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Letters to *Analytical Chemistry*

Separation of Peptides and Oligonucleotides Using a Monolithic Polymer Layer and Pressurized Planar Electrophoresis and Electrochromatography

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The rapid separation of mixtures of six peptides using porous polymer monolithic layers in electrophoresis and pressurized planar electrochromatography modes has been achieved. The separations in the former mode were performed on a generic hydrophobic poly(butyl methacrylate-*co*-ethylene dimethacrylate) layer with no ionizable functionalities and required 2 min. This layer also enabled the separation of three oligonucleotides. The separation in the pressurized planar electrochromatographic mode was carried out using a negatively charged layer prepared via cografting of 2-acrylamido-2-methyl-1-propanesulfonic acid and 2-hydroxyethyl methacrylate on top of the generic hydrophobic monolith and was completed in 1 min.

Although porous polymer monoliths have been known for 2 decades, they have been typically prepared in cylindrical formats such as analytical columns and capillaries and mostly used in high-performance liquid chromatography (HPLC) separations of large molecules including proteins, nucleic acids, and synthetic polymers.[1−4] An entirely new format of the organic polymer-based monoliths, thin layers, emerged only recently. For example, precise control of the morphology of the monolith that exhibits the desired combination of micro- and nanoscale roughness resulted in a facile and inexpensive approach to superhydrophobic polymer layers.[5] Porous polymer monolithic layers 100−200 μm in thickness supported by glass plates were also prepared and used in the unidimensional thin layer chromatography (TLC) separation of peptides and proteins.[6] While this method introduces an additional approach to the separation of peptides, the poor flow profile of classical planar chromatography limits the peak capacity.[7] In contrast, forced-flow techniques including rotational planar chromatography, overpressured layer chromatography, and planar electrochromatography eliminate some of the drawbacks of classical TLC.[8]

Electrochromatography is a separation method in which the mobile phase is driven through the stationary phase by electroosmotic flow (EOF). Pretorius introduced electrochromatography in both its incarnations, i.e., in columns and thin layers, 36 years ago.[9] Because of technical difficulties, it took almost 20 years before it was "rediscovered" in columns in the mid 1980s.[10] Monolithic columns quickly found their way to capillary electrochromatography since the in situ preparation of capillary columns eliminated the need for the tedious fabrication of frits in the capillary and reduced formation of bubbles.[10−13] At the same time, accelerated separations in planar electrochromatography carried out on a standard TLC plate have also been demonstrated.[14] However, this technique suffered from the limitations of EOF in an open bed, specifically, and depending on conditions, either accumulation of the mobile phase on the surface of the layer or drying of the layer.[15] Pressurized planar electrochromatography (PPEC) eliminates these limitations and, in addition, affords faster separations with enhanced efficiency.[8] Pressurization also provides a linear voltage drop and constant EOF.[16,17] Developments in

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planar electrochromatography have been summarized in recent excellent reviews.[18−20]

In this letter we present our initial results demonstrating the use of monolithic thin layers prepared from an organic polymer in the rapid separation of peptides. This subject is getting significant impetus by the current interest in proteomics. The reported separations are by PPEC and by electrophoresis in an apparatus for PPEC.

EXPERIMENTAL SECTION

Chemicals and Materials. 2-Acrylamido-2-methyl-1-propanesulfonic acid, butyl methacrylate, ethylene dimethacrylate, 2-hydroxyethyl methacrylate, benzophenone, 2,2-dimethoxy-2-phenylacetophenone, cyclohexanol, 1-decanol, *tert*-butanol, 3-(trimethoxysilyl)propyl methacrylate, acetic acid, trifluoroacetic acid, ammonium acetate, sodium acetate, fluorescamine, acetone, acetonitrile, methanol, water (used in the preparation of the monolithic layer), all of the highest available purity, were purchased from Sigma-Aldrich (St. Louis, MO). The methacrylate monomers were purified by passing through a short column packed with a basic alumina inhibitor remover (Sigma-Aldrich, St. Louis). Borofloat glass plates 13.3 cm × 3.3 cm, 1.1 mm thick were purchased from S. I. Howard Glass Co. (Worcester, MA). Solutions of standard peptides all from Anaspec Incorporated (San Jose, CA), with the exception of oxytocin acetate salt (from Sigma-Aldrich), were individually dissolved in Milli-Q deionized water (0.7 mg/mL) and then mixed. Oligonucleotides (a 5-mer, a 14-mer, and a 28-mer) fluorescently labeled with FAM fluorophore were purchased from Eurofins MWG/Operon (Huntsville, AL) and individually dissolved in Milli-Q deionized water (1 ng/nL) and then mixed. Peptide labeling was performed immediately before separation by mixing equal volumes of the peptide mixture with an acetone solution of fluorescamine (3 mg/mL).

Surface Modification of Glass Plates. The glass plates were rinsed with water, activated with 1 mol/L sodium hydroxide for 30 min, washed with water and 0.2 mol/L HCl for 30 min, rinsed with water again, and dried in a stream of nitrogen. The glass surface was then functionalized using 20 vol % solution of 3-(trimethoxysilyl)propyl methacrylate in ethanol adjusted to pH 5 using acetic acid. This solution was placed for 30 min at the activated surface of one plate covered with another one, thus functionalizing two plates at the same time. This functionalization was repeated twice. The plates were then washed with acetone and dried with nitrogen.

Preparation of the Hydrophobic Monolithic Layer. The polymerization mixture comprising butyl methacrylate (24 wt %), ethylene dimethacrylate (16 wt %), 1-decanol (30 wt %), cyclohexanol (30 wt %), and 2,2-dimethoxy-2-phenylacetophenone (1 wt % with respect to monomers) was deaerated by purging with nitrogen for 10 min. This mixture was filled into an assembled mold consisting of two modified glass plates clamped face to face and separated with two 125 μm thick Teflon strips (American Durafilm Co.) placed between them to define the thickness of the polymer layer. The mold was filled with the polymerization mixture using capillary action and exposed to UV light with an intensity of 12 mW/cm^2 for 15 min. Once the polymerization was completed, the mold was disassembled and the plate with the attached polymer layer was immersed in methanol for 1 h to remove the porogens. Finally, the plates were dried in a vacuum at 40 °C for 30 min.

Photografting of Surface Chemistry. The 125 μm thick hydrophobic layer was wetted with a solution containing 2 wt % 2-acrylamido-2-methyl-1-propanesulfonic acid, 13 wt % 2-hydroxyethyl methacrylate, and 0.25 wt % benzophenone in 3:1 (v/v) *tert*-butanol−water mixture. The wetted layer was then covered with a quartz plate and exposed to UV light for 10 min. The grafted plates were then washed with methanol and water to remove unreacted components and dried in nitrogen.

Instrumentation. The PPEC separations were performed in an apparatus that has been described previously[21] and which features a vertical plate holder. The same apparatus was used for electrophoresis with the exception of the plate holder. The original design of the holder included a single reservoir, which delivers run buffer into the bottom of the plate by electroosmotic flow. In contrast, electrophoresis features no flow of run buffer. To prevent the layer from drying it is necessary to have a plate holder with a reservoir at both the top and bottom. The new holder will be described elsewhere. The plates were sealed along the long edges with a 1:2 mixture of 734 Flowable Sealant (Dow Corning Corporation, Midland, MI) and toluene and were left to cure overnight. The pressurized zone was 10 cm in length for each holder, and the electric field was about 275 V/cm (at 3 kV) over a total distance of 11 cm.

Separation. The peptide solution (0.15 μL) was spotted 7 cm from the bottom of the plate. This allows peptides to migrate to either the anode or the cathode. The oligonucleotides, which all move toward the anode, were spotted 8 cm from the bottom of the plate. The spotting solvent was allowed to evaporate for 1 min, and the plate was then dipped for 5 s into the mobile phase consisting of acetonitrile in a phosphate buffer to within 2 mm of the applied spot. The plate was then removed, blotted dry on both the front and back, rotated 180°, and the procedure repeated. The plate was then placed into the plate holder and secured. The completed holder assembly was inserted into the PPEC apparatus, pressurized, and run under the desired conditions. The same procedure was used for both electrophoresis and for PPEC, with the exception that for PPEC the sample was spotted 4 cm from the bottom of the plate. Upon completion of the run, the plates were immediately removed, left to dry for 5 min, and then photographed and documented. The plates were then washed in a moving bath of 60% aqueous acetonitrile overnight, dried, and reused. Each plate can be used up to seven times with this procedure.

RESULTS AND DISCUSSION

Porous Polymer Layer. We have recently demonstrated that poly(butyl methacrylate-*co*-ethylene dimethacrylate) monoliths can be readily prepared in thin layer format using photoinitiated polymerization in a simple mold consisting of two glass plates

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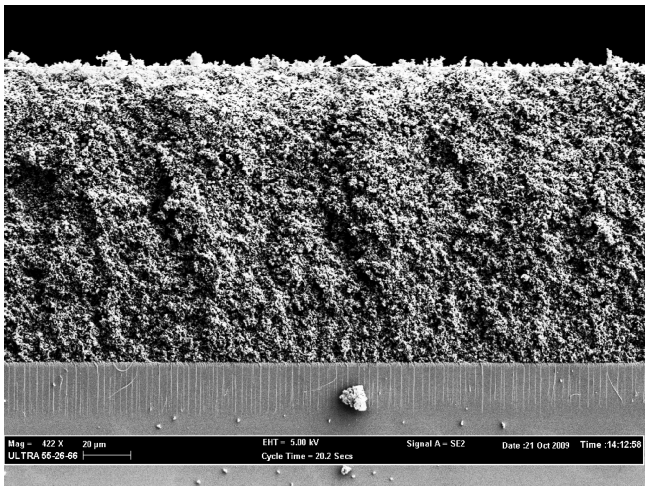

Figure 1. SEM image of the 125 μm thick porous poly(butyl acrylate-*co*-ethylene dimethacrylate) monolithic layer.

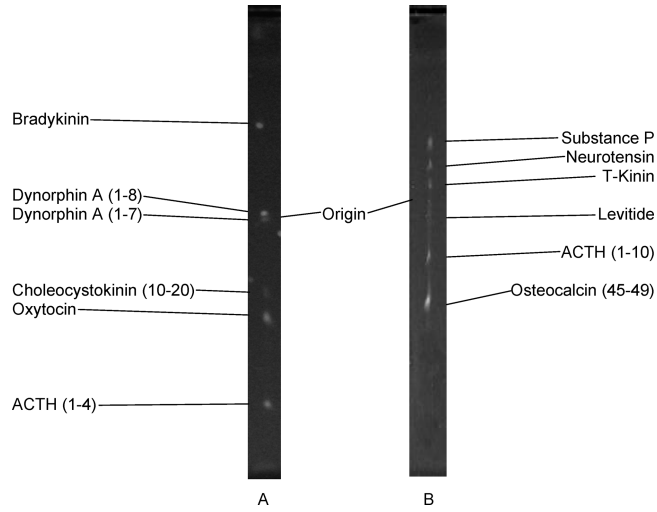

Figure 2. Electrophoretic separations of six peptides in 2 min at 20 °C on a 13 cm long hydrophobic poly(butyl methacrylate-*co*-ethylene dimethacrylate) layer. Conditions: run buffer 80% (a) or 70% acetonitrile (b) in 5 mmol/L phosphate buffer pH of 7.0; applied pressure 4.1 MPa; voltage 3 kV.

separated with Teflon film strips defining the thickness of the monolithic layer.[5] The reaction conditions described in the Experimental Section afford thin layers attached to a glass plate having a morphology shown in Figure 1. The generic monoliths consisting of a copolymer of 60% butyl methacrylate and 40% ethylene dimethacrylate do not contain any ionizable functionality. Their high hydrophobicity is characterized by a contact angle for water of 155°. Although the pore size of the thin layer is an important parameter, it cannot be measured directly since the amount of material on the plate is not sufficient for mercury porosimetry. However, polymer prepared from the same polymerization mixture in a vial exhibits a pore size distribution with a maximum at 1100 nm. These monoliths are well suited for the separations in the electrophoretic mode.

To achieve EOF, the monoliths need to be functionalized with ionizable functionalities. Photografting is a very powerful tool enabling tailoring surface chemistry of porous polymer monoliths.[22] We used cografting of 2-acrylamido-2-methyl-1-propanesulfonic acid and 2-hydroxyethyl methacrylate that we have developed earlier[23] to provide the layer with both ionizable and hydrophilic functionalities. This modification makes the layer highly hydrophilic with a contact angle close to 0°.

Separations Using Generic Monolith. First we attempted separation in the electrophoretic mode using generic monolith with no ionizable groups and found quite promising results. Optimization of the pore size and thickness of the monolith then led to layers that enabled successful separation of peptides. Parts a and b of Figure 2 show different 2 min long separations of six peptides using a run buffer consisting of 80 and 70% acetonitrile, respectively, in 5 mmol/L phosphate buffer at a nominal pH of 7.0. This pH refers to the value for the buffer solution before mixing with acetonitrile. The applied pressure was 4.1 MPa, the applied voltage 3 kV, and the temperature 20 °C. Attempts were made to improve the separation quality by adding 0.1% trifluoroacetic acid, but this resulted in a substantially higher electric current without any improvement in separation.

The applied pressure of 4.1 MPa was selected on the basis of early results, where the reproducibility was not very good. Subsequent improvement in the quality of the monolith layer resulted in decent reproducibility for the separation of the peptides, and this was monitored using a set of conditions that included an applied pressure of 4.1 MPa, as used in the current report. We have recently evaluated very low pressures and find that separation is poor when the layer is exposed to the atmosphere, as it dries in less than a minute. A decent separation is obtained when the monolith layer is contained in the holder in the normal fashion, with application of a pressure that we estimate to be below 0.1 MPa but which we cannot measure due to limitations of the current apparatus. Application of high pressure, however, results in compression of the layer and a substantial reduction in electrical current and Joule heating. This should be relevant when performing faster separations requiring higher voltages than used in this report.

Oligonucleotides can also be separated under similar conditions as illustrated in Figure 3. The separation of a 5-, 14-, and 28-mer is achieved in 4 min. The run buffer consisted of 70% acetonitrile in 5 mmol/L phosphate buffer at a nominal pH of 7.0. The applied pressure was 6.2 MPa, the applied voltage 3 kV, and the temperature 20 °C. Attempts to separate a 35-mer either separately or as a component of the mixture were not successful. Although we observed migration of the 35-mer, the spot shape was poor.

Separations Using Grafted Monolith. Our initial attempts to separate peptides using PPEC on a negatively charged layer grafted with pure 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) were unsuccessful. This problem was most probably caused by excessively high coverage of the generic monolith with sulfonic acid functionalities and has been solved by including 2-hydroxyethyl methacrylate in the grafting solution. The preliminary results show that a 10 min grafting with a solution containing both 2-acrylamido-2-methyl-1-propanesulfonic acid and 2-hydroxyethyl methacrylate afford charged monoliths exhibiting a good

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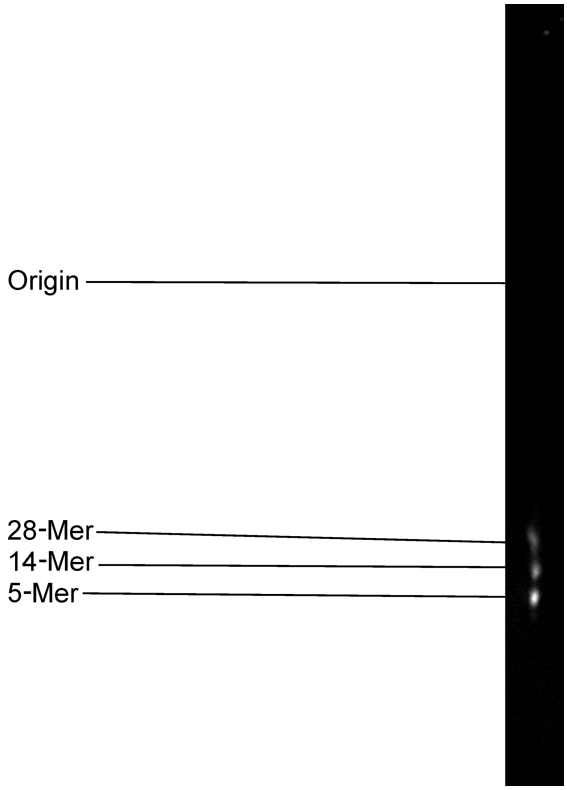

Figure 3. Electrophoretic separation of three oligonucleotides in 4 min at 20 °C on a 13 cm long hydrophobic poly(butyl methacrylate-*co*-ethylene dimethacrylate) layer. Conditions: run buffer 70% acetonitrile in 5 mmol/L phosphate buffer pH of 7.0; applied pressure 6.2 MPa; voltage 3 kV.

promise for separation of peptides. This is illustrated in Figure 4, which shows the separation of six peptides in 1 min. The conditions used allow two of the peptides to migrate against the electroosmotic flow. The run buffer was 70% acetonitrile in 5 mmol/L acetate buffer at a nominal pH of 4.7. The applied pressure was 4.1 MPa, the applied voltage 3 kV, and the temperature 20 °C.

CONCLUSIONS

Our initial results demonstrate that rapid electrochromatographic separations of peptides and oligonucleotides on planar monolithic polymer layers are possible. We believe that better and faster separations, with improved reproducibility, should be possible with adjustments to the structure and chemistry of the monolithic layer and the use of higher applied voltages. The current reproducibility of spot pattern is satisfactory. The demonstration of separations of two different types of analytes and using monoliths of two different chemistries illustrates the promise of this approach for separating molecules of biological interest.

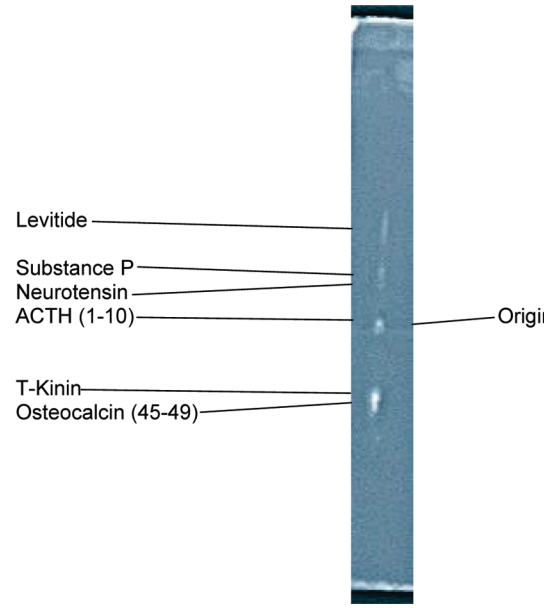

Figure 4. Electrochromatographic separation of six peptides in 1 min at 20 °C using a 12 cm long poly(butyl methacrylate-*co*-ethylene dimethacrylate) layer grafted with a mixture of 2-acrylamido-2-methyl-1-propanesulfonic acid and 2-hydroxyethyl methacrylate. Conditions: run buffer 70% acetonitrile in 5 mmol/L acetate buffer pH of 4.7; applied pressure 4.1 MPa; voltage 6 kV.

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