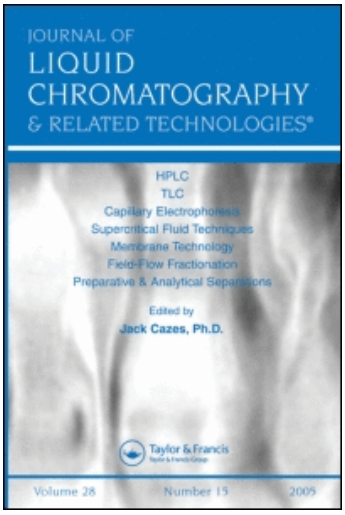

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Open Tubular Capillary Electrochromatographic Separation of Proteins and Peptides Using a TiO_2 Nanoparticle-Deposited Capillary by Liquid Phase Deposition

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Abstract: A titanium dioxide (TiO_2) nanoparticles (NP)-deposited column by the liquid phase deposition (LPD) method was exploited for open tubular capillary electrochromatographic (OTCEC) separation of proteins and peptides. With the phosphate buffer, the direction of electroosmotic flow (EOF) for the TiO_2 NP-deposited capillary was the same as that for the bare fused silica capillary, but the magnitude of EOF was much smaller at pH$\geq$7. Its OTCEC performance was then evaluated with four proteins (bovine serum albumin, ovalbumin, hemoglobin, and myoglobin) by varying parameters of the background electrolyte (BGE), such as pH value, composition, and concentration. Good separation was achieved with the phosphate buffer (40 mM, pH 9.0) and an applied voltage of 15 kV. In comparison with the bare fused silica capillary, the relatively high resolving power of the TiO_2 NP-deposited capillary would mainly result from the ligand exchange interaction between analytes and the phosphate adsorbed on the TiO_2. Finally, separation of the proteolytic digest mixture was also performed with the TiO_2 NP-deposited capillary at well defined conditions, indicating its potential applicability for biological analysis.

Keywords: Liquid phase deposition, Open tubular capillary electrochromatography, Protein and peptide separation, Titanium dioxide

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INTRODUCTION

In the last few years, capillary electrophoresis (CE) has become one of the most important techniques for the separation, detection, and determination of proteins and peptides owing to its excellent separation efficiency, rapid analysis, minimal need of samples and solvents, and high versatility in terms of separation modes.[1,2] The nature of the inner surface of the capillary, which relates closely to the direction and magnitude of electroosmotic flow (EOF) and influences the interactions with the analytes thus plays an important role in CE separations.[3] Fused silica capillary is by far the most common material used in CE, however, it is not appropriate for the direct separation of proteins and peptides without any modification on the inner surface of capillary or in the buffer solution. Because at acidic/neutral pH, a strong interaction would occur between the deprotonated silanol groups and the basic parts of biomolecules, while at basic pH, analytes with small differences in mobility would be quickly ejected through the capillary by a high EOF without sufficient resolution. In order to overcome these shortcomings, fabrication of metal oxide coatings (i.e., ZrO_2, TiO_2, Al_2O_3, and their composite) on the inner surface of the fused silica capillary has been introduced to construct open tubular capillary electrochromatography (OTCEC) as an effective approach to manipulate the EOF, minimize the adsorption of basic compounds or biomolecules, and improve the separation efficiency.[4–11] Hence, the preparation of metal oxide coatings has become an important and challenging topic in the development of OTCEC.

Since Tsai et al. prepared TiO_2- and Al_2O_3-coated capillaries for the OTCEC separation of proteins in 1994,[4] the sol-gel method has been a versatile approach to synthesize metal oxide coatings on the inner surface of the fused silica capillary. During the following years, Xie et al. have reported the preparation of ZrO_2-coated and MgO-ZrO_2-coated capillaries using the sol-gel process to control EOF and to achieve good separations of basic compounds,[5,6] and Xu et al. have introduced sol-gel based ZrO_2 and ordered mesoporous TiO_2 coating in OTCEC to separate alkaloids in nonaqueous systems.[7,8] Lately, many efforts have been devoted to investigate the separation of peptides and proteins on various TiO_2-coated capillaries (e.g., ODS modified TiO_2 and TiO_2 nanoparticles),[9–11] which are all prepared via the sol-gel method. Nevertheless, this method involving multiple preparation steps and relatively high heating temperature or long heating time to remove the residual organics, is inconvenient and may lead to undesirable cracks of the coating. Therefore, the development of a simple and reliable method is required for metal oxide coating fabrication in OTCEC.

Liquid phase deposition (LPD) is a low cost, facile, and environment friendly process for thin film preparation recently developed in many fields, such as integrated circuit processing, metal-oxide-semiconductor

technology, biosensor, photocatalytic, and antibacterial materials.[12–15] It consists of a ligand exchange equilibrium reaction of the metal-fluoro complex ions and F^- ions consuming reaction by boric acid (H_3BO_3) or aluminum metal (Al) as an F^- scavenger.[16] LPD was first developed for depositing SiO_2 thin film,[17] and was later used to prepare other metal oxide films, such as TiO_2, tin oxide, ZrO_2, or a variety of 3d transition metal oxides (V, Cr, Mn, Fe, Co, Ni, Cu, Zn, In, individually or combined).[18–23] Other inherent advantages of this method were nanoparticles deposited; feasibility to adjust the chemical and physical properties of obtained oxide films by experimental parameters, and easy application to various kinds of substrates with large surface area or complex morphology.[24–30] Finally, based upon the result that TiO_2 thin film was able to adhere with the SiO_2 substrate tightly via chemical bonds of Si-O-Ti formed during the deposition,[31] we can reasonably suppose that LPD will be successfully applied to prepare TiO_2 film within capillary columns.

Recently, we have prepared TiO_2 nanoparticles (NP)-deposited capillary by LPD to construct in-tube solid phase microextraction devices for successfully analyzing phosphopeptides from tryptic digest of phosphoproteins.[32] The TiO_2 film offers several attractive features, such as easy preparation with low cost, good trapping capability for phosphopeptides, and satisfactory stability. Considering the amphoteric nature of TiO_2 and its wide application in separation science, [4,8–11,33–36] we attempted to further evaluate the potential applicability of the TiO_2 NP-deposited capillary for OTCEC separation of proteins and peptides in this work. Its electroosmotic properties and separation mechanism in phosphate buffers were studied accordingly.

EXPERIMENTAL

Reagents and Materials

Fused silica capillaries with 50 μm i.d. × 365 μm o.d. were obtained from Yongnian Fiber Plant (Hebei, China). Ammonium hexfluorotitanate ($(NH_4)_2TiF_6$), boric acid (H_3BO_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), disodium hydrogen phosphate (Na_2HPO_4), phosphate acid (H_3PO_4), acetic acid (HAc), L-glutamic acid, trihydroxymethylaminomethane (Tris), ammonium bicarbonate (NH_4HCO_3), calcium chloride ($CaCl_2$), and thiourea were all purchased from Shanghai General Chemical Reagent Factory (Shanghai, China) and were of analytical reagent grade. Trypsin was purchased from Amresco (Solon, OH, USA). Bovine serum albumin (BSA), ovalbumin, hemoglobin, and myoglobin were purchased from Sigma (St. Louis, MO, USA). Double distilled water was used for all experiments.

Solution of proteins (BSA 2 mg/mL, ovalbumin 5 mg/mL, hemoglobin 2 mg/mL, myoglobin 1 mg/mL) were prepared in distilled water and stored in the refrigerator at −8°C. Before use, all samples and buffers were filtered through a 0.45 μm cellulose acetate membrane (Shanghai Bio Life Science & Technology, Shanghai, China).

Preparation of the TiO_2 NP-Deposited Capillary

The fused silica capillary was activated at ambient temperature by rinsing sequentially with 1 M NaOH for 2 h, water for 30 min, 1 M HCl for 4 h, and water for 2 h. After being connected to the nitrogen regulator, the capillary was placed in an oven at 160°C and purged with nitrogen for 10 h.

The TiO_2 NP-deposited capillary was prepared following the procedures described earlier.[32] Briefly, $(NH_4)TiF_6$ and H_3BO_3 were dissolved in a solution at concentrations of 0.1 and 0.3 M, respectively, and then mixed uniformly. Subsequently, the activated capillary was filled with the solution, sealed at both ends with silicone rubber, and then incubated in a thermostat water bath controlled at 35 ± 1°C for 16 h for LPD. Thereafter, the capillary was washed with water, dried at 120°C under a constant nitrogen flow, and then washed with 0.1 M NaOH and water in sequence. Finally, the calcination of the TiO_2 NP-deposited capillary was performed by heating at a rate of 1°C/min to 300°C and holding for 2 h to age the TiO_2 film.

Instrumental and OTCEC Conditions

The X-ray photoelectron spectroscopy (XPS) experiment was performed on a quartz plate and experienced the same LPD procedures as the TiO_2 NP-deposited capillary using an XSAM800 X-ray photoelectron spectroscope (Kratos, Manchester, UK), with Mg Kα radiation as the exciting source.

All the electrochromatographic experiments were performed on a CAPEL 105 CE System (Lumex, St. Petersburg, Russia) equipped with a UV-Vis detector at ambient temperature. Data collection and manipulation were carried out using Chrom&Spec 1.5 software for chromatography (Ampersand). The effective length of all capillaries used was 51 cm with total length of 60 cm. Thiourea was used as the EOF marker. Before analysis, a bare fused silica capillary was rinsed sequentially with 1 M NaOH (15 min), water (15 min), 1 M HCl (15 min), and water (15 min), followed by conditioning with buffer for 15 min; the TiO_2 NP-deposited capillary was directly preconditioned with the running buffer. Between runs, it was rinsed successively with 0.01 M NaOH, water, and buffer

at 2 min intervals. The UV absorbance detection was set at 220 nm. The OTCEC system was operated using "normal" polarity (the cathode was located on the detector side). The samples were injected by the hydrodynamic mode.

Preparation of Peptide Sample

The peptide mixture was derived from the tryptic digestion of myoglobin according to the procedure reported by Yu et al.,[37] with a little modification. Trypsin (0.5 mg) was dissolved in 1 mL 10 mM NH_4HCO_3 buffer (pH 8) containing 0.1 mM $CaCl_2$, which was then mixed with myoglobin at an enzyme to substrate ratio of 1:10 (w/w). The mixture was incubated at 37°C for 5 h and then digestion was stopped by the addition of 0.1 M acetic acid, pH 4.0. The proteolytic digest mixture was finally injected into the OTCEC separation system after filtration.

RESULTS AND DISCUSSION

Surface Chemistry Properties of the TiO_2 Film Deposited within the Capillary

In general, the surface chemistry of TiO_2 is very complicated. The heterogeneity of the surface mainly arises from the presence of Ti^{4+} sites (Lewis acid sites) and two kinds of hydroxyls.[38] As described in our previous work,[32] the TiO_2 film deposited on the inner surface of the capillary by LPD was ascertained to be in a nanocrystalline form of anatase by the results of scanning electron microscopy (SEM) and X-ray diffraction (XRD). Furthermore, the XPS experiment confirmed the successful removal of F^- ions adsorbed on the film and the existence of two kinds of hydroxyls. Figure 1a shows the XPS spectrum of the TiO_2 film in the range of 0–800 eV, on which no peaks attributed to F were observed; Figure 1b shows a high resolution XPS spectrum of the O_{1s} region, which can be decomposed into two contributions.[29] The main contribution is attributed to the Ti-O in TiO_2, which could form a bridged OH group in buffer solution, and the other is attributed to the O-H in Ti-OH, which is called terminal OH group.

The EOF in Phosphate Buffer

In order to further understand the surface property of the TiO_2 NP-deposited capillary, its EOF behavior was studied within the pH

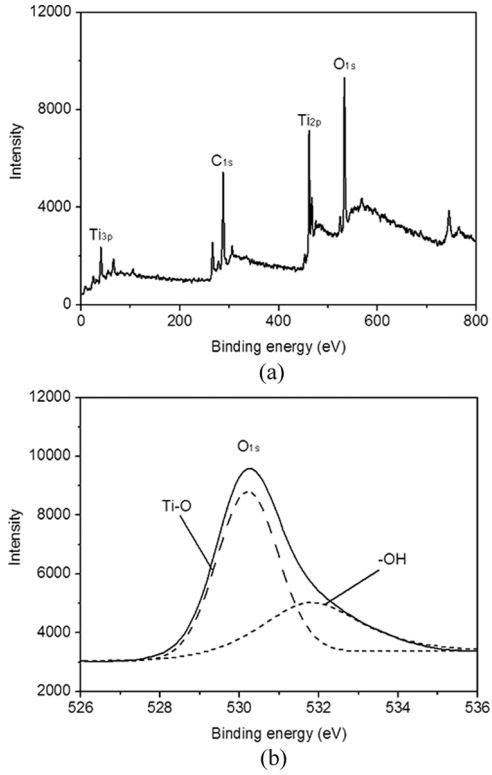

Figure 1. XPS spectrum of the TiO_2 film on a quartz plate (a) and the high resolution XPS spectrum of the O_{1s} region (b).

range of 2–10 in comparison with that of the bare fused silica capillary in phosphate buffer. As shown in Figure 2, the direction of EOF for the TiO_2 NP-deposited capillary is the same as that for the bare fused silica capillary, but an evidently smaller EOF is available at basic pH, which approximately levels off in the pH range of 7–10. This is consistent with the result obtained from a sol-gel based TiO_2 NP-coated capillary.[10,11] The significant suppression of EOF at pH$\geqq$7 indicates an efficient coverage of the capillary surface by TiO_2 film via LPD. Moreover, the constant cathodic EOF at pH less than the p*I* of TiO_2 (about 4–6) is likely ascribed to the preferential adsorption of phosphate ions from the buffer solution onto the surface of TiO_2. One reason is: the $H_2PO_4^-$ ions would adsorb onto the Ti^{4+} sites (existing in the form of $>Ti-OH_2^+$, >Ti-OH, $>Ti_2$-OH, and $>Ti_2$-O^-) via Lewis acid-base interaction. Furthermore, titania had anion exchange properties at acidic pH, the terminal OH groups have a tendency to be protonated on which $H_2PO_4^-$ was able to exchange with other negatively charged species.[39,40] Thus, both the adsorbed and

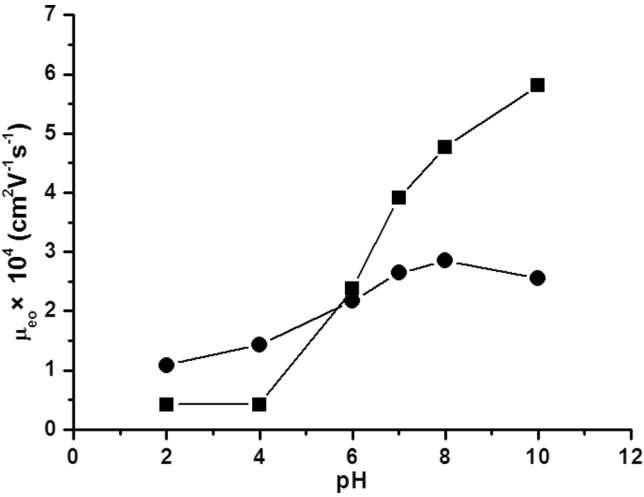

Figure 2. Effect of buffer pH on the EOF. Symbols: (■) bare fused silica capillary; (●) TiO_2 NP deposited-capillary. Column dimension, 60 (51) cm × 50 μm i.d.; marker, thiourea; hydrodynamic injection (30 mbar, 10 s); 40 mM phosphate buffer; voltage, +15 kV; detection, 220 nm.

exchanged phosphate ions make the titanium dioxide surface, as a whole, possessing a negative net charge and exhibiting a cathodic EOF.

Reproducibility

The reproducibility for the TiO_2 NP-deposited capillaries obtained by the LPD method was investigated by measurements of EOF. Capillaries from column to column and batch to batch underwent EOF measurement using 40 mM phosphate buffer within the pH range of 7–9 as buffer solution, the relative standard deviation (RSD) values of which were less than 12.0%. Stability of the TiO_2 NP-deposited capillary was also examined. Reproducibility tests were performed to determine both the intraday and interday variation in EOF mobility, the RSD values of which are lower than 2.7% and 8.8%, respectively. Considering the small dimension of the capillary columns, the variation is reasonable and can be accepted.

OTCEC Separation of Proteins

BSA (MW 66000, p*I* 4.7), ovalbumin (MW 45000, p*I* 4.7), hemoglobin (MW 64500, p*I* 6.8–7.0), and myoglobin (MW 17000, p*I* 7.4) were used as model analytes to evaluate the feasibility of protein separation on

the TiO_2 NP-deposited capillary in OTCEC mode. In order to understand the separation mechanism, as well as achieve the best separation efficiency, effects of several parameters on the separation, including the pH value, composition, and concentration of the background electrolyte (BGE), were investigated.

pH value of the BGE, which influences the charge properties of proteins and the inner surface of capillaries, relates closely to the migration time and the resolution of analytes. On the TiO_2 NP-deposited capillary using 40 mM phosphate buffer as BGE, no peaks of myoglobin and hemoglobin were observed at pH 6 within 2 h, complete resolution of four proteins was not achieved at pH 7 or 8, and the migration time of BSA and ovalbumin were both more than 60 min with significant peak broadening at pH 10. As shown in Figure 3a, when the pH value was adjusted to 9, four proteins are baseline separated and five peaks of

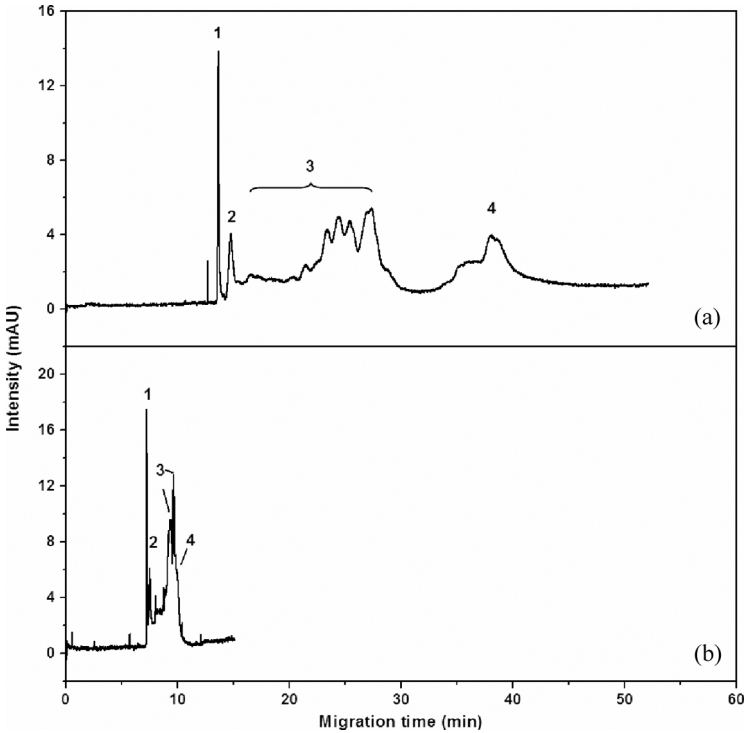

Figure 3. Separation of proteins at (a) TiO_2 NP deposited-capillary and (b) bare fused silica capillary. Column dimension: 60 (51) cm × 50 μm i.d. Hydrodynamic injection: (a) 30 mbar, 10 s; (b) 30 mbar, 5 s. BGE: 40 mM phosphate buffer at pH 9. Voltage, +15 kV; detection, 220 nm. Peak identification: (1) myoglobin; (2) hemoglobin; (3) OVA (glycoisoforms of OVA); (4) BSA.

ovalbumin (caused by an array of glycoisoforms, both with respect to sites of glycosyl chain attachment onto the peptide backbone as well as the sugar chains themselves[41]) were observed. A similar experiment had been performed on another TiO_2 NP-coated capillary prepared by the sol-gel method in a previous reported work,[11] on which the migration time of BSA and ovalbumin were much shorter than those on the TiO_2 NP-deposited capillary. Considering their electrophoretic behavior is almost the same on both capillaries, the explanation might be based on the fact that the TiO_2 NP-deposited capillary provided a larger ratio of the volume of stationary phase to that of the mobile phase, which consequently results in a greater capacity factor, and thus a longer retention time according to the chromatographic mechanism. Additionally, we also compared the TiO_2 NP-deposited capillary with a bare fused silica capillary under the same conditions, on which four proteins migrates quickly throughout the capillary (within about 10 min) without sufficient

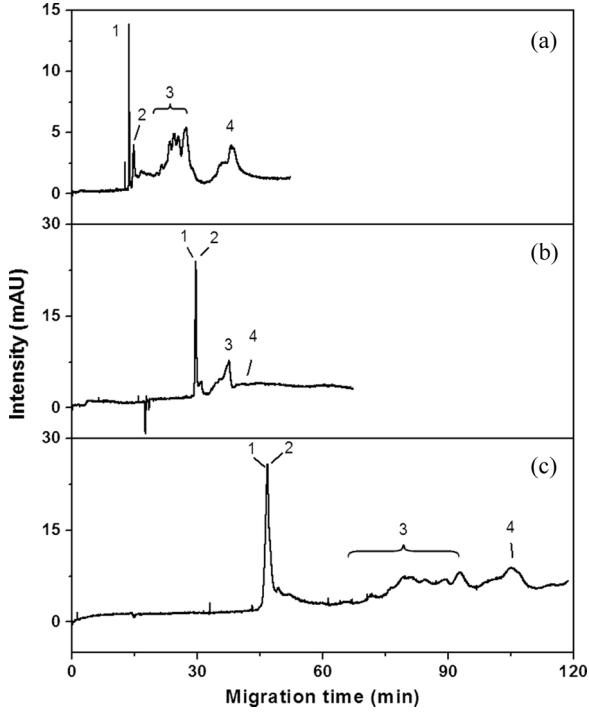

Figure 4. Separation of proteins using different types of BGE at pH 9. BGE (40 mM): (a) phosphate buffer; (b) glutamate buffer; (c) Tris-HAc buffer. Hydrodynamic injection: (a) 30 mbar, 10 s; (b, c) 30 mbar, 20 s. Other conditions are the same as those in Figure 3.

resolution (Figure 3b). Hence, it can be concluded that the TiO_2 film is effective to improve the selectivity, mainly resulting from the ligand exchange interaction between analytes and the phosphate adsorbed on TiO_2.

The ability of TiO_2 to ligand exchange originates from both the presence of Lewis acid sites (i.e., coordinative unsaturated Ti^{4+}) on the surface and the presence of water molecules and other easily displaced ligands coordinatively bonded to the sites.[42] The Lewis acid sites are able to coordinatively bond with water or some Lewis base molecules, which can be exchanged for others; the harder the base the easier the exchange. Thus, different types of BGE of the same concentration at pH 9 were introduced to further understand the contribution of TiO_2 film in separation, the Lewis basicity of which is as follows: phosphate >glutamate >acetate >Tris. The electrochromatograms are illustrated in Figure 4. Under this condition, proteins were all negatively charged. Competition for the coordinatively bonding sites consequently occurred between them and the anions of the buffer solution. As shown in

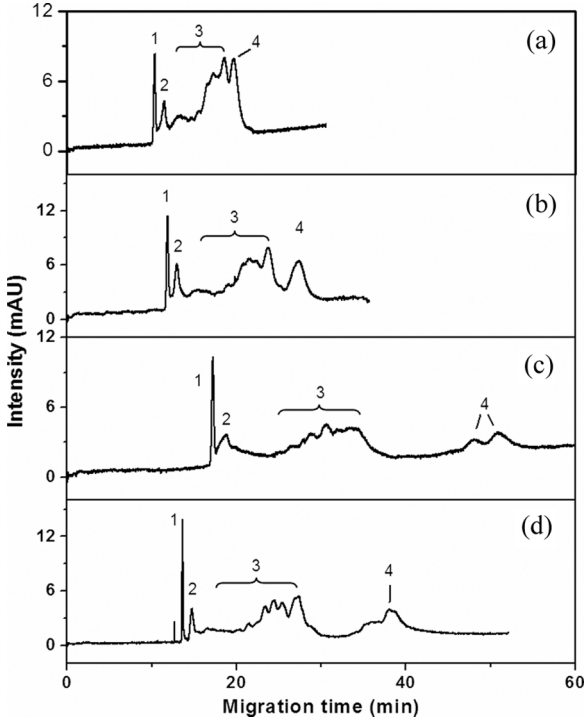

Figure 5. Separation of proteins at different concentrations of phosphate buffer of pH 9. Concentration: (a) 10 mM; (b) 20 mM; (c) 30 mM; (d) 40 mM. Hydrodynamic injection, 30 mbar, 10 s. Other conditions are the same as those in Figure 3.

Figure 4a, much better separation was achieved using phosphate buffer; myoglobin and hemoglobin were completely separated. When using glutamate and Tris-HAc as BGE, the smaller EOF, as well as the weaker affinity toward the ligand exchange sites, cooperatively resulted in longer analysis time and broadening of peaks (shown in Figure 4b and c).

Another evidence of the ligand exchange mechanism is obtained by the investigation of the effect of phosphate concentration on separation (Figure 5). When the buffer concentration increased from 10 mM to 30 mM, longer migration time of the proteins was observed according to the decrease in EOF mobility from $3.6 \times 10^{-4}\,cm^2\ V^{-1}\ s^{-1}$ to $2.9 \times 10^{-4}\,cm^2\ V^{-1}\ s^{-1}$. Interestingly, when the buffer concentration increased from 30 mM to 40 mM, the EOF mobility remained constant, however, the proteins migrated more quickly at higher concentrations of phosphate. Since there is a competition between phosphate ions and the analytes binding onto the surface of TiO_2, the equilibrium shift caused by higher concentration of phosphate might be a reasonable explanation.

Separation of Tryptic Digested Samples

Our experiments described above have illustrated that the TiO_2 film deposited on the inner surface of capillary could be used as an alternative means to manipulate the EOF and improve the separation efficiency. Consequently, we further attempted to apply the TiO_2 NP-deposited

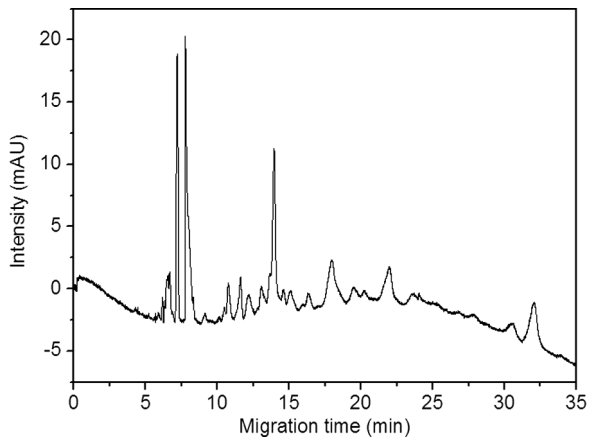

Figure 6. Separation of tryptic peptides of myoglobin on TiO_2 NP deposited-capillary. Column dimension, 60 (51) cm × 50 μm i.d.; hydrodynamic injection (30 mbar, 30 s); phosphate buffer (40 mM, pH 8); voltage, +23 kV; detection, 220 nm.

capillary to the separation of a more complex biological mixture, i.e., the tryptic digest of proteins. Proteolytic digest mixture was obtained according to a previously reported procedure[37] using trypsin as enzyme and myoglobin as substrate. A great number of peaks were detected when employing 40 mM pH 8 phosphate buffer as BGE at a voltage of 23 kV (shown in Figure 6). It is not our goal to identify all these peaks in this study; our result, however, has demonstrated the potential use of this column for biological analysis because of its favorable separation abilities.

CONCLUSIONS

In this paper, LPD was approved once again to be a simple and low cost technique to prepare metal oxide film within the capillary with satisfactory reproducibility and stability. Two kinds of hydroxyls (i.e., bridged OH and terminal OH), which relates closely to the electroosmotic properties of the capillary, was confirmed to be present on the surface of the resultant TiO_2 film by an XPS experiment. With the phosphate buffer, the direction of EOF for the TiO_2 NP-deposited capillary remained cathodic constantly within the pH range of 2–10, but the magnitude of EOF was much smaller than that of the bare fused silica capillary at $pH \geqq 7$. Its potential use for OTCEC separation of proteins was also described. This example clearly demonstrated that the deposited TiO_2 film can enable separations, which would not be feasible on the bare fused silica capillary under the same conditions owing to the ligand exchange interaction between analytes and the phosphate adsorbed on the TiO_2. Finally, the attempt of applying the TiO_2 NP-deposited capillary to the separation of proteolytic digest mixture indicated its feasibility in biological analysis, but there remains much to be understood before it can be used routinely.

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