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Field-free remobilization of proteins after isoelectric focusing in packed capillaries

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Abstract

Pressure-driven remobilization without an applied electric field is shown to be possible with capillary isoelectric focusing by using packed capillaries. The capillary dimensions are 100 μm i.d. and 2 cm in length, and the packing is made of 0.9 μm nonporous silica particles that are chemically modified with a brush layer of polyacrylamide. Both reversible and irreversible adsorption are shown to be negligible. The packed capillaries eliminate the problem of unwanted hydrodynamic flow between reservoirs. Three proteins are focused: trypsin inhibitor, carbonic anhydrase II and myoglobin. The time required for focusing in the packed capillaries is increased by only a factor of two compared to the open capillary, giving complete focusing in less than 15 min at 200 V/cm. The packed capillaries allow the use of higher electric fields, with resolution continually increasing up to at least 1500 V/cm. The packing obstructs diffusional broadening after the field is turned off: for trypsin inhibitor, $D=6.1(\pm 0.3)\times 10^{-8} \text{ cm}^2/\text{s}$ for the packed capillary vs. $D=28.8(\pm 0.3)\times 10^{-8} \text{ cm}^2/\text{s}$ for the open capillary. The broadening contributed by the packing during remobilization is from eddy diffusion, and it is described by its plate height, H , which is the variance per unit length: $H=^2/L=0.64 \mu\text{m}$. This limits the resolution to 0.1 pH unit for the 2-cm capillary having a pH range of 3-10, giving a theoretical peak capacity of 47.

Introduction

Capillary isoelectric focusing (cIEF) allows high-speed separations of proteins in only minutes, as detailed in recent reviews.¹⁻⁴ Resolution increases with the square root of applied voltage, and resolutions from 0.02 pH units to as low as 0.002 pH units have been achieved for high voltages.⁵⁻⁹ cIEF makes use of mobile ampholytes, which are low molecular weight species that quickly establish a pH gradient under an applied voltage. While gel IEF is usually used as part of a multidimensional separation, there are stand-alone analyses that have been developed for cIEF. As examples, proteins can be imaged inside capillaries to determine pI,⁸ the molecular weight can be estimated from the diffusion coefficient to effect a 2D analysis,¹⁰ and fluorescence imaging has been used for rapid analysis of doping drugs,⁶ therapeutic proteins,¹¹ and protein-DNA binding.¹²

For many applications, the proteins must be removed from the capillary for identification by mass spectrometry. The on-line integration of cIEF with mass spectrometry was first demonstrated for peptides, where replacing the base reservoir with acid gives chemical remobilization of the proteins by imparting a positive charge.¹³ A downside of chemical remobilization is that it changes the relative peak positions.^{14,15} Remobilization by pressurized flow avoids this problem. The electric field must be maintained during remobilization with pressurized flow to reduce band broadening caused by the Poiseuille flow profile. Pressure-remobilized proteins can be spotted on a MALDI plate,^{16,17} or electrosprayed into a mass spectrometer by using sheath flow of acidic solution to charge the proteins, although the ampholytes suppress electrospray ionization.^{13, 18,19} The remobilized

proteins can be flowed into a column for reversed-phase liquid chromatography (RPLC) for an additional separation dimension, where the RPLC separation also prevents suppression of ionization by separating the ampholytes from the proteins.²⁰ In practice, when two separations of high peak capacity are used sequentially as two dimensions, one dimension typically is sampled at a low frequency, i.e., nine cIEF fractions were analyzed by RPLC, with a pI resolution of 0.12 pH units (4).²¹ While cIEF-RPLC demonstrates a promising concept, a problem with the practical implementation is that the pH axis in cIEF is unpredictable because mobile ampholytes give shifting pH gradients, gradient compression, and sometimes band distortion from isotachopheresis.²²⁻²³ These instabilities make it extremely challenging to collect fractions at the intended pI values.

The present conundrum in proteomic applications of cIEF is that an immobilized pH gradient is needed to define the pI values of proteins, yet an immobilized gradient would not allow the electric field to remain on during pressure-driven remobilization. It is conceivable that packed capillaries could solve this problem, providing a high surface area to support immobilized ampholytes while also giving an even flow profile across the capillary diameter to enable remobilization without an electric field. On the other hand, the packing can introduce new problems, including eddy diffusion to broaden the bands during remobilization, adsorption of the proteins to shift the bands due to chromatographic retention, slower focusing from higher column resistance, and loss of protein through irreversible adsorption. The ability to immobilize a pH gradient in a packed capillary has been demonstrated: ampholytes were immobilized by binding them to a polymer monolith bearing epoxide groups.²⁴ Field-free remobilization has not yet been demonstrated.

The purpose of this work is to investigate whether capillaries packed with nonporous silica particles can be used for field-free pressure-driven remobilization, without significant loss of resolution. The silica particles are chemically modified with a brush layer of polyacrylamide made by atom-transfer radical polymerization,²⁵⁻²⁶ which uses a self-assembled siloxane monolayer to further reduce electroosmotic flow. The performance of cIEF in the packed capillary is compared with that for an open capillary having the same chemical modification on its inner walls, and both use the same carrier ampholytes to establish a pH gradient from 3 to 10 units. The speed, resolution, and range of electric fields are compared, and the resolution after field free, pressure-driven remobilization is studied for the packed capillary.

Experimental Section

Materials and Chemicals

0.9 μ m silica particles were purchased from Fiber Optic Center, Inc. (New Bedford, MA). Silica capillaries of 100 μ m i.d. were obtained from Polymicro Technologies (Phoenix, AZ). Trichloromethylsilane (Gelest, Inc., Morrisville, PA), (chloromethyl)phenylethyl-trichlorosilane (Gelest, Inc., Morrisville, PA), acrylamide (Sigma-Aldrich, St. Louis, MO), CuCl, 99.999% (Alfa Aesar, Ward Hill, MA), CuCl₂, 99% (Acros Organics, Morris Plains, NJ), carrier ampholytes pH 3-10 (Sigma-Aldrich, St. Louis, MO), urea (Mallinckrodt Baker, Phillipsburg, NJ), CHAPS (Bio-Rad Laboratories, Hercules, CA) and dithiothreitol (Bio-Rad Laboratories, Hercules, CA) were used as received without further purification. Tris (2-dimethylaminoethyl) amine was synthesized according to a procedure published before.²⁷ Protein standards including trypsin inhibitor, carbonic anhydrase II, myoglobin, cytochrome C, and β -galactosidase (all from Sigma-Aldrich, St. Louis, MO) were labeled with Alexa Fluor 546, Alexa Fluor 488, and Alexa Fluor 647 (Invitrogen Co., Carlsbad, CA) for fluorescence imaging, and separated from excess dye by Sephadex gel.

Capillary Preparation

To pack the capillaries, a 30% (w:w) slurry of silica particles in water was sonicated and wicked into freshly cleaned silica capillaries of 100 μm i.d., and then a pump was used to compress the slurry against a frit until a final pressure of 600 bar was achieved. The capillary was allowed to dry in air after the frit was removed. An 8% (v:v) solution of trichloromethylsilane and (chloromethyl)phenylethyl-trichlorosilane at a volume ratio of 1:20, respectively, was prepared in dry toluene. This solution was wicked into the dry, packed capillary and allowed to react overnight under nitrogen. The capillary was rinsed with dry toluene using an HPLC pump, and allowed to dry in an oven at 120 $^{\circ}\text{C}$ for two hours. A de-oxygenated solution of acrylamide, CuCl , CuCl_2 and Tris (2-dimethylaminoethyl) amine in DMF was prepared as described earlier,²⁷ and this solution was wicked into the capillary in an argon atmosphere and allowed to react for three hours. (CAUTION: acrylamide is a neurotoxin, care must be taken to avoid breathing the powder or contact of the solution with skin. Wear gloves, and weigh out the acrylamide under the hood.) The capillary was then rinsed with 0.1 M EDTA solution, water and then ethanol using an HPLC pump, and dried in a vacuum desiccator.

cIEF

The apparatus for cIEF is depicted in Figure 1. Overall, a 2-cm long capillary packed with 0.9 μm silica particles straddled two reservoirs made with PDMS. The PDMS reservoirs each had a double well connected by a narrow slot. The pair of PDMS reservoirs was press-fit onto the microscope slide after treatment with an UV ozone-plasma cleaner. The capillary fit into a tight notch in each reservoir to span across the reservoirs without leaking. A Pt electrode was inserted into each outer reservoir and connected to a power supply, while each end of the capillary was in an inner well. This minimized the introduction of bubbles into the capillary by allowing electrophoresis gases to dissipate in the outer wells. The capillary was imaged by placing the microscope slide on an inverted fluorescence microscope, and using fluorescence-labeled proteins in the experiments. The concentrations of H_3PO_4 and NaOH as anolyte and catholyte were optimized to minimize cathodic or anodic drift, and the optimal concentrations were found to be 20mM H_3PO_4 and 20mM NaOH .

A mixture of 0.002 mg/mL trypsin inhibitor, 0.002 mg/mL carbonic anhydrase II and 0.005 mg/mL myoglobin all of which were labeled with Alexa Fluor 546, 4% carrier ampholytes pH 3-10, 8 M urea, 2% CHAPS and 50 mM dithiothreitol was injected into the whole capillary. The catholyte reservoir was filled with 20 mM NaOH and the anolyte reservoir was filled with 20 mM H_3PO_4 . The electric field was supplied by a high-voltage power supply ("10A 25A" Series, Ultravolt, Ronkonkoma, NY), and controlled by LabView. (CAUTION, care must be taken to avoid touching the electrodes while the voltage is applied.) Care is needed to avoid bending the capillaries because this causes cracks to form. If the capillary had a crack, a gas bubble would form at the crack, which would cause electrophoretic migration to stop. An inverted optical microscope equipped with a Hg lamp (Eclipse Ti-S, Nikon Instruments Inc., Melville, NY) and a CCD camera (ProEM 512, Princeton Instruments, Trenton, NJ) was used for fluorescence detection. Fluorescence of labeled proteins was excited and then filtered using a filter cube (QMAX-Yellow, Omega Optical, Brattleboro, VT). The images were collected through a 2X objective (CFI Plan Apo 2X, Nikon Instruments Inc., Melville, NY) and Winview software (Princeton Instruments, Trenton, NJ) was used for data acquisition.

Results and Discussion

Figure 2 shows fluorescence images constituting raw electropherograms for a mixture of proteins labeled with Alexa Fluor 546, including myoglobin (pI=6.0), carbonic anhydrase II

($pI=5.6$) and trypsin inhibitor ($pI=4.4$) in 2-cm open vs. packed capillaries. Raw images showing how proteins focused from filling the entire capillary to narrow bands in packed capillary are provided in the supporting information as Figure S1. The pI values indicated in Figure 2 were the values after labeling. They were determined using commercial IPG strips, and differ from those of the parent proteins because the labeling dye changes the protein charges. Dyes are available that avoid a change in charge upon labeling, but the Alexafluor dyes were chosen for their stability and brightness. For both packed and open capillaries, the focusing was performed until there was no further improvement in resolution, which was on the scale of minutes. Images of the focused zones are shown in Figure 2 for two different electric fields: 200 V/cm and 500 V/cm. The images in Figure 2A for the case of 200 V/cm show that the electropherograms are similar for packed vs. open capillaries. For the open capillary, the pH gradient is shifted away from the reservoir and compressed, relative to that for the packed capillary. This commonly occurs for open capillaries and it is due to the incursion of catholytes and anolytes into the capillary²³. The packing apparently hinders this process. The images for the case of 500 V/cm in Figure 2B show that resolution is slightly increased for the case of the packed capillary, and the positions of the peaks are nearly the same. This amount of shifting is typical of that observed from run to run. The resolution for the packed capillary was determined using the main peak for trypsin inhibitor, which has a standard deviation of 0.28 μm (Figure S2 in supporting information), and the pH gradient of 0.27/mm in Figure 2B, shows that the resolution is 0.03 pH units. The resolution obtained by cIEF in packed capillary is comparable to the high resolution of open capillary cIEF for a comparable voltage⁶⁻⁹. For the open capillary, the broadening is too severe at 500 V/cm to be useful. The zones are apparently shaped by isotachopheresis, which sometimes occurs in open capillaries. The packing material apparently suffers less from heating at 500 V/cm.

To test whether resolution continues to improve with increasing electric fields for the packed capillary, trypsin inhibitor was focused at 200 V/cm, imaged, and then the field was increased stepwise by 100 V/cm and imaged for after focusing for each field. Trypsin inhibitor was used for this study because it was the cleanest protein sample. Figure 3 shows that the resolution is progressively increased with electric field, where the peak profile as a function of electric field is plotted for electric fields of 200, 500, 1000 and 1500 V/cm. Protein precipitation that might arise from Joule heating was not observed even at 1500 V/cm, which is probably due to higher thermal conductivity of densely packed silica particles.²⁸ The asymmetric peak at 200 V/cm becomes two peaks at 1000 and 1500 V/cm, with slightly better resolution at 1500 V/cm. The trypsin inhibitor peak was fit to a Gaussian at each electric field (supporting information, Figure S2), and the peak width decreases with the square root of electric field, but with a nonzero intercept (supporting information, Figure S3). The presence of an intercept suggests that the protein has at least one additional impurity that is not resolved. Higher fields were not tried. The results show that the packed capillary can be used at much higher electric fields to obtain higher resolution, and this is probably a consequence of the heat dissipation.

To test whether the better performance at higher voltage is due to better heat dissipation vs. less solution being inside of the packed capillary, it is instructive to examine the current vs. time during focusing. Figure 4 shows a plot of current vs. time for an open vs. packed capillary as focusing proceeds, with a fixed electric field of 200 V/cm. The current in the open and packed capillaries is on μA level, which is comparable to the value in regular cIEF as reported in the literature.^{19, 29} The current is initially three times higher for the open capillary, but the current decreases earlier. After 2 min, the current is a factor of two higher for the open capillary and remains approximately at that ratio. Given the relation for dissipated power as $P=I \cdot V$, the open capillary has about twice the dissipated power over the 20 min time of the experiment. The open capillary lost resolution at 500 V/cm, while the packed capillary still gained resolution at the three-fold higher field of 1500 V/cm. This

indicates that the packed capillary has better heat dissipation. The reason is likely that silica has higher thermal conductivity than water, and the packed capillary is mostly silica, with intimate contact with the water for maximum heat transfer. The packing also limits convection, further reducing heating effects.

The packing ought to slow the speed of focusing. Figure 4 compares the speed of focusing for the packed vs. open capillaries for the same electric field and capillary length: 200 V/cm and a 2-cm length. In both cases, the current dropped rapidly, and then decreased more gradually, consistent with what has been widely reported previously. The steep drop ended after 3 min for the open capillary and 6 min for the packed capillary, indicating that the packing increases the time for focusing by a factor of two. Complete focusing occurred in about double the time for the steep current drop, i.e., 6 min were required for the open capillary and 12 min were required for the packed capillary to complete the focusing at 200 V/cm. The two-fold slower rate for the packed capillary is not a detriment to practical applications. The focusing time of cIEF can be shortened to as little as a minute by using a higher voltage or a smaller length,^{30,31} and the packed capillary ought to follow suit. While the packing slows focusing, it is still much faster than gel IEF, where 15 hour runs are typical.

The packing is expected to obstruct protein diffusion, and this was measured by imaging the focused peaks as a function of time after the voltage was turned off. Again, trypsin inhibitor was analyzed because it was the cleanest protein. The supporting information (Figure S4) shows a plot of peak variance vs. time for trypsin inhibitor. The slope of such a plot is $2D$, and the data reveal a large difference in slope, giving $D=28.8(\pm 0.3)\times 10^{-8}$ cm²/s for the open capillary, and $D=6.1(\pm 0.2)\times 10^{-8}$ cm²/s for the packed capillary. The consequence of the small diffusion coefficient is that broadening during pressure driven remobilization is reduced. The time it would take for trypsin inhibitor to broaden by a factor of two would thus be 145 s. The packed capillary could thus be advantageous for remobilization into another separation dimension if other contributions to broadening are not overwhelming.

Figure 5 shows images of the trypsin inhibitor after pressure driven remobilization in the absence of electric field. For the packed capillary, illustrated in Figure 5A, the peak was flowed through a distance of 1.56 cm in 87.4 s, but total time that elapsed was 237 s because of the time required to attach the pressure fitting to the capillary. To account for diffusion during this time, a peak that simply diffused for the same time period of 237 s is shown for comparison. These two images show that the broadening from pressure remobilization is larger than the contribution from molecular diffusion alone. The additional broadening is from eddy diffusion, which arises from velocity differences due to heterogeneities in packing. To express how much broadening is caused by eddy diffusion from the packing, the intensity profiles of each of these two images was fit to a Gaussian (Figure S5 in the supporting information), and the difference in variance is attributable to eddy diffusion. Expressing this as the peak variance per length, which is the plate height, H , one gets $H=2/L=640$ nm. This allows one to predict how much broadening the material would impart for any length. For the open capillary, by contrast, pressure-driven flow with the same linear velocity as that for the packed capillary would completely disperse the peak. To illustrate this, a very small pressure was imposed from uneven reservoir heights, and a series of images is shown in Figure 5B, after focusing at 200 V/cm. The protein in the center of the capillary has bulged forward by hundreds of micrometers beyond the protein on the sides. This distortion is due to the Poiseuille flow profile. For the open capillary, even at this slow velocity, the band is broadened more in 1 mm than it is for 1.5 cm in the packed capillary at the higher velocity. This illustrates why the electric field is essential when using pressure-driven remobilization with open capillaries, but not for packed capillaries. There is no

parabolic profile from remobilization because the packing makes the profile even across the capillary diameter.

The remobilization of all three proteins by pressure-driven flow, after separation by cIEF, is shown in Figure 6, where the fluorescence is plotted vs. time at a fixed position in the capillary, which is 93% of the capillary length. The peak separation between myoglobin and trypsin inhibitor (pH=1.6) is 55 s, and the standard deviation of the trypsin peak is 0.9 s, therefore the resolution is 0.1 pH units. Thus, without an electric field to maintain focusing of the proteins, pressurized remobilization in the packed capillary reduces the resolution by the factor of 3 in this case. Further research to reduce eddy diffusion, which is possible with smaller particles,²⁸ as well as faster flow, could increase resolution further. Nonetheless, the resolution is comparable to that for open capillaries of comparable length and applied voltage when using an electric field during pressure driven remobilization.²¹

The issue of protein adsorption is important for enabling packed capillaries to be used. If reversible adsorption occurred, the protein bands would shift in position during remobilization due to protein chromatography. This possibility was tested by injecting a mixture of three proteins with different fluorescent labels to distinguish them. Pressure was then applied to the capillary in the absence of electric field, and when the proteins approached the end of the capillary, the pressure was released, and each of the three proteins was successively imaged using the respective optical filter for its dye label. These were not the same three proteins used in the cIEF, instead, three proteins of very different sizes were used to maximize the possibility of differences in adsorptivity: cytochrome C (12 kDa), trypsin inhibitor (20 kDa), and α -galactosidase (116 kDa). The results are shown in Figure 7. Images for each of the proteins are shown in Figure 7 A and plots of fluorescence intensity vs. peak position for three proteins are displayed in Figure 7B. The images show that the peaks for the three proteins are aligned to within 10 μ m after the migrating 1.3 cm, which means they shift by no more than 0.1%. For a pH gradient extending from 3 to 10 units over 2 cm, this precision is within 0.01 pH units. The asymmetric band shapes are attributed to injection artifacts because the peaks stayed the same shape over the distance of the capillary. This is corroborated by Figure 5A, which did not indicate a change in peak shape upon remobilization for the trypsin inhibitor. These results indicate that adsorption is not sufficient to significantly affect the peak positions. Irreversible adsorption is also insignificant. The images in the supporting information, Figure S1, illustrate that there is no noticeable background increase after the peaks have focused. The low adsorptivity is attributed to the polyacrylamide brush layer used as the stationary phase.^{25,27} These results indicate that the packing does not introduce deleterious effects from reversible or irreversible adsorption.

The packed capillaries have less protein injected by virtue of the lower free volume. To determine the free volume, the volume of water that wicks into the capillary was measured. The result of the measurement shows a free volume of 39%, which is virtually the same as the 40% free volume that one would expect from random packing of spheres. This reduces the amount of protein injected by a factor of 0.39, giving slightly worse the half of the sensitivity that one would obtain using an open capillary if the same resolution were achieved for both.

Conclusions

The results demonstrate that packed capillaries enable field-free remobilization with a resolution of 0.1 pH units. The focusing is rapid, the packing makes the capillaries simpler to use by blocking unwanted hydrodynamic flow, and they have less of a problem from heating compared to open capillaries. For whole-capillary imaging applications, where remobilization is not used, higher resolution could be achieved relative to open capillaries

because the packing enables the use of higher electric fields. These results indicate that it is worthwhile to investigate the use of an immobilized pH gradient in the packed capillaries to stabilize the gradient and facilitate direct coupling to mass spectrometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of apparatus. A 2-cm capillary straddles the two reservoirs, and the microscope objective collects the fluorescence for imaging. For the packed capillary, the particles have a polyacrylamide brush layer on top of a self-assembled monolayer.

Figure 2.

Raw images for cIEF of three proteins, comparing packed and open capillaries using A) 200 V/cm and B) 500 V/cm for focusing. All the images are on the same size scale. The tips of the capillaries, are aligned to the right. The region of pH gradient of 6-10 is not shown, since there was no protein focused in that region. The intensity range of the fluorescence intensity was adjusted for each image to maximize contrast. The dotted lines illustrate the gradient compression for the open capillary. The indicated pI's are for the proteins after labeling. The size scale is shown at the bottom.

Figure 3. Plot of the peak intensity vs. position for trypsin inhibitor at four different electric fields. The plot for the case of 1500 V/cm is dotted to facilitate readability of the graph.

Figure 4.

Plot of current vs. focusing time for the packed capillary (●) using the left-hand scale, and the open capillary (○) using the right-hand scale. The electric field was constant at 200 V/cm in both cases.

Figure 5.

Images of trypsin inhibitor after cIEF and pressure –driven remobilization. A) Packed capillary after focusing at 500 V/cm and flowing for the times and distance indicated. B) Open capillary after focusing at 200 V/cm and flowing for the times indicated, with the peak always within the same field of view.

Figure 6.

Fluorescence measured at a point near the end of the capillary vs. time during pressure-driven remobilization of the three-protein mixture from the packed capillary, after focusing at 500 V/cm. The red dotted line is a Gaussian fit to the trypsin peak, revealing a standard deviation of 0.9 s., which corresponds to 0.1 pH units.

Figure 7.

A) Images of proteins in the capillary, each with a different color label, are shown after they were injected together and then moved by pressurized flow through a distance of 1.3 cm from the beginning of the capillary. The dotted line shows that the centers of the zones are at the same position. The capillary diameter of 100 μm i.d. serves to show the size scale. B) Line profiles along the capillary axis to show the intensity vs. position from the images of part A.