

## Capillary Coating for Protein Separation Based on Si-O and Si-C Covalent Bond Formation for Capillary Electrophoresis With Laser-Induced Fluorescence Detection

Hossein Ahmadzadeh, Norman J. Dovichi, and Sergey Krylov

### Summary

Protein adsorption to capillary walls is one of the major complications in protein analyses with capillary electrophoresis (CE). Coating the capillary with different materials is used to reduce the adsorption. This chapter overviews different approaches used for capillary coating and concentrates on those utilizing Si-O and Si-C covalent bonds. The apparatus and methods are presented for capillary coating using Si-O and Si-C chemistry. Furthermore, procedures are described for monitoring the quality of coating.

### Key Words

Capillary electrophoresis; coating; covalent bond; laser-induced fluorescence; protein.

### 1. Introduction

The theory of capillary electrophoresis (CE) predicts that the efficiencies of separation for large biological polymers, such as nucleic acids and proteins, should be on the order of  $10^6$  theoretical plates because of their low diffusion coefficients. In practice, the results are close to the theoretical values only for nucleic acids, whereas for the proteins, the efficiencies are much below those estimated. The major reason for low quality of separation of proteins is their adsorption to the capillary wall, which results in peak broadening. This problem has hindered method development, and, therefore, the full potential of CE in protein separation has yet to be realized (1–3). One of the most efficient

ways to reduce the protein–wall interaction is to coat the inner wall of the capillary with a layer of an appropriate polymer. Such a polymer should first eliminate the negative charge (inherent to the silica surface) on the capillary wall, and second, it should create a hydrophilic layer (that reduces hydrophobic adsorption of proteins) on the surface.

Capillaries are typically coated with a polymer in two steps. Initially, a bifunctional reagent is covalently linked through the first functional group to silica on the surface of the capillary wall to form a sublayer with another functional group exposed and available for attaching the second layer. Then, a monomer is bound to the exposed functional group of the bifunctional reagent and polymerized as a top layer that is covalently linked to the sublayer. **Fig. 1** schematically depicts the general strategy of two-layer capillary coating.

Hjerten was the first to apply silane chemistry to coating capillaries for CE (**4**). He used a Si-O bond to create a sublayer and polymerized acrylamide (AA) as a top layer. The disadvantage of such a coating is its susceptibility to alkaline hydrolysis. Both the Si-O bond and polyacrylamide are hydrolyzed at pH >8.0. Such hydrolysis results in the destruction of the coating or the formation of a layer of polyacrylate that is capable of strongly adsorbing proteins and regenerating the electroosmotic flow (EOF). Novotny used the Grignard method to change the Si-O bond to a Si-C bond, which is much more resistant to alkaline hydrolysis (**5**). This method results in a physically more stable coating than the silane-coupling chemistry. However, because it still uses polyacrylamide, which is hydrolyzed at pH >8.0, this coating is inapplicable to protein separations at basic pHs.

Righetti et al. introduced and optimized the performance of several novel polymers of AA substitutes. The monomers studied were dimethylacrylamide (DMA), N-acryloylaminoethoxyethanol (AAEE), and acryloylaminopropanol (AAP; **6–19**). AAEE and AAP showed much less protein adsorption on the capillary wall than AA, whereas DMA was more adsorptive for proteins than AA. These properties are ascribed to the much higher hydrophilicity of AAP and AAEE compared with DMA. However, despite the use of the hydrophilic polymers, Righetti's method still involves a sublayer based on a Si-O bond that is vulnerable to alkaline hydrolysis. The chemical formulas of the monomers introduced by Righetti are shown in **Fig. 2**. The reaction steps in making Si-O and a Si-C bonds followed by polymerizing a monomer as a top layer are depicted in **Figs. 3** and **4**.

In **ref. 20** Ahmadzadeh and Dovichi reported coating of capillaries with AA, DMA, AAEE, and AAP based on either Si-O or Si-C sublayers. The procedures involved either the coupling of a silane bifunctional reagent (3-methoxypropyltrimethoxysilane) with the silanol groups (Si-O bond) or silanol chlorination followed by the Grignard-coupling of vinyl magnesium bromide

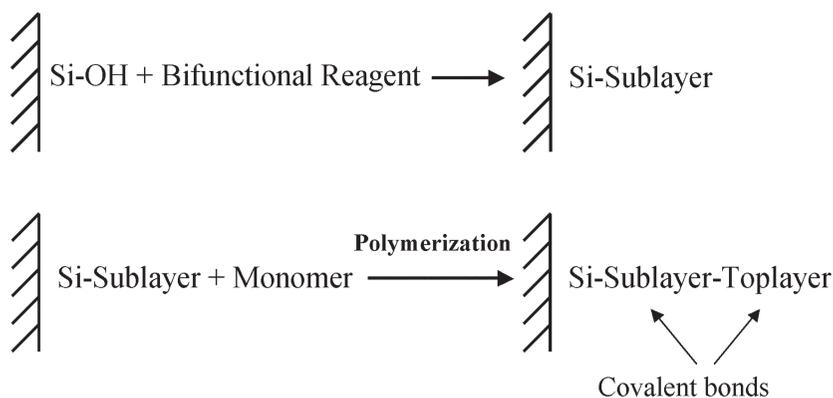


Fig. 1. Schematic diagram showing the strategy for permanent coating.

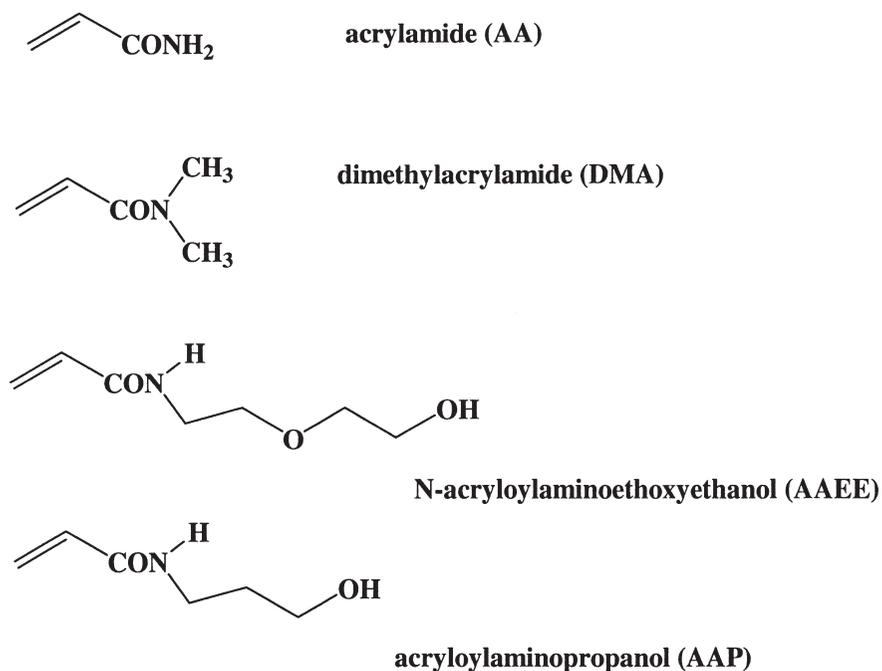


Fig. 2. Chemical formulas of the monomers introduced in **ref. 20**.

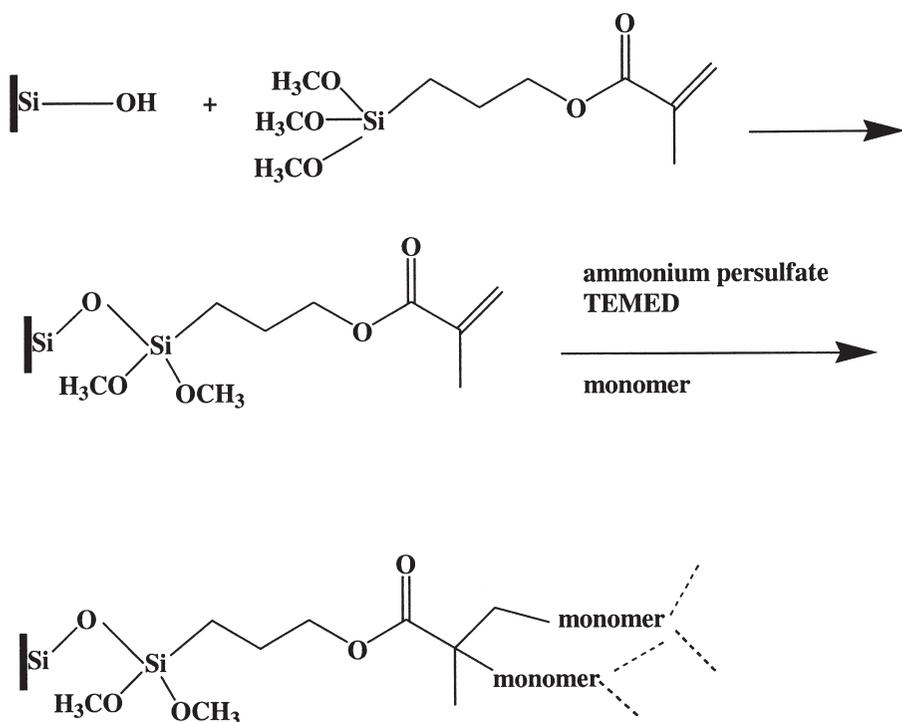


Fig. 3. Chemical equations and reaction steps for coating the capillaries based on Si-O bond formation and polymerization of a monomer of choice as a top layer.

(Si-C bond). The Grignard-based sublayer has been employed further to compare the performance of all four monomers. The procedures proved to be very practical for reducing protein adsorption on capillary walls in CE.

As aforementioned, the coating of capillaries can be based either on silanization or on the Grignard reaction (*see Figs. 3 and 4*). In the former method, 0.1 M NaOH is flushed through the capillary (4–5 m long, 50  $\mu\text{m}$  id) for 1 h followed by flushing with water for another hour. Finally, a 4% solution of 3-methacryloxypropyltrimethoxysilane in a 1:1 mixture of glacial acetic acid and water should be prepared, and the capillary has to be flushed with this solution for 20 min. The silanization reaction goes to the completion within 1 h. Next, the capillary should be flushed with water for 10 min. Ammonium persulfate (4  $\mu\text{L}$  of freshly prepared, 10%) and tetramethylethylenediamine (TEMED) (1  $\mu\text{L}$ ) should be added to 1 mL of a 3% solution of a monomer. This undegassed solution should immediately be flushed through the capillary. After 1 h, the polymerization reaction is complete and the gel, that is not

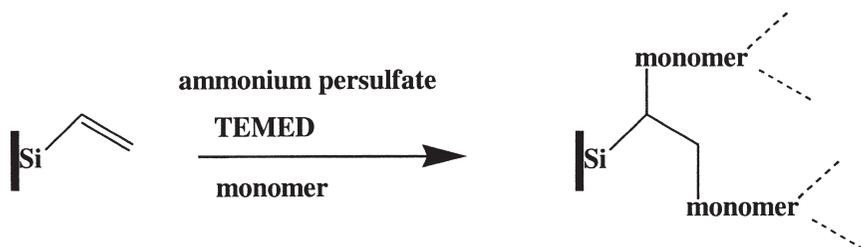


Fig. 4. Chemical equations and reaction steps for coating the capillaries based on Si-C bond formation and polymerization of a monomer of choice as a top layer.

immobilized on the walls, is flushed out and the capillary is filled with water. The capillary has to be stored in water, and before doing a CE run the water inside the capillary ought to be replaced manually with running buffer using a syringe with a proper fitting.

The instrumentation setup for capillary coating is shown in **Fig. 5**. The outlet of a low dead-volume fitting is connected to a 3-cm-long piece of 1/16-in outer-diameter (od) Teflon tubing. The capillary is threaded through the Teflon tube, the connector, and the tee. The capillary tip should be located near the middle of the Reacti-vial inside a disposable vial. For some solvents (like THF) and some reagents (like vinyl magnesium bromide), this disposable vial has to be made of glass. When the capillary is inside the disposable vial, which is, in turn, in the Reacti-vial, the top nut has to be tightened while the capillary is in place. It is recommended to flush the solutions through the capillary using nitrogen gas at 20 psi dehydrated with a moisture trap. The coating procedure has four steps that are described later in this chapter.

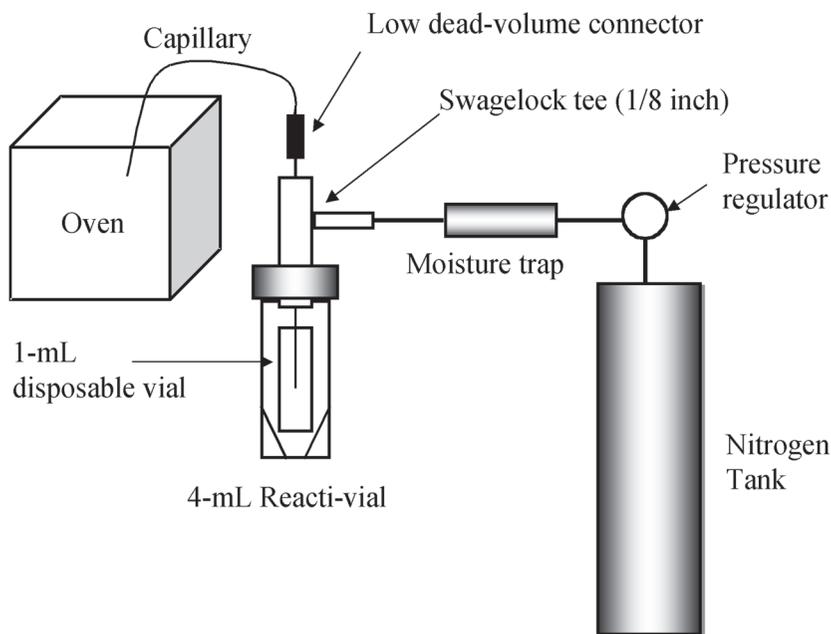


Fig. 5. Apparatus for delivering the reagents into the capillaries for the coating processes. Nitrogen gas pressure is used to force different reagents through the capillary. The reagents are held in a disposable vial placed inside the Reacti-vial. The fittings are used for the air-tight connection of the capillary to the reagent vials.

## 2. Materials

### 2.1. Capillary Coating Based on Si-O Bond Formation

1. Doubly distilled deionized water (*see Note 1*).
2. Concentrated acetic acid: add 0.5 mL of concentrated acetic acid to 0.5 mL of water (solution A).
3. 0.1 M NaOH.
4. Silane reagent, 3-methacryloxypropyltrimethoxysilane: add 40  $\mu\text{L}$  of the silane reagent to 1 mL of solution A (solution B).
5. A 3% solution of a monomer in water.
6. TEMED.
7. Ammonium persulfate (APS) 10% in water, freshly prepared.

### 2.2. Capillary Coating Based on Si-C Bond Formation

1. Doubly distilled deionized water (*see Note 1*).
2. Type 4-A molecular sieves (*see Note 2*).
3. Thionyl chloride (*see Note 3*).
4. 1 M Vinyl magnesium bromide (*see Note 4*).

5. Anhydrous THF (*see Note 4*).
6. A 3% solution of a monomer in water, freshly prepared.
7. TEMED.
8. APS 10% in water.

### 2.3. Fluorescent Labeling of Proteins and Peptides

1. 2.5 mM Sodium tetraborate (10 mM borate), pH 9.4 (*see Note 1*).
2. 1 nM Protein solution (*see Note 5*).
3. Dry 5-furoyl quinoline-3-carboxaldehyde (FQ) 100 nmoles (*see Note 6*).
4. 25 mM KCN solution in either water or 10 mM borate (*see Note 7*).

### 2.4. In-House CE With Laser-Induced Fluorescence Detector

1. An in-house constructed CE instrument (*see Note 8*).
2. A laser-induced fluorescence (LIF) detector based on a sheath-flow cuvet (*see Note 8*).
3. Fused-silica capillaries, 50  $\mu\text{m}$  id, 150  $\mu\text{m}$  od (Polymicro Technologies, Phoenix, AZ).
4. A 0–30 kV dc power supply (CZE 1000, Spellman, Plainview, NY).
5. A 488-nm argon-ion laser to be operated at 12 mW (Model 2211-55 SL, Uniphase, San Jose, CA or Model Innova 90-4, Coherent, Mountain View, CA).
6. A  $\times 6.3$  microscope objective (Melles Griot, Nepean, ON, Canada) and a  $\times 60$ , 0.7 NA microscope objective (Mo-0060LWD, Universe Kokagu, Oyster Bay, NY).
7. An interference filter centered at 615 nm with a 45-nm transmittance window (Omega Optical, Brattleboro, VT).
8. A photomultiplier tube (PMT) (R1477, Hamamatsu, Middlesex, NJ).
9. A 16-bit data acquisition board (NB-MIO 16 XH-18, National Instruments, Austin, TX).
10. A personal computer.

### 2.5. Capillary Coating Apparatus

**Fig. 5** shows a simple apparatus that could be constructed to deliver reagents into the capillary. Reagents should be prepared in 1-mL disposable vials. Each reagent vial has to be placed inside a 4-mL Reacti-vial. A hole should be drilled through the lid of the Reacti-vial to accommodate a one-eighth inch stainless steel Swagelock tee. This tee is held in place with the aid of copious amounts of Teflon tape. One side of the tee is connected with Teflon tubing to a nitrogen cylinder. A guard column filled with molecular sieves (type 4-A), as a moisture trap, should be connected to the nitrogen tank to remove the water impurity from nitrogen gas before it is being used to purge the capillaries. The top side of the Swagelock tee has to be connected to a one-sixteenth inch adaptor. This adaptor is connected to a low dead-volume fitting for the capillary. For the temperature-controlled steps, the capillary is placed inside an oven.

### 3. Methods

#### 3.1. Capillary Coating Procedures

##### 3.1.1. Method 1: Coating Based on Si-O Bond Formation

###### 3.1.1.1. CAPILLARY PRETREATMENT

1. Cut a 4–5-m long piece of fused-silica capillary, 50  $\mu\text{m}$  id.
2. Condition the capillary by flushing with 0.1 M NaOH for 3 h using 20 psi nitrogen pressure.
3. Flush the capillary with water for 1 h using 20 psi nitrogen pressure.

###### 3.1.1.2. SILANE REACTION FOR Si-O BOND FORMATION

1. Flush the capillary with silane solution B (*see Subheading 2.1.4.*) for 1 h using 20 psi nitrogen pressure.
2. Flush the capillary with water for 10 min using 20 psi nitrogen pressure.

###### 3.1.1.3. POLYMERIZATION STEP

1. Cut the capillary into 1-m long sections.
2. Coat each section with a monomer (*see Note 9*). For this, prepare a 3% solution of a monomer by diluting the stock solution in water. Add 1  $\mu\text{L}$  of TEMED and 4  $\mu\text{L}$  of 10% ammonium persulfate to 1 mL of the monomer, and then immediately flush the polymer through the capillary at 60 psi for 5 min.
3. After 1 h of polymerization, flush the gel that is not immobilized on the capillary wall out of each capillary and fill the capillaries with water.
4. Store these coated capillaries in water, and flush the running buffer through them prior to use (*see Note 10*).

##### 3.1.2. Method 2: Coating Procedure Based on Si-C Bond Formation

###### 3.1.2.1. CAPILLARY PRETREATMENT

1. Cut a 4–5-m long piece of fused-silica capillary, 50  $\mu\text{m}$  id.
2. Condition the capillary by flushing 0.1 M NaOH for 3 h at a 20 psi nitrogen pressure.
3. Flush the capillary with water for 1 h using 20 psi nitrogen pressure.
4. Flush the capillary with methanol for another hour using 20 psi nitrogen pressure.
5. Dry the capillary in an oven at 140°C for 8 h or overnight by flushing nitrogen gas through it at a pressure of 5 psi.

###### 3.1.2.2. CHLORINATION OF SILANOL GROUPS (*SEE NOTE 11*)

1. The following day, while the capillary is inside the oven and nitrogen is flowing through it, reduce the oven temperature to 65°C and then flush thionyl chloride under 20 psi nitrogen pressure for 30 min. Check the outflow to identify any plugged capillaries, and test for acidity using pH paper (*see Note 3*).

2. After 30 min, seal one end of the capillary with a GC septum while the outlet end remains connected to the vacuum line for an additional 15 min to remove the excess of thionyl chloride. This step should be done while the capillary is inside the oven. Then, seal the outlet end of the capillary using a GC septum, and heat the capillary in the oven for 8 h or overnight (*see Note 12*).

#### 3.1.2.3. GRIGNARD REACTION (SI-C BOND FORMATION)

1. Prepare a fresh solution of 0.25 M vinyl magnesium bromide in dry THF (solution C) under nitrogen atmosphere (*see Note 13*).
2. Submerge a freshly cut inlet end of the capillary into solution C and uncap the outlet end of the capillary, cut a few centimeters from the outlet end and place it in a tube containing methanol. Apply nitrogen pressure (20 psi) to the inlet end of the capillary to rinse it with solution 1 for 30 min (*see Note 14*).
3. Cap the capillary at both ends using a GC septum, and heat it for 6–8 h or overnight at 70°C.
4. Uncap the capillary and cut 5–10 cm off both ends.
5. Rinse the capillary with anhydrous THF for 30 min followed by water for another 30 min (*see Notes 15 and 16*).

#### 3.1.2.4. POLYMERIZATION STEP

1. Cut the capillary into 1-m long sections.
2. Coat each section with a monomer (*see Note 9*) as top-layer using the following procedures. Prepare a 3% solution of the monomer by diluting the stock solution in water. Add 1  $\mu\text{L}$  of TEMED and 4  $\mu\text{L}$  of 10% APS to 1 mL of the monomer, and then immediately flush the polymer through the capillary at 60 psi for 5 min.
3. After 1 h of polymerization, flush the gel out of each capillary using nitrogen and replace the polymer with water.
4. Store these coated capillaries in water, and flush a run buffer through them prior to use (*see Note 10*).

#### 3.1.3. Method 3: FQ Labeling of Proteins and Peptides (*see Note 17*)

1. Add 7.5  $\mu\text{L}$  of  $10^{-7}$  M protein solution to 100 nmol of dry FQ, and then add 2.5  $\mu\text{L}$  of 5 mM KCN in water (**21**).
2. Stop the reaction after 30 min by adding 740  $\mu\text{L}$  of the run buffer (*see Note 18*).

#### 3.1.4. Method 4: EOF Mobility Measurement (*see Note 19*)

To evaluate the quality of coating, measure the EOF for each capillary by the current monitoring method (**20,22**) using the following procedures.

1. Fill the capillary with 10 mM borate at pH 9.4, apply high voltage and monitor the current.
2. After the current stabilizes, change the run buffer to 8 mM borate. The current will decrease and reach a constant value after a certain period of time ( $\Delta t$ ). Measure this time (*see Note 20*).

3. Calculate the velocity of EOF ( $v_{\text{EOF}}$ ) and the mobility of EOF ( $\mu_{\text{EOF}}$ ) using the following formulas:

$$v_{\text{EOF}} = L/\Delta t \quad (1)$$

$$\mu_{\text{EOF}} = v_{\text{EOF}}/E = L^2/V\Delta t \quad (2)$$

where  $L$  is the capillary length and  $V$  is the applied voltage.

#### 4. Notes

1. Prepare all aqueous solutions with Milli-Q deionized water and filter them using a 0.2- $\mu\text{m}$  filter. Then degas the buffers to prevent the formation of air bubbles during electrophoresis.
2. The molecular sieve is a moisture trap for  $\text{N}_2$  gas that is used to deliver the reagents into the capillary. If not trapped, the traces of water will cause the precipitation of vinyl magnesium bromide and, hence, plug the capillary.
3. Do not return the excess of thionyl chloride to the original bottle. The excess should be decomposed by the addition of water to the solution under a fume hood.
4. THF is a solvent for vinyl magnesium bromide. It has to be absolutely dry for the reaction to be successful. Traces of water will cause precipitation inside the capillary and, hence, failure of the process. Keep THF in a desiccator.
5. Dissolve the proteins in water if stacking is desired and dissolve it in the run buffer otherwise.
6. Prepare a stock solution of 10 mM FQ in methanol; aliquot 10  $\mu\text{L}$  of the solution into 500- $\mu\text{L}$  microcentrifuge tubes and remove the solvent under vacuum using a Speed Vac (Savant Instruments Inc., Farmingdale, NY). The dried FQ (100 nmoles) aliquots should be stored at  $-20^\circ\text{C}$ . These precautions are necessary because it has been observed that FQ slowly degrades while in solution even if the solution is stored at  $-20^\circ\text{C}$ .
7. Potassium cyanide is highly poisonous. It reacts rapidly with acids to form lethal HCN gas. Stock solutions should be made in a basic buffer and an experimenter should be aware of any change to acidic pH during the experiment. The waste containing KCN should be neutralized by adding a 1% solution of NaOH followed by slowly adding bleach.
8. An in-house constructed CE-LIF instrument with a detector based on a sheath-flow cuvet is described in detail elsewhere (21–24). Here we are briefly outlining the construction of the instrument. Unless otherwise stated, fused-silica capillaries were 40 cm long, 50  $\mu\text{m}$  id, and 141  $\mu\text{m}$  od. The electric field was applied to an inlet end of the capillary from a 0–30 kV dc power supply. The excitation was provided by the 488-nm line of an argon-ion laser operated at 12 mW. The laser beam was focused approx 30  $\mu\text{m}$  from the outlet tip of the capillary with a  $\times 6.3$  microscope objective. Fluorescence was collected by a  $\times 60$ , 0.7 NA long-working-distance microscope objective, filtered with a spatial filter, and a 615 DF 45

band-pass filter to remove stray and scattered light. Fluorescence was imaged onto a photomultiplier biased at 1 kV. The photocurrent was passed through a current-to-voltage converter and a low-pass filter ( $RC = 47$  ms) and then digitized with a 16-bit data acquisition board connected to a personal computer.

9. This step is valid for each monomer, AA, DMA, AAEE, or AAP.
10. If the gel is left inside the capillary for a period longer than 3 h in this step, a gel-filled capillary that is suitable for capillary gel electrophoresis is obtained. If the reaction time is too long (much more than 3 h) then the capillaries can be irreversibly plugged.
11. Before the chlorinating step, check the color of thionyl chloride. Replace the solution with a fresh one if it is faint.
12. In some cases this step can be repeated to obtain a more rugged coating.
13. To prepare this solution, add 0.75 mL of dry THF and 0.25 mL of 1 M vinyl magnesium bromide in THF into a tube filled with nitrogen gas.
14. If the capillary is plugged and no flow is observed, place the capillary back inside the oven for a short period of time. If it is still plugged, check for blockage using a microscope and cut the capillary to appropriate lengths to remove the blocked parts of the capillary and save the rest.
15. The Grignard step could be repeated to get more rugged capillaries.
16. Initially, about 10% of the capillaries could be plugged during the Grignard reaction step. We observed that the failure rate dropped to zero in winter, when the relative humidity in the laboratory decreased and as experienced was gained in handling the reagents. Novice experimenters in humid environments may expect difficulty with this sublayer coating step.
17. The labeling protocol is a modification of the procedure first introduced by Novotny in **ref. 18**.
18. This FQ-labeled protein, with a final concentration of  $10^{-9}$  M, could be used to evaluate the coated capillaries. In earlier studies, we showed that a 30-min reaction time was optimal for obtaining the highest intensity of the fluorescence signal (**20**).
19. EOF measurements can be used to evaluate the quality of coating; the lower the EOF mobility the better the quality. For Si-O based coatings, the reduction of EOF should be in the order of 100 times as compared to uncoated capillaries. For Si-C based coatings, the EOF should be 1000 times less than that for uncoated capillaries.
20. We have proved that this current monitoring method to measure EOF is as accurate as the neutral marker method, especially for coated capillaries (*see ref. 20*).

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