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One-step coating of silica capillaries for selective protein retention by Cu(II)-IDA IMAC

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ABSTRACT

A simple protocol to obtain Cu(II)-IDA (iminodiacetic acid)-modified capillaries was developed for immobilized metal ion affinity chromatography (IMAC). It consisted in the synthesis of IDA-silane used for a one-step coating of fused silica capillaries. The approach prevented the hydrolysis of silica potentially induced by two step coatings (γ-GPTMS, then IDA) employed in the conventional method of bonding iminodiacetic acid. The IDA content was quantified using a model relating the electroosmotic flow generated in IDA-modified capillaries to the charges induced by IDA species. The retention behavior of holotransferrin and bovine serum albumin on these IMAC columns was then investigated. Holotransferrin revealed a high affinity for Cu(II)-supports through a specific interaction with Cu(II) ions whereas albumin did not show any retention. The use of such columns for sample pretreatment before an HPLC analysis was proved to be successful.

1. Introduction

Since its introduction by Porath et al. in 1975, immobilized metal ion affinity chromatography (IMAC) has developed into a robust and versatile analytical tool [1]. Its use has been rapidly increasing in protein chemistry and includes many applications such as protein and peptide purification and selective enrichment or protein characterization as reviewed by Gaberc-Porekar and Menart [2] and Chaga [3]. More recently, several research groups reported the use of silica capillaries as supports to create miniaturized devices capable of either separating or concentrating proteins by IMAC. The columns were either packed with particles [4], used as monolithic blocks [5–7] synthesized *in situ* or as coated capillaries [8–11].

Columns packed with commercial beads offer the advantage of well-defined supports. However, the time-consuming packing process, the high pressure drop generated as well as the difficulties encountered in frit formation constitute severe limitations of particle packed columns for IMAC. To overcome these problems, alternatives, such as the use of coated capillaries were considered. Various protocols leading to the immobilization of IDA through the use of small organic spacers, e.g. γ-glycidoxypropyltrimethoxysilane, were proposed [8,9].

Little attention has been paid to the characterization of the coating technology. The reason could be the minute amounts of stationary phase in such miniaturized systems. The modification of the capillary surface was monitored either by electroosmotic flow measurements [8] or by determination of the activity of an enzyme immobilized on the surface through interactions with the metal [9]. Other attempts to characterize IMAC phases synthesized directly in capillaries were also reported. In most cases, the presence of IDA on the capillary surface was indirectly confirmed after Cu(II) immobilization. This was done by the measurement of the electroosmotic flow [6,8], visual observation of a color attributed to Cu(II) [5], quantification of Cu(II) after releasing of the ion [6,11] or even by comparison of the retention of some peptides in the absence and in the presence of metal [5]. Chuang et al. investigated the functionalization of a polystyrene monolithic layer by IDA in its ester form and monitored the degree of hydrolysis of the latter using electroosmotic flow measurements [7].

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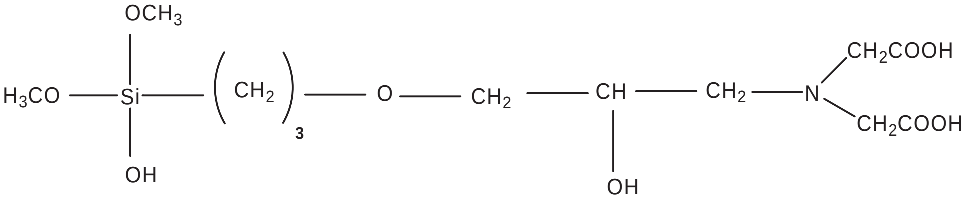

Fig. 1. Compound **1**: IDA-silane.

The goal of this work was to develop a simple procedure for a one-step coating to produce iminodiacetic acid modified capillaries allowing Cu(II) immobilization. A method based on electroosmotic mobility measurements, was proposed for the evaluation of the IDA content. Preliminary results of the behavior of two model-proteins (holotransferrin and serum albumin) on such IMAC capillaries were shown for sample pretreatment before HPLC.

2. Materials and methods

2.1. Chemicals and reagents

The reagents used were of analytical grade. Methanol (Carlo Erba, Val de Reuil, France) and sodium hydroxide (Merck, Frankfurt, Germany) were used for the capillary conditioning. Ultra pure water, obtained from a Milli-Q system (Millipore, Billerica, MA) was used for the preparation of all the solutions. Benzyl alcohol and sodium ethylenediaminetetraacetate Na_2EDTA (>99%) were purchased from Merck. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA) and dissolved in water for the preparation of the running buffer at the concentration of 25 mM at pH 7.4. Phosphate solutions were prepared from $Na_2HPO_4{\cdot}12H_2O$ (Merck), $NaH_2PO_4{\cdot}2H_2O$ (Prolabo, VWR, Radnor, PA) and phosphoric acid (H_3PO_4 85%, Carlo Erba) mixed in different proportions to obtain the desired pH. All the buffer solutions were freshly prepared and filtered using 0.45 μm filters (Analypore, Thermo Fisher Scientific, Waltham, MA).

γ-Glycidoxypropyltrimethoxysilane (γ-GPTMS 98%) used in the synthesis was purchased from Aldrich (Sigma–Aldrich). Iminodiacetic acid (97%), copper (II) nitrate and sodium nitrate (>99%) were obtained from Fluka. Human holotransferrin (>98%) and bovine serum albumin (>96%) were purchased from Sigma (Sigma–Aldrich). They were prepared in the migration buffer (HEPES 25 mM, pH 7.4). Acetonitrile (99.9%, HPLC gradient quality) and trifluoroacetic acid TFA (98%) were purchased from Sigma–Aldrich.

2.2. Instrumentation

^1H NMR (300 MHz) spectra were recorded with a Bruker Avance (Billerica, MA) spectrometer in the absence of an internal standard at 25 °C. Samples were prepared in D_2O. IR analysis was performed on a Perkin-Elmer (Waltham, MA) IR spectrophotometer. ESI-MS measurements were performed on an ESI-TOF (LCT, Waters, Vernon Hills, Il) with a cone voltage of 35 V. The sample prepared in water was infused with a 40 mL/h flow rate.

All capillary electrophoresis experiments were carried out on a Beckman Coulter P/ACE MDQ (Brea, CA) capillary electrophoresis instrument using the 32 Karat software (Beckman Coulter). Untreated fused silica capillaries (30 cm × 75 μm i.d., 375 μm o.d.) were purchased from Thermo Fisher Scientific. HPLC was performed with a Waters (IET) system consisting of a pump model 626, a work station model 6005, an UV detector model 486 and a LC-Packings (Dionex, Sunnyvale, CA) splitter. Proteins eluted from the IMAC capillary were injected in the HPLC system via a Rheodyne (Cotati, CA) injection valve model 7125 equipped with a 5 μL PEEK injection loop (Upchurch Sci., IDEX, Oak Harbor, WA). Separations were performed in a 15 cm × 300 μm i.d. C_4 column (Dionex). UV detection was performed at 214 nm.

2.3. Synthesis of IDA-silane

IDA (8.30 g or 60 mmol) was dissolved in 40 mL of water. pH was adjusted to 8 by the addition of 8 M NaOH. The flask containing the solution was placed in an ice bath to drop the temperature to 0 °C. Then, 6.7 mL (30 mmol) of γ-GPTMS were gradually added under stirring. Then, the temperature was raised to 65 °C and the reaction mixture was left to react for 20 h. At the end of the reaction, the solvent was evaporated. Compound **1** was recovered as a white solid and stored at 4 °C (Fig. 1). Analytical data for compound **1**: ^1H NMR (δ, ppm) 4.16 (t, 1H, J = 6.5 Hz, $-CH_2CH(OH)CH_2-$), 3.77 (m, 4H, $-CH_2OCH_2-$), 3.57 (s, 6H, $-SiOCH_3$), 3.49 (s, 4H, $-NCH_2COOH$), 3.26 (d, 2H, J = 6.6 Hz, $-CHCH_2N-$), 1.65 (m, 2H, $-SiCH_2CH_2-$), 0.64 (t, 2H, J = 6.5 Hz, $-SiCH_2-$). IR (infrared) (ν, cm^{-1}): 3300 (–SiOH); 2940 ($-CH_2-$); 1600 ($-COO^--$); 1095 (–C–O); 104 (–Si–O–C). MS (ESI): m/z = 355.13 (M^+).

For the sake of comparison, IDA-silanes were also synthesized according to two literature procedures: according to Guo et al. [9] and Anspach [12].

According to Guo et al. [9], 1.48 g (11 mmol) of IDA was dissolved in 17 mL of water and pH was adjusted to 11 by the addition of 8 M NaOH. The solution was then cooled down to 0 °C. 500 μL (2.3 mmol) of γ-GPTMS was slowly added under stirring. The solution was then heated at 65 °C for 6 h under stirring and placed into an ice bath to decrease the temperature to 0 °C again. Two consecutive additions of 500 μL γ-GPTMS were performed using the same procedure and the mixture was finally left to react overnight at 65 °C under stirring. pH of the solution was then adjusted to 6 with 10 M HCl. After evaporation of the solvent, compound **2** was recovered as a white solid and stored at 4 °C. Analytical data for compound **2**: ^1H NMR (δ, ppm) 4.17 (t, 1H, J = 6.5 Hz, $-CH_2CH(OH)CH_2-$), 3.79 (m, 4H, $-CH_2OCH_2-$), 3.59 (s, 3H, $-SiOCH_3$), 3.51 (s, 4H, $-NCH_2COOH$), 3.28 (d, 2H, J = 6.6 Hz, $-CHCH_2N-$), 1.68 (m, 2H, $-SiCH_2CH_2-$), 0.65 (t, 2H, J = 6.5 Hz, $-SiCH_2-$).

According to Anspach [12], 2.91 g of IDA (21 mmol) was dissolved in 23 mL of water and pH was adjusted to 11 by the addition of sodium hydroxide pellets. The temperature was then dropped to 0 °C by placing the mixture in an ice bath and 5 g (21 mmol) of γ-GPTMS was slowly added. The reaction mixture was allowed to warm up to room temperature and maintained at this temperature for 4 h. Subsequently, the temperature was raised to 65 °C and the reaction mixture was stirred overnight. Finally, the pH of the solution was adjusted to 7 with 10 M HCl. After evaporation of the solvent, compound **3** was recovered as a white solid and stored at 4 °C. Analytical data for compound **3**: ^1H NMR (δ, ppm) 4.23 (t, 1H, J = 6.5 Hz, $-CH_2CH(OH)CH_2-$), 3.79 (m, 4H, $-CH_2OCH_2-$), 3.68 (s, 1H, $-SiOCH_3$), 3.6 (s, 4H, $-NCH_2COOH$), 3.27 (d, 2H, J = 6.6 Hz, $-CHCH_2N-$), 1.77 (m, 2H, $-SiCH_2CH_2-$), 0.75 (t, 2H, $J = 6.5$ Hz, –SiCH_2–). Note that no gelation (as a result of possible polymerization of silanols formed by the methoxy groups hydrolysis) was observed during the synthesis of compounds **1**–**3**.

2.4. Preparation of Cu(II)-IDA-modified capillaries

Capillaries were activated with 0.1 M NaOH for 1 h, and then filled with IDA-silane solution (compound **1**, 16%, w/w, pH 8) during 15 min under a 400 kPa nitrogen flow. Afterwards, they were sealed at both ends and heated at 100 °C for 2 h. Finally, they were flushed with water for 2 min under a 400 kPa nitrogen flow to remove the excess of the unreacted silane.

A 20 mM Cu(II) solution was prepared by dissolving Cu(II) nitrate in a 25 mM solution of HEPES, pH 5. This solution was passed through the IDA-modified capillary for 20 min under 138 kPa. The capillary was then washed using HEPES 25 mM, pH 7.4 to remove the excess of Cu(II). IDA-modified capillaries were also prepared using compounds **2** and **3**. However they showed very poor capacity of immobilization of Cu(II).

2.5. Capillary electrophoresis measurements

Prior to the first use, a silica capillary was successively rinsed with methanol for 5 min (138 kPa), water for 2 min (138 kPa), 1 M NaOH for 5 min (138 kPa) and water for 3 min (138 kPa). Prior to the runs, coated and uncoated capillaries were equilibrated with the running buffer by a 15 min rinse under 138 kPa. For the coating characterization, hydrodynamic injections of benzyl alcohol (0.05%, v/v) at 1.38 kPa for 5 s, corresponding to 1.5% of the capillary volume (20 nL), were performed and migrations were carried out in phosphate buffers at different pHs at 12 kV and 25 °C. In all other experiments, runs were performed in a 25 mM HEPES buffer at pH 7.4 under a constant voltage of 15 kV and at 25 °C. Electroosmotic mobility measurements were performed by hydrodynamic injections of water (at 1.38 kPa for 5 s). Proteins were hydrodynamically injected at 3.45 kPa for 5 s, corresponding to 3.75% of the capillary volume (50 nL).

Detection was performed by UV at 200 nm. For this purpose, a detection window was made at 20 cm from the injection end by removing the polyimide coating using a heated wire (Innova Tech).

2.6. HPLC separation of proteins

The optimized gradient consisted of isocratic elution for 10 min (water/acetonitrile, 80/20 containing 0.1% trifluoroacetic acid) followed by a 0.36% acetonitrile/min slope gradient elution. Holotransferrin standard solutions used contained 50, 80, 100 or 125 ng of holotransferrin in 100 mM EDTA.

3. Results an discussion

3.1. Synthesis and characterization of IDA-silane

IDA coating onto silica was often reported as a two-step procedure involving first a coating of γ-GPTMS [8,13,14]. However, the alkaline medium required for the nucleophilic substitution by IDA leads to the risk of hydrolysis of a considerable amount of siloxane bonds. To prevent it, IDA-silane was synthesized prior to coating. However, hydrolysis of the methoxysilane groups which will be later involved in the coating can still occur. Therefore, different syntheses were investigated using different pHs (8 and 11) and stoichiometries IDA:γ-GPTMS (varying from 1:1 to 2:1) in order to minimize the hydrolysis.

The presence of methoxysilanes was revealed by a singlet around 3.6 ppm in the ^{1}H NMR spectra. These groups were quantified using the intensity of Si$C\mathbf{H}_2$ groups as reference. The results, summarized in Table 1, show that the use of a pH of 11 leads to a significant hydrolysis of methoxysilanes. It could also be decreased by using an excess of IDA. As a consequence, the reaction performed at a lower alkaline pH in the presence of an excess of IDA was adopted (compound **1**). Therefore, IR spectra for compounds **2** and **3** were not given since they were not eligible for the use in the coating (not enough methoxy groups). In the case of compound **1**, the ^{1}H NMR spectrum indicated that only one methoxysilane was hydrolyzed. It was confirmed by mass spectrometry. Indeed, the monoisotopic mass of compound **1** was found to be 355.13 Da (expected value for $[M+H]^+$ 356.13 Da). This might be explained by an oxidation process which can occur in the ESI source and lead to M^+ through the loss of one electron as already reported [15,16].

Table 1
Influence of the conditions of IDA-silane synthesis on the percentage of hydrolyzed methoxysilanes.

Compound	Molar ratio (spacer:ligand)	pH	Hydrolyzed $-OCH_3$ (%)
1	1:2	8	33
2	1:1.6	11	67
3	1:1	11	89

The absence of bands corresponding to the epoxide, i.e. –C–O– and –C–H– vibrations (1240–1260 and 2990–3050 cm^{-1}) was also noted in the IR spectrum, which confirms the transformation of the γ-GPTMS into the IDA-silane. The similarity of the ^{1}H NMR spectra shows that this compound can be stored in solid form at 4 °C for at least a year.

3.2. Coating of the capillary

Coating experiments were performed on ten different capillaries using compound **1**. The efficiency of the procedure was evaluated by measurements of the electroosmotic flow at pH 7.4 in both unmodified and IDA-modified capillaries. The electroosmotic mobility was calculated using the following equation:

$$\mu_{eo} = \frac{L \times l}{t_{eo} \times V} \quad (1)$$

where L is the total length of the capillary, l the effective length of the capillary, t_{eo} the migration time of the neutral marker and V the applied voltage.

In comparison with unmodified capillaries, an average decrease of 10% in the electroosmotic mobility of IDA-modified capillaries was observed. The relative standard deviation, less than 4%, indicated the good reproducibility of the procedure.

Capillary electrophoresis experiments were realized to estimate the percentages of IDA and silanols species in the capillaries. Indeed, the surface charge density of the supports varies with the degree of dissociation of functional groups on the surface as a function of pH of the electrolyte. According to Hayes et al. and Kitagawa and Tsuda [17–19], the electroosmotic velocity (ν_{eo}) is related to the total surface charge density regardless of its source, as expressed as:

$$\sinh(\nu_{eo}) = \sigma_t C^{-1} \quad (2)$$

where σ_t is the surface charge density and C is a constant since the applied voltage was constant in our experiments.

Tsuda and co-workers [20] exploited this relationship to determine pK_a of charged coatings using electroosmotic flow variations. According to them, the surface charge density in silica unmodified capillaries can be expressed as follows:

$$\sigma_t = \sigma_n + \sigma_{\mathrm{SiOH}} \quad (3)$$

where σ_n is the naturally induced surface charge density and σ_{SiOH} is the charge induced by all the residual silanols.

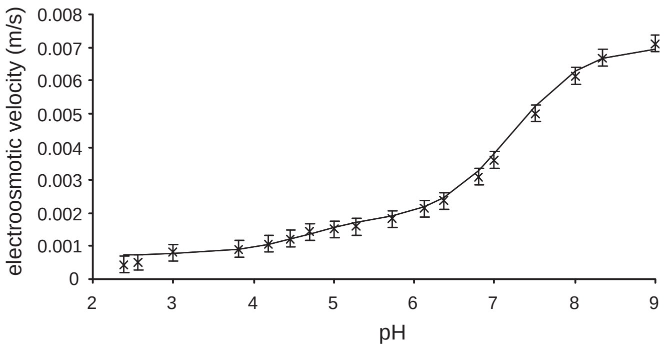

Fig. 2. Dependence of electroosmotic velocity (benzyl alcohol as neutral marker) on pH for uncoated capillaries. Experimental and theoretical velocities are presented as dotted and continuous lines, respectively. Conditions: fused silica capillary: 30 cm (effective length 20 cm) × 75 μm i.d. fused silica, applied voltage: 12 kV, electrolyte: 10 mM Na^+ phosphate buffer, T = 25 °C, detection: UV at 200 nm, sample injection: 1.38 kPa, 5 s.

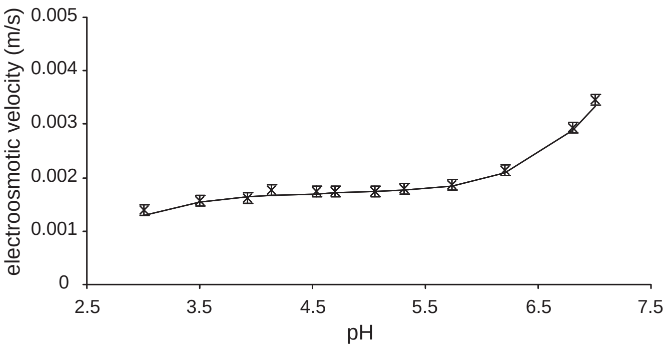

Fig. 3. Dependence of electroosmotic velocity (benzyl alcohol as neutral marker) on pH for IDA-modified capillaries. Experimental and theoretical velocities are presented as dotted and continuous lines, respectively. IDA-modified fused silica capillary. Conditions as in Fig. 1.

Therefore σ_t can be expressed as:

$$\sigma_t = \sigma_n + e[\mathrm{SiOH}]_1 + e[\mathrm{SiOH}]_2 \qquad (4)$$

where e, $[\mathrm{SiOH}]_1$ and $[\mathrm{SiOH}]_2$ are the elementary charge and the concentrations of the two types of deprotonated silanols, respectively.

The experimental electroosmotic velocities were measured in phosphate buffers at different pHs ranging from 2.4 to 8.9 for the uncoated silica capillary. They were corrected to obtain similar ionic strengths. Calculations of the theoretical electroosmotic mobilities were performed using the Sigma Plot software (version 8.0) with 0.00001 and 100 as tolerance and number of iterations, respectively. Fig. 2 presents the plot of both experimental and theoretical electroosmotic velocities as a function of pH.

It can be clearly seen that the fused silica presents two types of silanols, named SiOH_1 and SiOH_2 with two different dissociation constants, $K_{(\mathrm{SiOH})_1}$ and $K_{(\mathrm{SiOH})_2}$, respectively. These two silanol types were already described by Ong et al. [21] as being acidic and basic silanols corresponding respectively to isolated ($\mathrm{pK(SiOH)}_1 = 4.9$) and vicinal silanols ($\mathrm{pK(SiOH)}_2 = 8.5$). A successful modeling of the electroosmotic velocity was obtained for $\mathrm{pK(SiOH)}_1 = 4.6 \pm 0.1$ and $\mathrm{pK(SiOH)}_2 = 7.2 \pm 0.2$, with a correlation coefficient of 0.998. Moreover, the modeling allowed the calculation of the percentages of the two types of silanols. Therefore, an uncoated capillary presented 17 ± 3% of acidic silanols and 83 ± 2% of basic silanols. These results are in agreement with the percentages of silanols calculated by Ong et al. in planar fused silica. They found 19% for acidic silanols and 81% for basic ones [21]. Fan et al. [22] calculated the relative surface densities of silica ($S_{\mathrm{SiOH}_1} = 7.4 \times 10^{12}\ \mathrm{cm}^{-2}$; $S_{\mathrm{SiOH}_2} = 3.2 \times 10^{13}\ \mathrm{cm}^{-2}$) which lead to the same percentages.

In IDA-modified capillaries, Eq. (3) becomes:

$$\sigma_t = \sigma_n + \sigma_{\mathrm{SiOH}} + \sigma_{\mathrm{IDA}} \qquad (5)$$

where σ_{IDA} is the charge induced by all the charged forms of IDA and Eq. (2) becomes:

$$\sinh(\nu_{eo}) = (\sigma_n + \sigma_{\mathrm{SiOH}} + \sigma_{\mathrm{IDA}})C^{-1} \qquad (6)$$

As for the uncoated capillaries, experiments were carried out to measure the electroosmotic velocities in electrolyte pHs ranging from 3.3 to 7.4. The pH range used in the study of IDA-modified capillaries was narrower than that used in the study of uncoated capillaries in order to maintain the coating. The reproducibility of the observed electroosmotic velocities was found to be 1.5% (n = 10).

The experimental electroosmotic velocities were plotted versus pH for the IDA-modified capillary as illustrated in Fig. 3. Note that the influence of pH on the electroosmotic flow in the modified capillaries is less pronounced than that in uncoated ones. Eq. (6) was used to calculate theoretical electroosmotic velocities using a rational regression (Fig. 3). For these calculations, the pKs of the silica were maintained constant (4.6 and 7.2, respectively). This consideration was based on the work of Tsuda and coauthors who found identical dissociation constants for silanols in both unmodified and octadecylsilyl-modified silica gels [20]. This approach helped maintaining reasonable the number of parameters to fit. Correlation coefficients above 0.994 were accepted. The modeling succeeded for $\mathrm{pK}_1 \leq 2.5$; $\mathrm{pK}_2 = 2.8 \pm 0.3$; $\mathrm{pK}_3 \geq 9$. These calculated dissociation constants (pK_1 1.8; pK_2 2.8; pK_3 9 for IDA-modified capillary shown in Fig. 4) were in agreement with those determined elsewhere for IDA by Kolpachnikova et al. (pK_1 1.76; pK_2 2.70; pK_3 9.73) [23] and Zachariou et al. (pK_1 1.79; pK_2 2.55; pK_3 9.26) [24]. Moreover, the modeling led to the following percentages of species: no acidic silanols, 81 ± 4% of basic silanols and 19 ± 5% of IDA species.

3.3. Assessment of the potential of Cu(II)-IDA-modified capillaries for protein retention

The concept of miniaturized immobilized metal affinity columns has been little explored. Tsukagoshi et al. separated HSA and γ-Ig on Cu(II)-IDA-modified capillaries [10]. Cai and El Rassi preconcentrated carbonic anhydrase in Zn(II)-IDA-modified capillaries [8]. In this work, the retention of two proteins, holotransferrin and albumin, was investigated.

Prior to use, the modified capillaries were rinsed with a solution of Cu(II) and the immobilization of Cu^{2+} was monitored by measuring the electroosmotic mobility decrease between IDA-modified and Cu(II)-IDA-modified capillaries. An average decrease of 20%, attributed to a decrease of the negative surface charge, was observed. This process was found to be reproducible as demonstrated by the relative standard deviation calculated from 5 different capillaries, which was found to be less than 5%.

A series of 12 consecutive injections of 0.875 mg/mL holotransferrin followed by a voltage application in capillary electrophoresis was performed on Cu(II)-IDA-modified capillaries. For each injection, the peak area was measured. Since the electroosmotic velocity was found to decrease as injections were performed indicating a change in the capillary surface, migrations times were found to vary. As a consequence, unretained holotransferrin was evaluated from corrected peak areas (area divided by migration time) (Fig. 4). It can be noticed that the corrected surface area increases continuously from an area corresponding to around 5% of the

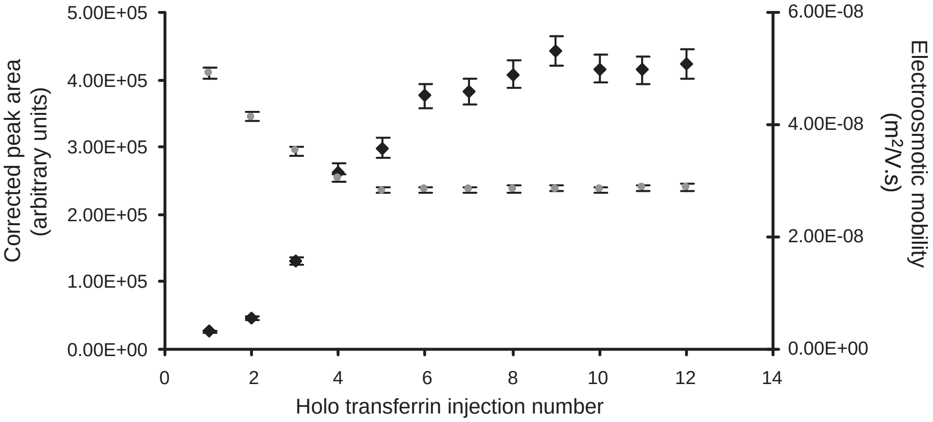

Fig. 4. Variations of eletroosmotic mobility (●) and corrected peak areas (♦) as a function of the number of holotransferrin injections. Conditions: Cu(II)-IDA-modified fused silica capillary: 30 cm (effective length 20 cm) × 75 μm i.d., applied voltage: 15 kV, electrolyte: 25 mM HEPES buffer (pH 7.4), $T = 25\,^{\circ}$C, detection: UV at 200 nm, sample injection: holotransferrin at 0.875 mg/mL, 3.45 kPa, 5 s.

holotransferrin injected to reach, after the tenth injection, a stable corrected peak area corresponding to the totality of the quantity injected. On the contrary, several successive injections of holotransferrin on an IDA-modified capillary before Cu(II) immobilization were found to produce identical electropherograms without any change of corrected peak areas. This behavior reflects a non reversible retention of the holotransferrin on the Cu(II) support.

Substantial washes of the Cu(II)-IDA-modified capillary (380 volumes of the capillary) with a concentrated Na^+ electrolyte (0.1 M $NaNO_3$) did not lead to any release of the protein, which indicates the existence of a non purely electrostatic interaction involving Cu(II). If the interaction IDA-Cu(II) were purely electrostatic, Na^+ would have replaced Cu(II).

In parallel, four consecutive injections of bovine serum albumin were performed in a Cu(II)-IDA-modified capillary. Reversible retention was observed as indicated by constant corrected peak areas equal to those obtained when injecting the same protein in an IDA-modified capillary.

3.4. Protein separation and quantification by HPLC

The potential of the IMAC selection was assessed by implementation in a complete protein analysis procedure. For this purpose, a series of 10 consecutive injections (~50 nL) of a solution of holotransferrin (1 mg/mL) and bovine serum albumin (1 mg/mL) followed by a voltage application by capillary electrophoresis (HEPES 25 mM, pH 7.4) was performed in a Cu(II)-IDA-modified capillary. The capillary was then rinsed with 100 mM EDTA under 138 kPa and the obtained solution was injected and separated in HPLC. The HPLC conditions were previously optimized using a mixture of holotransferrin and bovine serum albumin prepared in 100 mM EDTA. Injections were performed in triplicate and the relative standard deviation of the peak areas was 10%, whereas that of the retention times was 0.4%.

The detection and quantification limits for holotransferrin were 15.8 ng and 50 ng, respectively. Fig. 5 presents the separation of a mixture of holotransferrin and bovine serum albumin before and after a selection step using IMAC. A single peak corresponding to holotransferrin was obtained after injection of the sample in the Cu(II)-IDA-modified capillary. The absence of a peak corresponding to bovine serum albumin shows the capacity of the elaborated supports to select holotransferrin through its specific interaction with Cu(II). Quantification of holotransferrin by HPLC-UV enabled us to determine the loading capacity of these supports equal to 100 ng for a 1.2 μL column.

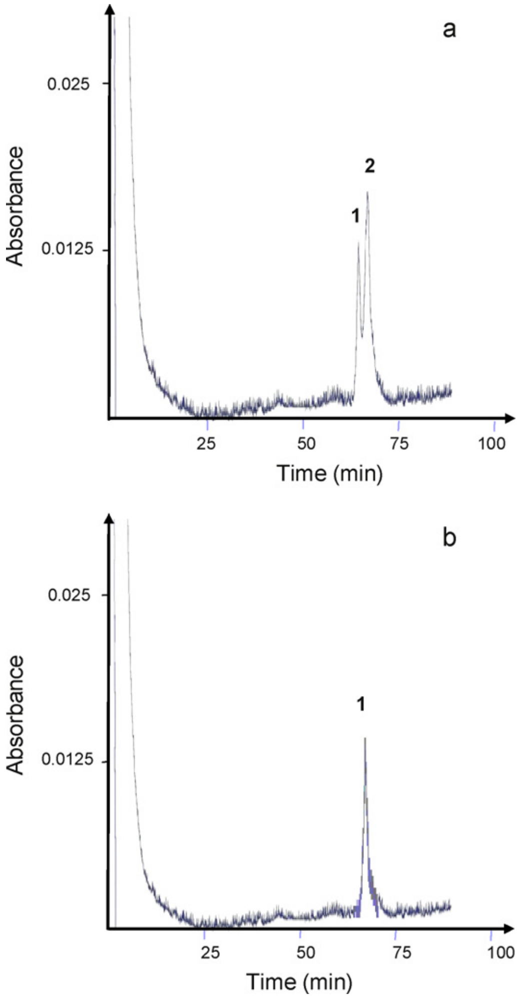

Fig. 5. Chromatograms of solutions containing holotransferrin (1) and bovine serum albumin (2). (a) 5 μL of a protein solution (50 ng each) in 100 mM EDTA and (b) 5 μL (×2) of a protein solution pretreated in an IMAC capillary. Column: C_4 (15 cm × 300 μm i.d.), eluent: water/acetonitrile (0.1% TFA), 25 °C, detection: UV at 214 nm.

4. Conclusion

A simple and reproducible one-step method was proposed to obtain miniaturized IDA-modified supports for IMAC. The percentages of the silanols and IDA functional groups present on the surface were estimated by electroosmotic velocity measurements and subsequent modeling. Experiments carried out on proteins demonstrated the potential of these supports to select Cu(II)-binding proteins such as the holotransferrin. Moreover, the compatibility of IMAC with HPLC was shown through an application to a mixture of holotransferrin and bovine serum albumin.

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