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Short communication

Preparation of an iminodiacetic acid-modified capillary and its performance in capillary liquid chromatography and immobilized metal chelate affinity capillary electrophoresis

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Abstract

We prepared iminodiacetic acid (IDA)-modified and Cu(II)–IDA-modified capillaries through polymerization of *N*-(vinylbenzylimino) diacetic acid. The fundamental performance of these capillaries was examined in capillary liquid chromatography (LC) and immobilized metal chelate affinity capillary electrophoresis (IMACE). Copper(II), cobalt(II), and hematin were detected at different retention times by means of capillary LC with a chemiluminescence detector, during which the IDA-modified capillary was used. The difference in the retention times was attributed to the difference in the interaction between metal ions or complex and IDA moieties on the inner wall of the capillary. In addition, human serum albumin (HSA) and human serum γ-globulin (HγG) were separated and detected using IMACE with an absorption detector, during which the Cu(II)–IDA-modified capillary was used. The separation of HSA and HγG was achieved through the interaction between proteins and Cu(II) chelate moieties on the inner wall of this capillary.

Keywords: Capillary columns; Affinity capillary electrophoresis; Immobilized metal chelate affinity capillary electrophoresis; Albumin; Globulin; Proteins

1. Introduction

Liquid chromatography (LC) is one of the most widespread and useful separation techniques. Immobilized metal chelate affinity chromatography (IMAC) of proteins was introduced in 1975 by Porath et al. [1]. Since then, it has been applied successfully to many kinds of proteins and is becoming a standard tool for the isolation of proteins [2–5]. IMAC has been especially useful for group separation between two classes of proteins with and without affinity for heavy metal ions.

On the other hand, in the 1980s, micro bore liquid chromatography (ca. 1 mm i.d.) was studied in earnest [6,7] in order to save on packing-sorbents, reagents, and samples, all of which are expensive and valuable. Recently, as the technique of capillary electrophoresis (CE) has developed, capillary electrochromatography (50–100 μm i.d.) has received much attention [8,9]. Certainly, capillary electrochromatography has progressed further in achieving down-sizing than microbore liquid chromatography. However, the inconvenience of the complicated procedures required for packing sorbents remains.

We proposed to aggressively introduce specific modifications or coatings into the capillary inner wall in order to improve separation selectivity in CE [10]. In a previous study, phenylboronic acid (PhBA) moieties were introduced onto the inner wall of a capillary through polymerization of *m*-acrylamidophenylboronic acids after activating the wall using a silane coupling reagent. The PhBA-modified capillary thus obtained successfully separated a model mixture sample of nucleosides (adenosine and deoxyadenosine, or uridine and deoxyuridine) in CE on the basis of interaction between *cis*-diol groups and PhBA moieties on the capillary wall.

In the present study, we prepared iminodiacetic acid (IDA)-modified and Cu(II)–IDA-modified capillaries through polymerization of *N*-(vinylbenzylimino)diacetic acid. In order to examine their performance as an analytical device, the modified capillaries were used in capillary LC with a chemiluminescence (CL) detector and immobilized

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metal chelate affinity capillary electrophoresis (IMACE) with an absorption detector, which were novel concepts for separation and detection.

2. Experimental

2.1. Reagents

All of the reagents used were commercially available and of special grade. Ion-exchanged water was distilled for use. Copper(II) sulfate [Cu(II)], cobalt(II) chloride [Co(II)], hematin, luminol, human serum albumin (HSA), and human serum γ-globulin (HγG) were purchased from Nacalai Tesque. [3-(Methacryloyloxy)propyl]trimethoxysilane, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and ammonium peroxodisulfate were purchased from Tokyo Kasei Kogyo.

2.2. Preparation of IDA-modified capillary

An IDA-modified capillary was produced according to preparation of a PhBA-modified capillary [10]. A new capillary of 75 μm i.d. was treated with 1 M NaOH for 30 min and washed with distilled water and then acetonitrile. A solution containing 4 ml of acetonitrile, 16 μl of [3-(methacryloyloxy)propyl]trimethoxysilane, and 32 μl of acetic acid was passed through the capillary for 60 min. The capillary was then left for 90 min at room temperature, and washed with acetonitrile. *N*-(Vinylbenzylimino)diacetic acid (2.5 mg), which was synthesized according to the previous report [11], was dissolved in 1 ml of distilled water. To the solution were added 7.5 μl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 15 μl of 10% ammonium peroxodisulfate solution. The solution was passed through the capillary for 10 min using a syringe and left overnight at room temperature. Finally, the capillary was washed with distilled water for 1 h.

2.3. Preparation of Cu(II)–IDA-modified capillary

A 30 mM Cu(II) solution (pH 4), prepared by dissolving Cu(II) sulfate in a dilute acetic acid solution, was delivered into the IDA-modified capillary for ca. 30 min to allow chelate formation between Cu(II) and the IDA moieties on the inner wall. A migration buffer was then passed through the capillary for ca. 30 min in order to remove any excess Cu(II) to finally produce the Cu(II)–IDA-modified capillary.

2.4. Apparatus and procedure of capillary LC with CL detector

Unmodified and the IDA-modified capillaries (70 cm × 75 μm i.d.) were used. A 10 mM phosphate buffer (pH 7.0, 8.2, and 10.8) as the mobile phase was fed by siphoning through the capillary. Cu(II), Co(II), and hematin [Fe(III)–porphyrin complex, which is well known as a catalyst for luminol CL reaction] stock sample solutions were prepared to 1 mM by dissolving in their corresponding buffers, which included potassium sodium tartrate at 10 times as much as metal compound concentration. The stock solutions were diluted as needed. A sample was injected into the capillary by siphoning for 15 s from a 35 cm height, after which a phosphate buffer or the mobile phase was successively delivered through the capillary by siphoning (flow rate; ca. 0.07 μl min^{-1}). The sample zone finally reached the CL detection cell.

The concept of the CL detection cell was originally proposed by us in a previous report [12]. The detection cell is made of PTFE, with a 4 cm outer diameter, 2.5 cm height, and 8 ml inner volume, to which CL reagent (a phosphate buffer of pH 10.8 including 1 mM luminol and 10 mM H_2O_2) is added. An optical fiber (core diameter; 2 mm) and a capillary are fixed to the cell; the fiber is set up straight to the capillary with a distance of ca. 0.3 mm between them. When the sample emerges from the capillary, it reacts with the CL reagent at the capillary outlet to produce visible light. The CL is detected by a photomultiplier tube equipped with a CL detector (Model EN-21, Kimoto Electric).

2.5. Apparatus and procedure of CE with absorption detector

Unmodified, the IDA-modified, and the Cu(II)–IDA-modified capillaries [70 cm (50 cm effective length) × 75 μm i.d.] were used. A 50 mM phosphate buffer (pH 7.0, 8.0, or 9.0) was used as the migration buffer. The capillary was filled with migration buffer in advance. HSA and HγG as model samples were introduced into the capillary for 20 s from 20 cm height by siphoning. A high voltage of 15 kV was applied, and they migrated and were detected at 210 nm with a modified SPD-6AV spectrophotometric detector (Shimadzu).

3. Results and discussion

3.1. Chromatographic behavior of metal ions and hematin in the IDA-modified capillary

As preliminary experiments for capillary LC, Cu(II), Co(II), and hematin samples were introduced into an unmodified capillary which was combined with the CL detector. They were migrated with a phosphate buffer of pH 10.8. All were detected at ca. 13 min with high sensitivity; they could be determined over the ranges 1×10^{-7} to 1×10^{-3}, 1×10^{-8} to 1×10^{-3}, and 1×10^{-9} to 1×10^{-6} M, respectively. Cu(II) and Co(II) were also detected at ca. 13 min with pH 7.0 and 8.2, but hematin did not dissolve at pH 7.0 and 8.2 and the measurement was not performed.

Next, Cu(II), Co(II), and hematin were examined using the IDA-modified capillary with pH 10.8. The hematin peak appeared faster than those of Cu(II) and Co(II); hematin

was detected at ca. 13 min, while Cu(II) and Co(II) were detected at ca. 20 min. The results meant that Cu(II) and Co(II) could interact with IDA moieties on the capillary inner wall through a complex formation reaction, while hematin hardly interacted with the moieties as hematin is an Fe(III)–porphyrin compound, which is inert to complex formation with the IDA ligand.

3.2. Separation of metal ions and hematin by capillary LC with CL detector

On the basis of the observed phenomena, chromatographic separation of a mixture of Cu(II), Co(II), and hematin was tried using the modified capillary with a phosphate buffer of pH 10.8. That is, we proposed the novel concept of capillary LC with a CL detector using an IDA-modified capillary. The mixture of Cu(II), Co(II) and hematin was used as a model because they indicate catalytic activities for luminol CL reaction as well as have different sizes and different configurations of coordination sites. The obtained chromatogram is shown in Fig. 1. The signal of hematin and those of Cu(II) and Co(II) were completely separated. However, the Cu(II) and Co(II) signals overlapped at around 20 min.

The retention times of Cu(II) and Co(II) were examined in detail using the modified capillary with pH 7.0, 8.2, and 10.8 (data not shown). The retention times increased with increasing pH, and the Co(II) signal appeared faster than that of Cu(II) under all pH conditions. The difference in retention times of Cu(II) and Co(II) between pH 8.2 and 10.8 was larger than that between pH 7.0 and 8.2. This data is consistent with the facts that the stability constant of Cu(II) with IDA moieties is larger than that of Co(II) [13], which was clarified by the orders of the Irving–Williams series and their metal ion radii, and that the values of pK_{a1} and pK_{a2} for IDA are reported to be 2.89 and 9.89 [14]. However, unfortunately, Cu(II) and Co(II) were not completely separated on the chromatogram.

Although the present capillary LC with the CL detector may require more detailed examination in order to optimize the analytical conditions, this technique is an important step forward in the development of a promising way for separation and determination of metal compounds with high sensitivity, easy operation, and extremely simple instrumentation that does not need any light source or spectroscopes.

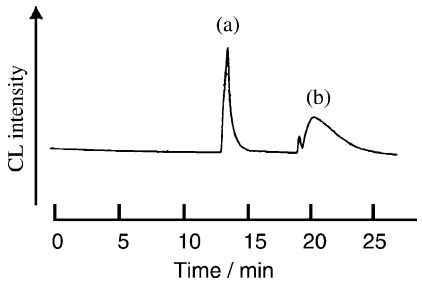

Fig. 1. Chromatogram obtained using IDA-modified capillary and CL detector: (a) hematin and (b) Cu(II) and Co(II). Conditions: capillary: 70 cm × 75 μm i.d. fused silica, mobile phase: 10 mM phosphate buffer (pH 10.8), sample injection: siphoning (for 15 s at 35 cm height), CL reagent: 10 mM phosphate buffer (pH 10.8) including 1 mM luminol and 10 mM H_2O_2, and sample concentration: 1.0×10^{-6} M hematin and 1.0×10^{-5} M Cu(II) and Co(II).

3.3. Electroosmotic flow of the modified capillaries

In order to confirm modification of the capillary inner wall, the migration velocity of acetone as a neutral marker was examined. Fig. 2 shows The migration velocities obtained using unmodified, IDA-modified, and Cu(II)–IDA-modified capillaries decreased in this order (Fig. 2). Thus, the inner wall of the capillary was coated or modified through the polymerization procedure of *N*-(vinylbenzylimino)diacetic acid, that is, the silanol groups of the inner wall were blocked through the polymerization. Nonetheless, there existed sufficient electroosmotic flow in the modified capillaries, in contrast to negligible flow in the case of ordinary polyacrylamide-modified capillaries previously reported [15], and this difference is most likely due to dissociation of IDA sites on the inner wall. In addition, the difference in the migration velocities between the IDA-modified and Cu(II)–IDA-modified capillaries must be attributed to the negative charge due to dissociated IDA sites decreasing through the chelate formation with Cu(II).

3.4. Separation of HSA and HγG by IMACE with an absorption detector

The concept of IMAC was first described by Porath et al. [1]. Nowadays, packing sorbents containing an IDA ligand at the end of a spacer for LC are commercially available. The sorbents possess a chelate formation ability against such metal ions as Cu(II), Zn(II), and Co(II). Chromatography using sorbents possessing metal ions produces a unique

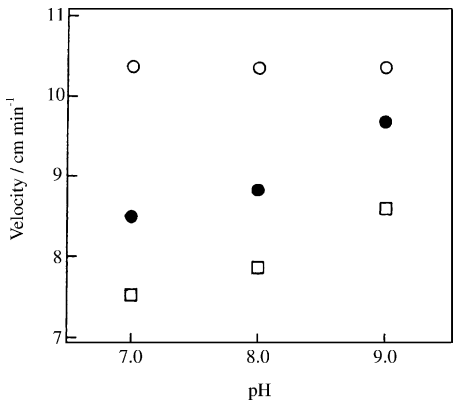

Fig. 2. Migration velocities of acetone as a neutral marker when using (○) unmodified, (●) IDA-modified, and (□) Cu(II)–IDA-modified capillaries. Conditions: capillary: 70 cm (effective length 50 cm) × 75 μm i.d. fused silica, applied voltage: 15 kV, migration buffer: 10 mM phosphate buffer (pH 7.0, 8.0, and 9.0), sample injection: siphoning (for 20 s at 20 cm height), and detection: 270 nm.

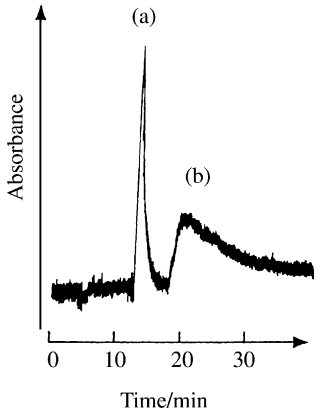

Fig. 3. Electropherogram of HSA and HγG obtained using Cu(II)–IDA-modified capillary: (a) HAS and (b) HγG. Conditions: capillary: 70 cm (effective length 50 cm) × 75 μm i.d. fused silica, applied voltage: 15 kV, migration buffer: 10 mM phosphate buffer (pH 7.0), sample injection: siphoning (for 20 s at 20 cm height), detection: 210 nm, and sample concentration: 8.0×10^{-5} M.

separation performance for proteins through the interaction between proteins and metal chelate moieties. Metal chelate moieties allow stronger adsorption of histidine and cysteine-containing proteins, which are usually the more basic proteins. Globulins which possess higher isoelectric points than albumins show stronger affinity for metal chelate moieties than albumins. As far as we know, the concept of metal chelate affinity separation has been little pursued with CE. Haupt et al. demonstrated IMACE using soluble polymer-supported ligands for metal ions [16]. Jiang et al. studied the structure–function relationship in glycosylated α-chymotrypsin by IMACE [17], while another group developed on-line Fe(III) IMAC–CE–electrospray ionization MS and applied it to sub-picomoles analysis of phosphopeptides [18,19].

In the present study, samples of HSA and HγG were subjected to CE using unmodified, IDA-modified, and Cu(II)–IDA-modified capillaries, in which a migration buffer of pH 7.0 was used. We also examined the separation procedure at pH 8. However, HγG did not migrate toward the capillary outlet reproducibly. The most likely explanation is that strong binding must have occurred between the protein and metal chelate site. The comparatively weak binding obtained at pH 7 allowed the migration of HγG and its separation from HSA in CE mode. The migration times of HSA and HγG were examined by using the unmodified, IDA-modified, and Cu(II)–IDA-modified capillaries (the data not shown). The HSA sample was eluted at ca. 9.5, 10, and 12 min, respectively, with the above three capillaries. The change in the migration times of HSA was due to the change in electroosmotic flow, but not due to the change in affinity between the protein and surface of the inner wall. On the other hand, the HγG sample was eluted at ca. 9.8, 10.3, and 20 min, respectively, with the three capillaries. Clearly, HγG has stronger affinity for Cu(II)–IDA moieties on the inner wall of capillaries than HSA.

On the basis of the above phenomenon, we were able to successfully separate a mixture of HSA and HγG with the present IMACE using the Cu(II)–IDA-modified capillary (Fig. 3). First, HSA was detected at ca. 12 min and, then, HγG was detected at ca. 20 min. IMACE features extremely small reagent volumes and sample amount, compared with ordinary IMAC. In addition, the modified capillary was able to be stood for about 2 weeks without any use after preparation with no lowering of separation ability. The coated capillary was repeatedly used with less than 10% relative standard deviations for migration time and CL intensity within 30 electrophoresis measurements.

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