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Cycloaliphatic epoxy resin coating for capillary electrophoresis

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Abstract

Coating the interior surface of a fused-silica capillary with a polymeric material has long been used in capillary electrophoresis (CE) to reduce or eliminate electroosmotic flow and suppress adsorption. A cycloaliphatic epoxide-based resin was bonded to silane treated capillaries and crosslinked with a curing agent. The epoxy resin coating significantly reduced electroosmotic flow over a pH range of 3–10. This coating was sufficiently hydrophilic to suppress protein adsorption. The epoxy resin coated capillary was used to separate several acidic and basic proteins and peptides. Separation efficiencies greater than 400 000 theoretical plates were achieved. The relative standard deviations in migration times for proteins were $<0.8\%$. Speed and simplicity are important advantages of the coating procedure compared to other published coating methods.

Keywords: Coated capillaries; Epoxy resin coating; Proteins; Peptides

1. Introduction

Capillary electrophoresis (CE) has emerged as a fast, efficient, and high-resolution separation technique for the separation of biomolecules. However, in applying capillary electrophoresis to the separation of proteins [1–3], care must be taken to minimize adsorption on the capillary column wall. Adsorption can lead to asymmetrical peaks, poor efficiency, modified electroosmotic flow and irreproducible migration velocity [4–6]. Much research has been devoted in the past to control surface–molecule interactions, including the use of extreme pH conditions [7–9], high-ionic-strength buffers [10,11], buffer additives [12–16], and modification of the surface of the fused-silica capillary wall [17–23].

Chemical modification of the fused-silica surface is the most desirable method for protein separation because of the increased flexibility in the choice of separation conditions. Poly(vinyl alcohol)-coated capillaries were shown to effectively screen silanol groups, resulting in high efficiency separations. This coating suppressed the electroosmotic flow (EOF) and was stable over a wide pH range [19,24]. Hjertén [25] was the first to chemically bond polyacrylamide to a fused-silica capillary. The resultant coating was effective in eliminating EOF as well as protein adsorption. Schmalzing et al. [26] attached the linear polyacrylamide layer via a covalently bound polyvinylsiloxane film. The acrylamide layer was crosslinked to increase the stability of the coating. High efficiency separations were obtained. Several other researchers have reported the use of different meth-

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ods to chemically bond acrylamide [27,28]. Recently, Srinivasan et al. [18] crosslinked polyacrylamide and polyvinylpyrrolidone on a silane treated surface. Malik et al. [20] used a surface derivatizing agent and crosslinking reagent to bind the polymer on the fused-silica surface. The resultant coating gave high efficiency separations of proteins with great reproducibility.

Epoxy resins have been used in many commercial applications requiring polymer films due to the variety of chemical structures that are available and their excellent adhesive properties. Changing the chemical structure of the curing agent and the curing conditions results in resins with versatile properties such as toughness, chemical resistance, flexibility, hardness, adhesive strength, heat resistance and electrical resistance. This versatility makes them useful in a variety of applications such as floorings, protective coatings, and low-pressure molding resins for textiles and fiber reinforced plastics [29]. An epoxy resin was first used as a polymer coating by Towns et al. [30], which involved a multi-step in-situ polymerization process. In the initial step, an organosilane was used to derivatize the surface of the fused-silica capillary. This was followed by deposition of a film of multifunctional oxirane that was polymerized by using either boron trifluoride or a tertiary amine. Another film of glycidol was then deposited on this coated capillary which was derivatized using diazomethane. Thus, the whole method required several steps and the derivatization process was long, inconvenient, and cumbersome.

Other efforts to use epoxy-based materials were reported by Liu et al. [31,32], which involved the use of bisphenol epoxy resin cured with aliphatic and aromatic amines. These coatings provided successful separations of proteins over the pH range of 3–10, although high concentration buffers were utilized, since at lower concentration some interactions still occurred between proteins and residual silanol groups. Also, since the epoxy resin was cured using amines, a reversal in EOF occurred at pH lower than 4. Ren et al. [33] treated the column with epoxy resin before coating with poly(ethylene–propylene glycol). This coating gave reproducible separations of basic proteins and was stable over the pH range of 4–11.

Most investigations of epoxy resin coatings for CE have involved the most common form of epoxy resins, the reaction products of epichlorohydrin and bisphenol A [31,33]. This paper describes a simple procedure to prepare a uniform and inert epoxy coating on fused-silica capillaries using a cycloaliphatic epoxide-based resin that can be cured with acid anhydride in the presence of imidazole. The cycloaliphatic epoxide-based resin was investigated because of its binding to the silica surface either through van der Waal forces or covalent bonds at active sites, and because of the presence of amide and ester groups which make it hydrophilic. This epoxy coating was evaluated for model proteins and peptides with respect to efficiency, stability and migration time reproducibility.

2. Experimental

2.1. Apparatus

Capillary electrophoresis was performed using a Crystal CE 300 system (ATI, Madison, WI, USA) equipped with an on-line Crystal 100 variable-wavelength UV–Vis absorbance detector. The absorbance wavelength used was 214 nm unless otherwise specified. Data were collected using a ChomPerfect chromatography workstation (Justice Laboratory, Palo Alto, CA, USA) and a computer. Fused-silica tubing (Polymicro Technologies, Phoenix, AZ, USA) of 50–75 μm I.D. and 360 μm O.D. was used for preparation of capillary columns.

2.2. Reagents and materials

Epoxy resin, acid anhydride and imidazole were purchased from Ciba Specialty Chemicals (Brewster, NY, USA). Peptide and protein standards and Tris–HCl were obtained from Sigma (St. Louis, MO, USA). Sodium phosphate, phosphoric acid, γ-glycidoxypropyltrimethoxysilane and dimethyl sulfoxide (DMSO) were purchased from Aldrich (Milwaukee, WI, USA). Deionized water, used to prepare the buffer solutions and to rinse the columns, was prepared using a Milli-Q water system (Millipore, Milford, MA, USA). All buffer solutions were filtered through a 0.45-μm cellulose acetate membrane (Corning, Corning, NY, USA), and degassed thoroughly prior to use.

2.3. Tryptic digest

A myoglobin digest was prepared by dissolving 1 mg of myoglobin in 1 ml of 50 m*M* Tris–HCl and 1 m*M* $CaCl_2$ (pH 7.6). Trypsin was added to this mixture so that the protease/protein ratio was 1:20. The mixture was then incubated at 37 °C for 10 h. The reaction was terminated by the addition of 6 μl of 10% formic acid.

2.4. Capillary preparation

The capillary was first rinsed with 1 *M* NaOH for 30 min and then deionized water for an additional 30 min. The capillary was dried using a nitrogen purge while heating at 100 °C for 60 min in a GC oven. The rinsed and dried capillary was then treated with γ-glycidoxypropyltrimethoxysilane for 30 min. The remaining γ-glycidoxypropyltrimethoxysilane was then removed by purging with nitrogen while heating at 90 °C for 60 min. The capillary was rinsed with methylene chloride and then methanol for 15 min each. After this, the column was filled with 1% epoxy solution in methylene chloride (cycloaliphatic epoxide–tetrahydrophthallic anhydride–imidazole, in the ratio of 100:114:0–2.5, w/w, respectively) and left at room temperature for 30 min before removing the solvent using vacuum. During evaporation, the capillary was immersed in an oil bath to maintain constant temperature. The epoxy resin was then bonded to the surface of the fused-silica capillary by heating at 100 °C for 1 h and then 140–220 °C for 2–6 h inside a GC oven with a nitrogen purge through the column. Before installing the column in the CE system, it was successively rinsed with methylene chloride, methanol, deionized water and separation buffer for 30 min each.

2.5. Electrophoresis conditions

DMSO was used as a neutral marker. The capillary column was rinsed with separation buffer for 6 min between runs. Hydrodynamic injection was used to introduce the sample into the separation capillary. The injection time varied between 2 and 6 s. Concentrations of proteins in solutions ranged between 0.5 and 1 mg ml^{-1}. When the separation buffer was changed, the capillary was rinsed with deionized water (10 min) followed by run buffer (30 min).

3. Results and discussion

3.1. Curing agent, temperature and time

The extent of crosslinking of the epoxy polymer depends on three factors: curing agent, temperature and time. The cycloaliphatic epoxide can be crosslinked through its oxirane rings by the use of acidic anhydrides such as tetrahydrophthalic anhydride. Thus, a series of experiments were performed to find the optimum conditions for obtaining a highly crosslinked uniform layer of epoxy resin coating. To enable sufficient crosslinking, the ratio of tetrahydrophthalic anhydride used was slightly higher than the cycloaliphatic epoxide. Columns were prepared with imidazole amounts of 0, 0.5, 1.0, 1.5, 2 and 2.5 (parts by mass). Absence of imidazole resulted in significantly longer curing times. Imidazole in 2 parts by mass aided in completion of crosslinking and reduced the curing time considerably without any effect on efficiency. Amounts lower than this did not show much effect on crosslinking and curing time.

Columns were prepared using crosslinking temperatures of 140, 160, 180, 200 and 220 °C. Optimum curing conditions of 100 °C for 1 h, followed by 200 °C for 3 h, were determined from the above experiments. A temperature below 160 °C decreased the efficiency due to incomplete crosslinking which resulted in a non-uniform film of epoxy resin on the capillary surface.

3.2. Surface modification

The reaction scheme used in the curing of the epoxy polymer is shown in Fig. 1A. The capillary surface was first treated with γ-glycidoxypropyltrimethoxysilane to deactivate the silanol groups via both chemical reaction and hydrogen bonding. The bound groups facilitated binding of the epoxy polymer layer to the silica surface through stable Si–O–Si–C bonds. When curing at 200 °C for 3 h, the epoxy resin became highly crosslinked, giving a stable and homogeneous polymer coating. The capil-

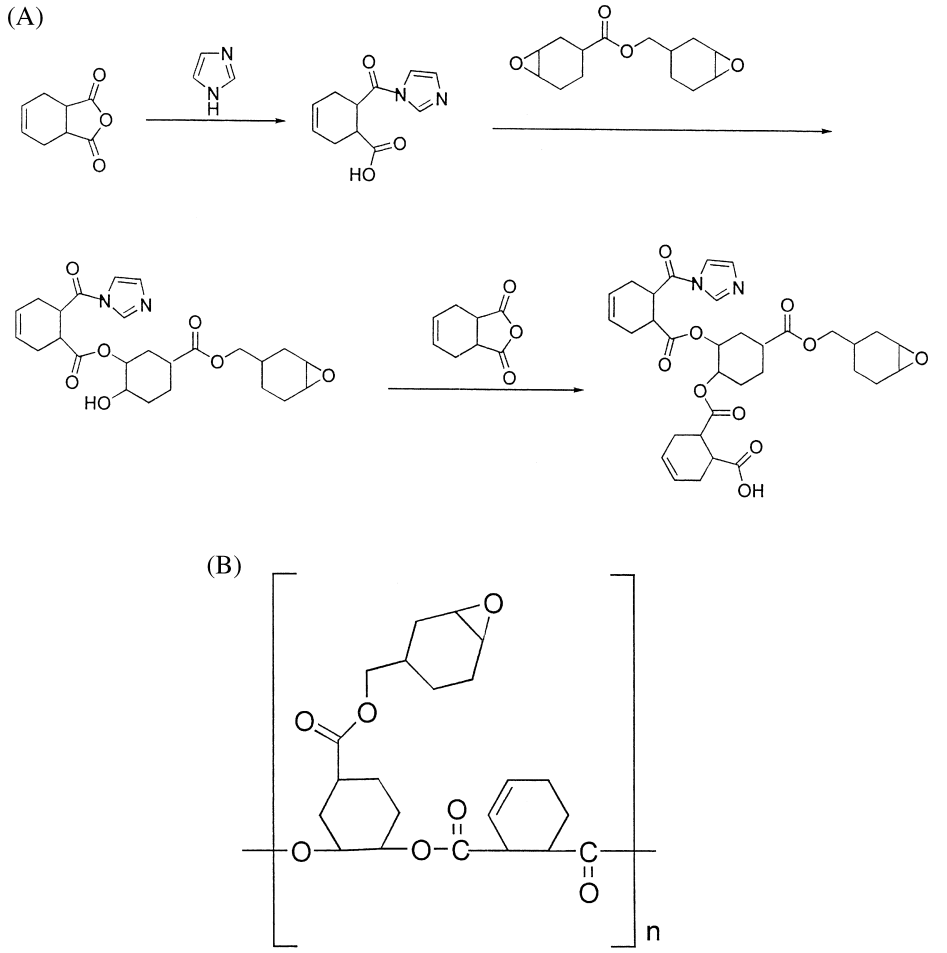

Fig. 1. (A) Reaction scheme used in the preparation of the epoxy polymer coating; (B) chemical structure of the epoxy coating.

lary coating procedure consisted of two treatments. The capillary was first treated with γ-glycidoxypropyltrimethoxysilane which helped to reduce the electrostatic interactions between the silica surface and the protein molecules. The final treatment involved coating with epoxy resin which served as a hydrophilic shield for protection against hydrophobic interactions which otherwise existed between the capillary wall and the proteins. Fig. 1B shows the chemical structure of the epoxy polymer.

3.3. Electroosmotic flow

The electroosmotic flow is dependent on the magnitude of the zeta potential across the double layer surface, which is dependent on the charge density at the capillary surface. An increase in silanol ionization at high pH results in an increase in EOF. Thus, the magnitude of EOF reflects the properties of the column inner surface and the effect of surface treatment. The electroosmotic flow in the coated capillary was measured as a function of pH and compared to results obtained with an uncoated capillary (Fig. 2). For an untreated fused-silica capillary, the EOF significantly increased with an increase in pH from 3 to 10 compared to the gradual increase in the epoxy resin coated capillary. It can be seen that the total EOF decreased significantly in the epoxy resin coated capillary, indicating that most of the silanol groups were covered.

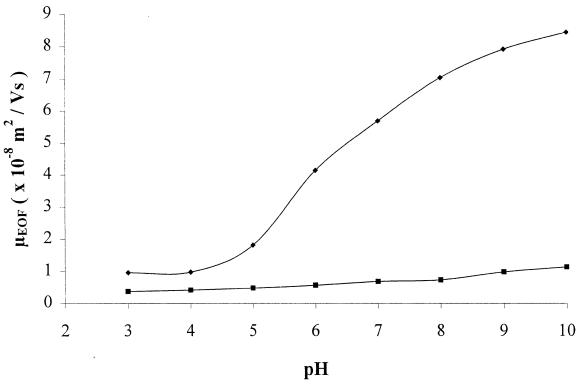

Fig. 2. Electroosmotic flow as a function of pH for untreated and epoxy resin polymer coated fused-silica capillary columns. Conditions: 64 cm×50 μm I.D. fused-silica capillary columns, 20 m*M* phosphate buffer, DMSO neutral marker, 18 kV applied voltage, UV detection (215 nm). ♦, untreated column; ■, epoxy resin coated column.

3.4. Basic protein separations

The surface inertness of the epoxy resin coating was evaluated by separating various basic proteins by CE. Since basic proteins are positively charged, they show maximal interaction with the negatively charged silanoate groups on the fused-silica capillary surface, leading to poor peak shape, reduced efficiency and irreproducible migration time. Fig. 3 shows an efficient CE separation of five basic proteins. The separation conditions were specifically chosen so that protein molecules were positively charged and strongly interacted with the negatively charged capillary surface. This separation was achieved using 50 m*M* Tris–HCl buffer at pH 4.7 without the addition of buffer additives. An efficiency greater than 400 000 plates was achieved, which is greater than previously reported for epoxy polymer coatings [34]. Some tailing was observed which could be due to residual adsorption of the basic proteins on the unshielded silica surface or on the epoxy coating.

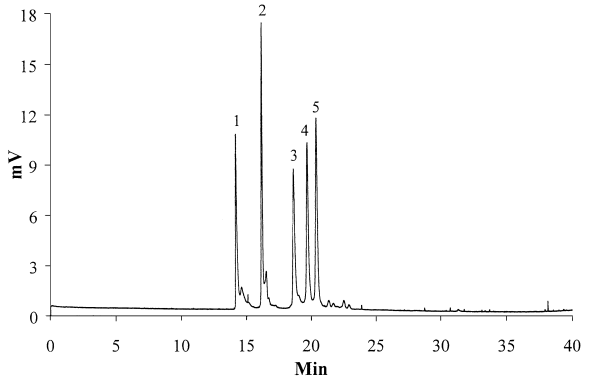

Fig. 3. Capillary electropherogram of basic proteins. Conditions: 64 cm×75 μm I.D. epoxy resin coated fused-silica capillary column, 50 m*M* Tris–HCl buffer (pH 4.7), 17 kV applied voltage, UV detection (215 nm). Peak identifications: 1=cytochrome *c*, 2=lysozyme, 3=ribonuclease A, 4=trypsinogen, 5=α-chymotrypsinogen A.

The coated capillary was evaluated for run-to-run and day-to-day reproducibility. Efficiency and RSD values for migration times are listed in Table 1. The migration time reproducibility was <0.8% RSD (n= 22) from run to run, and the day-to-day reproducibility was <2% RSD (evaluated for 5 days, 22 runs each day). The small RSD values verify that there is no significant adsorption of proteins onto the column surface or leaching of the coating into the buffer solution. The column-to-column migration time reproducibility was also evaluated for eight columns using lysozyme as test solute, and an RSD value of <2.5% was obtained. These low values suggest that the coating method is very reproducible. The epoxy polymer coated column was also evaluated for stability over time, and no loss in efficiency was observed after continuously operating a column for more than 100 injections. This coating was found to be stable at pH as high as 10.

3.5. Acidic protein separations

The epoxy coated capillary was suitable for the separation of acidic proteins. Acidic proteins have isoelectric point (p*I*) values less than 7. Fig. 4 shows a CE separation of acidic proteins using the epoxy coated capillary. As the proteins were negatively charged at the pH of the separation buffer, the separation was carried out in the reversed polarity mode (cathode at the injection end), so that the negatively charged proteins migrated on the basis of their electrophoretic mobilities past the detection point. Excellent resolution is shown for the separation of β-lactoglobulin A and B. These two proteins differ by only two amino acids at positions 64 (glycine for aspartic acid) and 118 (alanine for

Table 1
Reproducibility of migration times for basic proteins[a]

Protein	Efficiency ($\times 10^{-5}$ plates)	t_r (min)	RSD (%)	
			Run-to-run[b]	Day-to-day[c]
Lysozyme	4.22	16.23	0.78	1.66
α-Chymotrypsinogen A	2.06	20.43	0.57	1.44
Cytochrome *c*	1.92	14.22	0.66	1.57
Trypsinogen	1.93	19.74	0.53	1.39
Ribonuclease A	1.55	18.69	0.75	1.64

[a] Conditions as in Fig. 3.
[b] $n=22$.
[c] $n=5$.

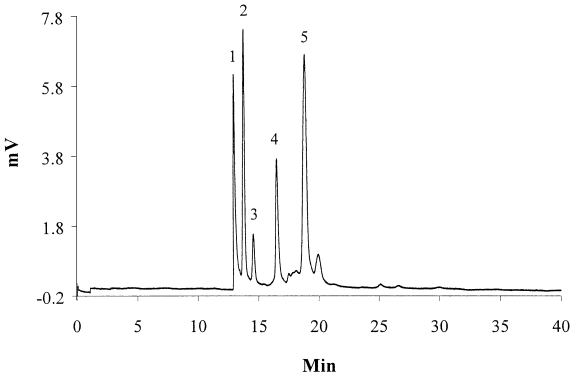

Fig. 4. Capillary electropherogram of acidic proteins. Conditions: 40 m*M* phosphate buffer (pH 6), −18 kV applied voltage, UV detection (254 nm); other conditions as in Fig. 3. Peak identifications: 1=β-lactoglobulin B, 2=β-lactoglobulin A, 3=α-lactalbumin, 4=ovalbumin, 5=pepsin.

valine) in a total sequence of 182 [35]. This substitution induces a net charge difference to provide different electrophoretic velocities. The RSD of migration time was found to be <1% and can be seen in Table 2.

Table 2
Reproducibility of migration times for acidic proteins[a]

Protein	Efficiency ($\times 10^{-5}$ plates)	t_r (min)	RSD[b] (%)
β-Lactoglobulin B	0.74	12.95	0.71
β-Lactoglobulin A	0.76	13.79	0.75
α-Lactalbumin	0.64	14.57	0.59
Ovalbumin	0.70	16.50	0.64
Pepsin	0.44	18.85	0.69

[a] Conditions as in Fig. 4.
[b] $n=22$.

3.6. Standard peptide separations

Natural peptides are composed of ~20 amino acids linked through peptide bonds and, sometimes, disulfide bonds. The great variation in sequence of amino acids in a polypeptide chain results in the extraordinary diversity of peptides. Peptides provide important information for fundamental studies and diagnosis of many pathological conditions. Some peptides differ by only a few amino acids; therefore, an efficient separation technique such as CE is necessary for their separation. Fig. 5 shows the CE separation of a series of synthetic peptides. All nine peptides were separated with baseline resolution and

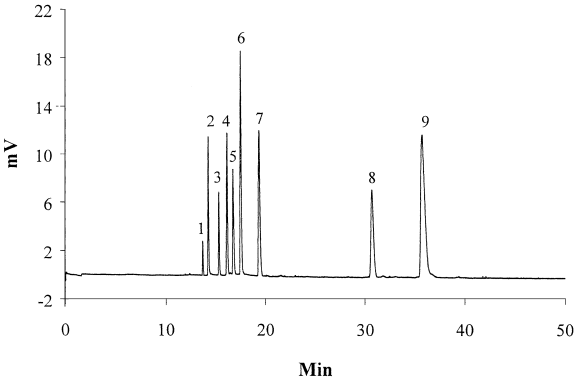

Fig. 5. Capillary electropherogram of standard peptides. Conditions: 50 m*M* Tris–HCl buffer (pH 4.8), 15 kV applied voltage, UV detection (215 nm); other conditions as in Fig. 4. Peak identifications: 1=Arg–Gly–Asp–Ser, 2=Gly–Gly–Tyr–Arg, 3=Arg–Val–Tyr–Ile–His–Pro–Phe, 4=Arg–Pro–Pro–Gly–Phe, 5=Arg–Pro–Pro–Gly–Phe–Ser–Pro, 6=Asp–Arg–Val–Tyr–Ile–His–Pro–Phe, 7=Tyr–Gly–Gly–Phe–Leu, 8=Phe–Gly–Gly–Phe, 9=Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg.

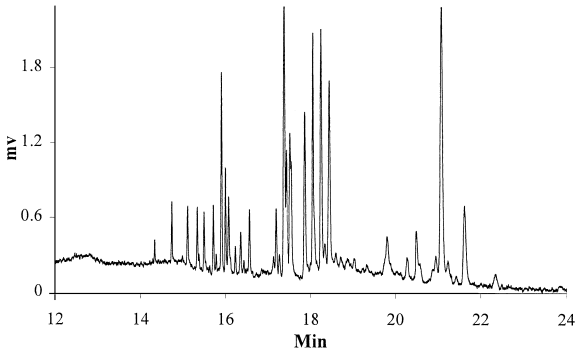

Fig. 6. Capillary electropherogram of tryptic digest of myoglobin. Conditions as in Fig. 5.

excellent peak shape. This is a result of the stable and inert properties of the epoxy coating.

3.7. Tryptic digest of myoglobin

Peptide mapping serves as a powerful tool for protein identification, structure elucidation, sequence determination and analysis of protein microheterogeneity, including post-translational modifications such as phosphorylation and glycosylation. Due to the great complexity of peptide maps, multidimensional techniques such as CE combined with MS are gaining importance. For obtaining a good separation in the first dimension in CE, a coated column is required. Fig. 6 shows an electropherogram of a tryptic digest of myoglobin. Enzymatic digestion of myoglobin was performed using trypsin [36], and the resultant mixture was separated using the epoxy resin coated column.

4. Conclusions

A new type of coating for CE was developed using a cycloaliphatic epoxide-based resin cured with acid anhydride in the presence of imidazole. The presence of imidazole reduced the curing time and aided in completion of crosslinking. This method produced a stable hydrophilic crosslinked layer that was able to suppress protein adsorption. This coating gave superior results compared to previously reported epoxy-based coatings. Efficiencies greater than 400 000 plates were achieved for the basic protein lysozyme. This hydrophilic epoxy-based coating was efficient, stable, easy to prepare and very reproducible. Thus, this epoxy coating was suitable for protein and complex peptide mixtures.

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