSA), fluorescein isothiocyanate labeled BSA (FITC-BSA), amyloglucosidase (AMG), myoglobin (Mb) and ribonuclease A (RNase A) were purchased from Sigma (St. Louis, USA). PEG (M_n = 6000) and polyvinyl alcohol (PVA, M_n = 77 000) 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 ($Na_2HPO_4 \cdot 12H_2O$) 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.

Preparation of the PEG coated capillary

The preparation of the PEG covalently coated capillary was as follows: a new bare fused silica capillary was rinsed with 0.1 M NaOH for 30 min and deionized (DI) water for 10 min. Then 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 1 min and air dried for 5 min. Subsequently, the capillary was flushed with aqueous solution of PEG (4 mg mL^{-1}) for 5 min, and then flushed with DI water for 1 min and air dried for 5 min. A self-assembled DR/PEG bilayer coating was completed. The coating cycle was repeated for several times to obtain multilayer DR/PEG coated capillary. Afterwards, the coated capillary was exposed to 365 nm UV light with an intensity of 350 μW cm^{-2} for 15 min in order to form the covalently linked PEG capillary coatings.

Instruments and conditions

UV-vis spectrometer (TU-1810, China) was used for monitoring the LBL self-assembly coating process. The photo-crosslinking of the DR/PEG 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) and X-ray photoelectron spectroscopy (XPS, Axis Ultra spectrometer, Kratos Analytical, USA) were 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 measurements were carried out using a method reported elsewhere.[21] 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. Protein adsorption within capillaries was measured as follows: FITC-BSA was dissolved in 40 mM phosphate buffer (pH = 7.0) at a concentration of 0.05 mg mL^{-1}, and pumped through the capillaries for 30 min at a flow rate of 5 μL min^{-1}. Then, the capillaries were rinsed thoroughly with the buffer and subsequently analyzed using an inverted fluorescent microscope (Axiovert 200, Zeiss, 488 nm excitation and 530 nm detection). Fluorescent images of various samples were taken and quantified using NIH-Scion Image viewer. Bare silica capillaries analyzed under the same light exposure were used as background controls.

Results and discussion

Formation of DR/PEG coatings on the inner wall of capillary

As shown in Fig. 1a, UV-vis spectroscopy is used to monitor the LBL self-assembly process. The absorbance of the DR/PEG film at 380 nm, which derives from the characteristic π–π* transition absorption of the diazo group of DR, increases linearly with the number of assembly cycles (Fig. 1a, inset). This indicates

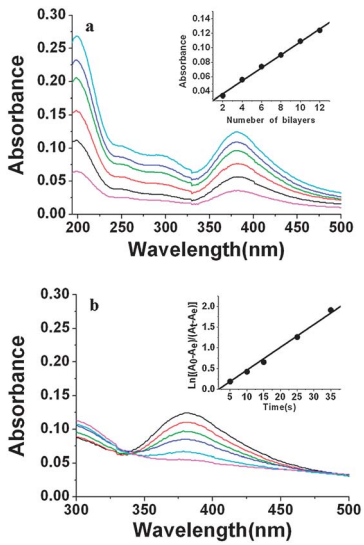

Fig. 1 (a) UV-vis spectra of the assembly from DR and PEG. Number of assembly cycles (bottom to top): 1, 2, 3, 4, 5 and 6. The inset plot shows that the absorbance of the films at 380 nm changes linearly with the number of assembly cycles; (b) UV-vis spectra of DR/PEG multilayer coatings at different UV irradiation times. Irradiation time (s) (top to bottom): 0, 5, 10, 15, 25 and 35. Irradiation intensity (at 365 nm): 350 μW cm^{-2}. Inset: relationship between $\ln[(A_0 - A_e)/(A_t - A_e)]$ and irradiation time.

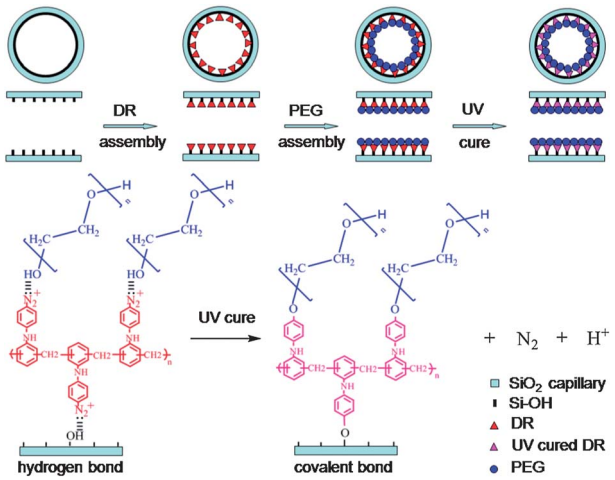

Fig. 2 Schematic illustration of the preparation of DR/PEG covalent coating on capillary surface.

that the LBL assembly is carried out successfully and uniformly. The driving force of the assembly comes from the hydrogen bond between the diazo group of DR and hydroxyl group of PEG.

DR is a non-toxic photoactive component often used in cell culture supports,[29,30] and the diazo groups involved in the DR/PEG multilayer films will be decomposed under UV irradiation, which results in a gradual decrease in the absorbance of the film at 380 nm (Fig. 1b). The photoreaction that takes place in the multilayer films, which originates from the diazo decomposition, is a first-order reaction: $\ln[(A_0 - A_e)/(A_t - A_e)]$ changes linearly with irradiation time (Fig. 1b, inset), where A_0, A_t and A_e represent the absorbance of the film before irradiation, after irradiating for time t, and at the end of irradiation (35 s), respectively. As illustrated in Fig. 2, following the decomposition of the diazo group in the film under UV irradiation, the hydrogen bonds convert into covalent bonds.[31] The unique photo-crosslinking reaction of DR has been applied to the fabrication of covalently attached self-assembly films,[32] hollow microcapsules,[33] and bio chips.[34] For example, Shi and coworkers reported the fabrication of stable, multilayer ultrathin films by self-assembly of DR with single-walled carbon nanotubols (SWNTols) followed by cross-linking under UV irradiation.[35] Yang *et al.* fabricated stable DR/chiral polyaniline composites (CPAC) shell on polystyrene (PS) colloids by self-assembly and UV cross-linking. After the PS core was removed by chemical etching, stable DR/CPAC hollow spheres were obtained.[36] Yu *et al.* prepared stable ultrathin DR/deoxyribonucleic acid (DNA) micropatterns by self-assembly and photolithography, which could find important application in biochips intended for gene therapy and drug identification.[37]

AFM images (Fig. 3) show the surface morphology of the bare and DR/PEG covalently coated capillaries. The inner surface of bare capillary is very smooth with an average surface roughness (Ra) of 0.22 nm, and after surface modification with 2 and 4 layers of DR/PEG covalent coatings, the Ra increases to 1.61 and 2.07 nm, respectively. The average thickness for the 2 and 4 layers of DR/PEG covalent coatings is about 3.22 and 5.12 nm, respectively. The increased thickness with layer numbers

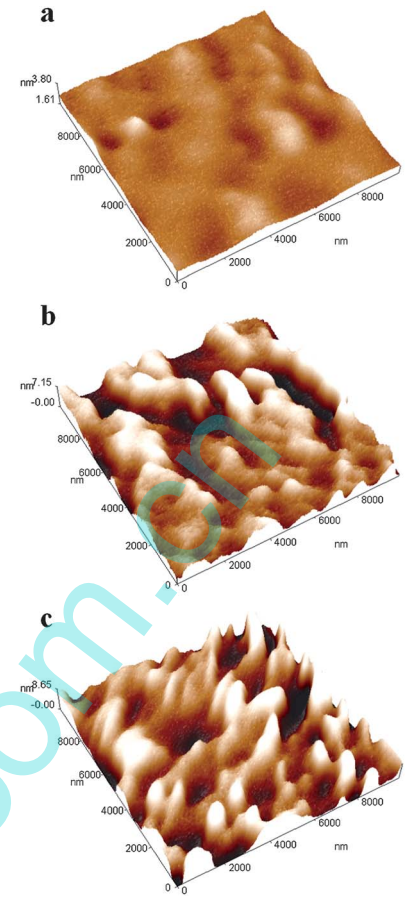

Fig. 3 AFM images of inner surface of (a) bare capillary, (b) 2 layers of DR/PEG covalently coated capillary, and (c) 4 layers of DR/PEG covalently coated capillary.

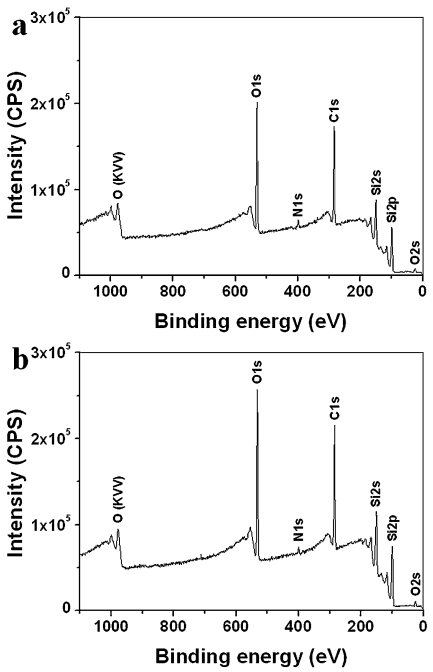

Fig. 4 X-ray photoelectron spectrum of inner surface of (a) 2 layers of DR/PEG covalently coated capillary, and (b) 4 layers of DR/PEG covalently coated capillary.

indicates that the LBL coating process is carried out successfully. Since the 2-layer capillary coating is smoother than the 4-layer coating and facile to fabrication, it is adopted for the following performance studies.

It is well know that X-ray photoelectron spectroscopy is a powerful analytical technique for surface studies,[37] because it can supply both qualitative and quantitative information about the chemical composition of the first few nanometers of the surface, and it is particular sensitive to the top atomic layer. Furthermore, the measured chemical shift in the binding energy reflects the chemical state of the atom. Therefore, this approach can be utilized to investigate the DR/PEG multilayer coatings. It's clear from Fig. 4a and b that the C 1s and N 1s signals come from the DR/PEG coatings, and the Si 2s and Si 2p signals come from the exposed silica surface, which indicates that the fused silica surface might not be fully covered by the DR/PEG coatings, and there should be a few pinholes (Fig. 3b and c) in the coatings to leak the silica signals out. According to corrected area ratio of Si 2p signal to C 1s signal by atomic sensitivity factors, the surface coverage rate for the 2 layer coatings is roughly calculated to be ~80%.

Performance of the DR/PEG covalent coatings for CE analysis of proteins

Fig. 5 compares the EOF at different buffer pH in bare, DR/PEG non-covalently and covalently coated capillary columns. The magnitude of EOF in a capillary is dependent on the net surface charge density of chargeable groups. For uncoated capillaries, the dissociation of silanol groups is responsible for the generation of EOF, which increases with the increase of buffer pH, due to the strong dissociation of silanol groups in a high pH environment. By contrast, when the capillary is coated with DR/PEG, especially the covalently coated DR/PEG, the EOF is low. For example, the EOF of the DR/PEG covalently coated column at pH 4.0 is 4.51×10^{-9} m^2 V^{-1} S^{-1} that is less than one fourth of that of the uncoated one (19.63×10^{-9} m^2 V^{-1} S^{-1}), because the silanol groups on capillary surfaces which are responsible for the generation of EOF are mainly reacted and shielded by the DR/PEG coating (Fig. 2).

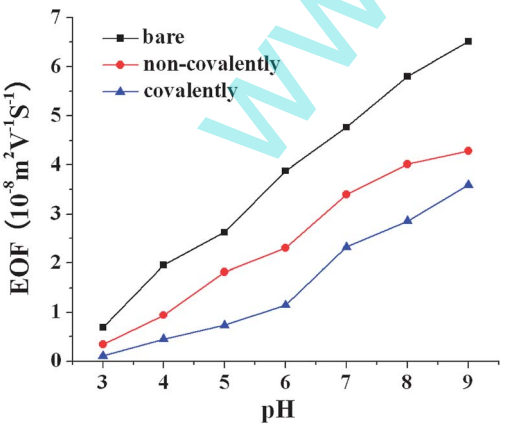

Fig. 5 Influence of pH on EOF of bare, DR/PEG non-covalently and covalently coated capillary columns. Test conditions: buffer, 40 mM phosphate; injection, 20 s with a height difference of 20 cm; applied voltage, +15 kV; UV detection, 214 nm; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25 °C.

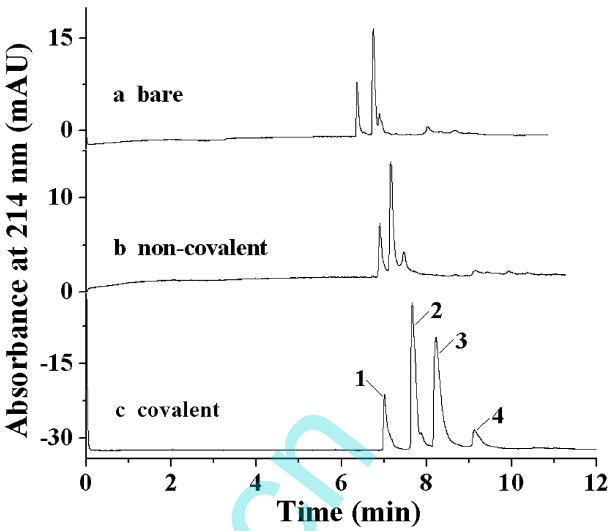

Fig. 6 Separation of proteins using the bare capillary (a), PEG non-covalently coated capillary (b) and 2-layer PEG covalently coated capillary (c). Separation conditions: buffer, 40 mM phosphate (pH = 3.0); injection, 20 s with a height difference of 20 cm; applied voltage, +18 kV; UV detection, 214 nm; sample, 0.5 mg mL^{-1} for each protein; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25 °C. Peak identification: (1) Cyt-c; (2) Lys; (3) BSA; (4) RNase A.

Fig. 6a–c show CE separation results for four proteins by using the bare capillary, DR/PEG non-covalent, and DR/PEG covalent capillary coatings in the optimized conditions, respectively. The bare capillary performs a strong adsorption to the proteins, and thus a bad separation result with only two characteristic peaks is obtained. Although the separation performance of DR/PEG non-covalent capillary coating is better than that of bare capillary, effective separation of the proteins cannot be achieved, and the stability of the coating is very poor due to lack of strong bondings to the capillary. Compared with them, the PEG covalent capillary coating has the best separation performance, and a stable and baseline separation of the Cyt-c, Lys, BSA and RNase A is achieved within 10 minutes.

Fig. 7 gives a measure of the potency of PEG coating in preventing FITC-BSA adsorption to the silica wall. Compared with bare capillary, the non-covalently coated capillary shows

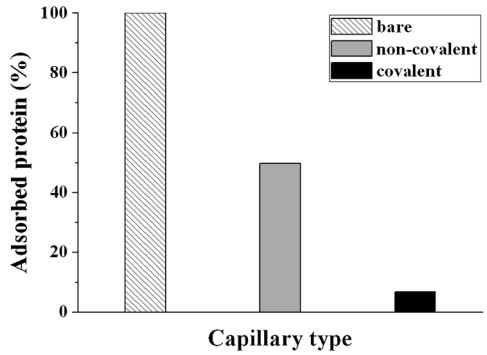

Fig. 7 Efficacy of PEG coating in preventing protein adsorption to the capillary wall.

Table 1 Chemical stability of the 2-layer DR/PEG covalent coatings[a]

Rinse solvent	EOF before rinsing ($\times 10^{-9}$ m^2 V^{-1} S^{-1})	EOF after rinsing 15 min ($\times 10^{-9}$ m^2 V^{-1} S^{-1})	Degradation ratio (%)
0.1 M NaOH	1.642	1.602	−2.44
0.1 M HCl	1.602	1.630	1.75
Acetone	1.630	1.675	2.76
DMF	1.675	1.639	−2.15

[a] EOF test conditions: the same as Fig. 5, and buffer pH = 3.0.

Table 2 Separation performance of the 2-layer DR/PEG covalent coatings[a]

Protein	Migration time RSD (%)			
	Run to run ($n = 5$)	Day to day ($n = 7$)	Capillary to capillary ($n = 5$)	Continuous 200 times running
Cyt-c	0.98	1.51	2.32	1.89
Lys	0.46	1.56	2.37	1.65
BSA	0.56	2.23	3.41	2.40
RNase A	0.79	2.18	3.39	2.38

[a] Separation conditions: the same as Fig. 6.

~50% inhibition of protein adsorption, while the covalently coated capillary can inhibit protein adsorption by as much as ~93%. The superior anti-protein-fouling property of the covalent coatings should come from the strong binding force of the PEG layers to the silica wall.

The 2-layer DR/PEG covalent coatings prepared by this method have very good stability and repeatability. Table 1 shows the coatings can resist the long time flush of 0.1 M NaOH, 0.1 M HCl, acetone and DMF, and the degradation ratio of EOF is less than 3%, which demonstrates that the DR/PEG covalent coatings have excellent stability and good tolerance to the strong alkaline, acid and organic solvent.

Table 2 shows that the run-to-run ($n = 5$) RSD of migration time for the proteins is less than 1%, day-to-day ($n = 7$) RSD is less than 2.5%, and capillary-to-capillary ($n = 5$) RSD is less than 3.5%. After a continuous 200 times running in a coating column, the RSD of migration time for the proteins are all less than 2.5% (Table 1), and the separation performance of the DR/PEG covalent coatings is not deteriorated. Therefore, the DR/PEG covalently coated capillaries are robust and may be used in heavy duty analysis.

Fig. 8b shows the separation of five proteins-BSA, Cyt-c, Lys, AMG, and Mb in 15 minutes using the DR/PEG covalent coatings under an optimized condition. The isoelectric point (pI) of the five proteins varies from 4.0 to 11.0, and the Mw of the proteins varies from 12 000 to 143 500. Compared with the bare capillary (Fig. 8a), the DR/PEG covalently coated capillary performs a much better separation for the five proteins. Compared with the silane-coupled PEG covalent coating reported elsewhere,[20] the DR/PEG coating shows better performance in the range of proteins separated.

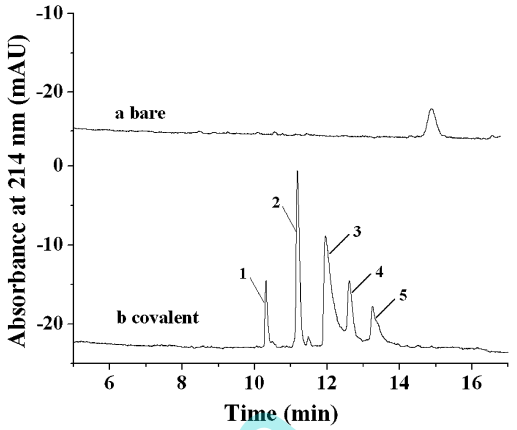

Fig. 8 Separation of five proteins using the bare capillary (a) and 2-layer PEG covalently coated capillary (b). Separation conditions: buffer, 40 mM phosphate (pH = 3.0); injection, 20 s with a height difference of 20 cm; applied voltage, +12 kV; UV detection, 214 nm; sample, 0.5 mg mL^{-1} for each protein; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25 °C. Peak identification: (1) Cyt-c; (2) Lys; (3) BSA; (4) AMG; (5) Mb.

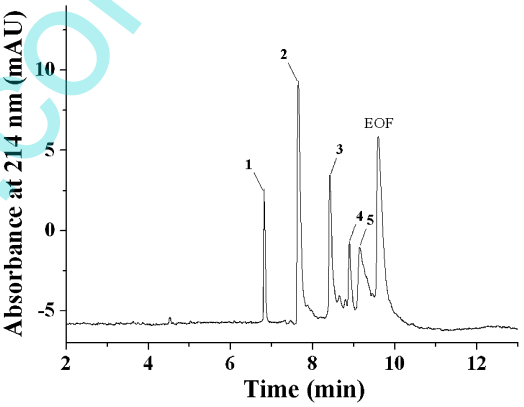

Fig. 9 Separation of five proteins using the 4-layer DR/PVA covalently coated capillary. Separation conditions: buffer, 40 mM phosphate (pH = 3.0); injection, 20 s with a height difference of 20 cm; applied voltage, +15 kV; UV detection, 214 nm; sample, 0.5 mg mL^{-1} for each protein; capillary, 75 μm ID × 50 cm (41 cm effective); capillary temperature, 25 °C. Peak identification: (1) Cyt-c; (2) Lys; (3) BSA; (4) AMG; (5) Mb.

Additionally, our recent study verified that the unique photochemistry reaction of DR can also be applied to make other DR/polymer covalent capillary coatings based on self-assembly. For example, the 4-layer of anti-protein-fouling DR/polyvinyl alcohol (PVA) covalent capillary coatings are fabricated successfully using same method as the DR/PEG coatings, and a satisfactory separation of five proteins is achieved (Fig. 9). The EOF mobility *vs.* pH graph (ESI, Fig. S1†) and the corresponding reproducibility data (ESI, Table S1†) of the DR/PVA coatings are quite similar to those of the DR/PEG coatings. Due to the replacement of highly toxic and moisture sensitive silane coupling agent by DR in the covalent coating preparations, this method may provide a green and easy way to make the covalently coated capillaries for all kinds of CE applications.

Conclusion

In this work, a new type of covalently linked PEG capillary coating is prepared successfully using photosensitive DR as coupling agents combined with the LBL self-assembly technique. The hydrogen bonding between the DR and PEG is converted into covalent bonding after treatment with UV light through the unique photochemistry reaction of DR. The covalently bonded coatings suppress protein adsorption on the inner surface of the capillary, and thus baseline separation of Lys, Cyt-c, BSA and RNase A is achieved within 10 minutes at optimized separation conditions. Compared with the bare capillary or non-covalently bonded DR/PEG coatings, the covalently linked DR/PEG capillary coatings not only improve the CE separation performance for proteins, but also exhibit good stability and repeatability. Moreover, for the replacement of highly toxic and moisture sensitive silane coupling agent by DR in the covalent coating preparation, this method may provide a green and easy way to make the covalently coated capillaries for all kinds of CE applications.

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